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Influence of feeding frequency on growth, hematology, blood biochemistry, liver histology, and intestinal microbiota in the Oscar (*Astronotus ocellatus*)

Influência da frequência de alimentação no crescimento, hematologia, bioquímica sanguínea, histologia hepática e na microbiota intestinal no Oscar (*Astronotus ocellatus*)

Nelson Mauricio Lopera-Barrero[1]; Felipe Pinheiro de Souza[2]; Ed Christian Suzuki de Lima[2]*; Lucas Mendonça Odebrecht[3]; Victor César Freitas Pandolfi[2]; Cindy Namie Seino Leal[2]; Ulisses de Pádua Pereira[4]; Andréia Carla Eugenio Pupim[5]; Paulo César Meletti[6]; Eduardo José de Almeida Araújo[7]

Highlights

Fish fed once daily showed higher hepatosomatic index values.
Fish fed once daily showed lower hepatocyte nuclei count.
Fish fed once daily showed lower serum albumin levels.
Fish fed four times daily showed a higher taxa richness in the intestinal microbiota.
Briefly, feeding frequencies of two, three and four times daily were most suitable.

Abstract

The Oscar (Astronotus ocellatus) is an important Amazonian species with high production potential in fish farms and commercial value for aquarists. However, limited information on the feeding characteristics, such as the frequency of daily feeding, is available for this species in captivity. Therefore, the objective

[1] Prof. Dr., Department of Animal Sciences and Postgraduate Program in Animal Science, Universidade Estadual de Londrina, UEL, Londrina, PR, Brazil. E-mail: nmlopera@uel.br
[2] Students of the Masters or Doctorate Course of the Postgraduate Program in Animal Science, UEL, Londrina, PR, Brazil. E-mail: felipeps1991@gmail.com; edchris7@hotmail.com; vcfpand@gmail.com; cindynamies@gmail.com
[3] Student of Veterinary Medicine Course, Centro Universitário Filadélfia, UniFil, Londrina, PR, Brazil. E-mail: lucasmodebrecht@hotmail.com
[4] Prof. Dr., Department of Preventive Veterinary Medicine and Postgraduate Program in Animal Science, UEL, Londrina, PR, Brazil. E-mail: upaduapereira@uel.br
[5] Laboratory Technician of Department of Histology, UEL, Londrina, PR, Brazil. E-mail: pupim@uel.br
[6] Prof. Dr., of Department of Physiological Sciences, UEL, Londrina, PR, Brazil. E-mail: pmeletti@uel.br
[7] Prof. Dr., Department of Histology, UEL, Londrina, PR, Brazil. E-mail: eduardoaraujo@uel.br
* Author for correspondence

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of this study was to evaluate the effects of feeding frequency on growth, hematology, blood biochemical parameters, liver histology, and intestinal microbiota parameters of juvenile Oscars. Four treatments were tested, consisting of fish fed at a feeding rate of one (T1), two (T2), three (T3), or four (T4) times daily with 1% of their biomass. There were no significant differences ($p > 0.05$) between treatments for growth and hematological parameters. T1 showed lower serum albumin values than T2 and T4 ($p < 0.05$) and higher hepatosomatic index values than T4 ($p < 0.05$), which was corroborated by the hepatocyte nucleus count. Intestinal microbiota analysis revealed higher taxon richness in T4 than in T1 ($p < 0.05$). Lower feeding frequencies (T1) positively influenced the deposition of hepatic energy reserves and decreased albumin production, possibly because of lower peptide absorption in the same treatment. Based on these results, feeding frequencies of two (T2), three (T3) and four (T4) times daily were the most suitable for juveniles of this species.
Key words: Aquarium hobby. Fish. Fish farming. Intestinal microbiota. Nutrition. Ornamental aquaculture.

Resumo

O Oscar (Astronotus ocellatus) é uma importante espécie amazônica de alto potencial produtivo em pisciculturas e valor comercial para aquariofilistas. No entanto, limitadas informações sobre suas características alimentares, como frequência de alimentação diária, estão disponíveis para espécies em cativeiro. Portanto, o objetivo do presente estudo foi avaliar o efeito da frequência alimentar no crescimento, hematologia, parâmetros bioquímicos do sangue, histologia hepática e microbiota intestinal de juvenis de Oscar. Foram testados quatro tratamentos, consistindo de peixes alimentados com taxa de arraçoamento de uma (T1), duas (T2), três (T3) ou quatro (T4) vezes ao dia com 1% da sua biomassa. Não houve diferenças significativas ($p > 0,05$) entre tratamentos para crescimento e parâmetros hematológicos. T1 apresentou valores de albumina sérica menores que T2 e T4 ($p < 0,05$), e maiores valores de índice hepatossomático em relação à T4 ($p < 0,05$), o que foi corroborado pela contagem de núcleos de hepatócitos. A análise da microbiota intestinal revelou maior riqueza de táxons em T4 do que em T1 ($p < 0,05$). As menores frequências de alimentação (T1) influenciaram positivamente a deposição das reservas energéticas hepáticas e a diminuição da produção de albumina, possivelmente devido à menor absorção de peptídeos no mesmo tratamento. Com base nesses resultados, frequências alimentares de duas (T2), três (T3) e quatro (T4) vezes ao dia foram as mais adequadas para os juvenis desta espécie.
Palavras-chave: Aquarismo. Aquicultura ornamental. Microbiota intestinal. Nutrição. Peixe. Piscicultura.

Introduction

Ornamental fish culture is widely distributed globally (Dey, 2016), and its beginning is confused with that of aquaculture (Ribeiro et al., 2009). Cultivating these fishes involves several sectors of international trade, fisheries, and aquaculture and is one of the most economical and profitable areas for fish production (Ghosh et al., 2008). Brazil stands out internationally as a producer and exporter of ornamental fish owing to its considerable species diversity (Sousa et al., 2018; Tavares-Dias et al., 2010).

Among the species with the greatest potential for production and commercialization is the Oscar (Astronotus ocellatus). This species belongs to the Cichlidae family, is native to the Amazon basin, is known worldwide for its importance as an ornamental fish (Gutierre et al., 2016), and is used as a food resource at its place of origin (Tavares-Dias et al., 2014). Research evaluating the ornamental fish production chain has shown that this species is among the most bred and commercialized in Brazil (Assis et al., 2014; Cardoso et al., 2012). However, some important aspects related to the farming of this fish need to be elucidated.

One of the factors that can significantly influence fish farming is feeding frequency. Feeding frequency is fundamental for adequate food management; when it is inadequate, it hinders growth, increases stress, and predisposes fish to illnesses (Guo et al., 2018; Ribeiro et al., 2012; Wu et al., 2021), resulting in large economic losses. According to Tian et al. (2015), high and low feeding frequencies can result in growth retardation and reduced feed efficiency. Therefore, establishing an ideal feeding frequency based on the requirements of each species and growth assessment is essential for increasing earnings and improving production.

Studies evaluating the effects of different dietary frequencies on ornamental fish species have shown that feeding frequency influences productive performance, production costs, reproductive parameters, and survival (Abe et al., 2016; Fujimoto et al., 2016; Karadal et al., 2017; Kasiri et al., 2011; Pinheiro et al., 2023; Ribeiro et al., 2012). However, no studies have evaluated this effect on farmed Oscar. In addition, few studies have evaluated the influence of feeding frequency on metabolic and hematological parameters, which can be analyzed to understand the effects of feeding frequency on the growth and health of these fish. Studies on other species have shown the effects of different feeding frequencies on the hepatosomatic index (HSI) and blood biochemical parameters (Aderolu et al., 2017; Baloi et al., 2014; Gao et al., 2022; Guo et al., 2018; Wu et al., 2021).

Therefore, determining the most effective feeding frequency for Oscars would help both aquaculture and aquarists. Improving the growth rate by controlling the feeding frequency would add greater value for commercialization of aquaculture, which is desirable for the large-scale production of any fish species. Although the improvement in fish growth parameters is not as important for aquarists as for fish farmers, maintaining the health and well-being of these animals is essential for expressing desirable phenotypic characteristics and ensuring greater longevity of individuals in aquariums.

Another limitation of these studies is the impact of feeding frequency on the gut microbial community structure. Intestinal microbiota plays an important role in nutrients and vitamins metabolism, immune response regulation, and pathogenic microorganisms' competition (Eichmiller et al., 2016; Vatsos, 2016) within a "hidden organ" (Eichmiller et al., 2016) that should be analyzed for productive purposes. Microbiota characterization enables the evaluation of the effects of diet, environment, and interaction with pathogens on microbial composition in several fish species (Eichmiller et al., 2016; Lima et al., 2024; F. P. Souza et al., 2020a; Suphoronski et al., 2019; Tarnecki et al., 2017).

However, no study to date has evaluated the gut microbiota of Oscars or how feeding frequency can modulate microbial community activity. Intestinal bacteria depend on their host for nutrient supply and energy (Fetissov, 2016), and certain bacterial phyla can capture additional energy from food during periods of food restriction, providing a competitive advantage over other phyla (Tarnecki et al., 2017; Xia et al., 2014). Therefore, studies are required to elucidate how food management, especially feeding frequency, influences the intestinal microbiota of fish.

The objective of the present study was to analyze the influence of feeding frequency on performance, hematological, blood biochemical, and liver histological parameters, and to evaluate the intestinal microbiota (gene 16S-rRNA, region V3-V4) of Oscar juveniles using next-generation sequencing (NGS).

Material and Methods

Experimental conditions

All animal procedures were approved by the Ethics Committee on Animal Use of the State University of Londrina (approval number CEUA/UEL-14939.2019.82).

Oscar juveniles (Astronotus ocellatus) (n = 80) were purchased from a local supplier, transported in plastic bags with 2/3 of the volume filled with water and 1/3 with oxygen, and taken to the acclimatized laboratory (27–28 °C) of the Center of Study and Research in Aquaculture and Genetics (Núcleo de Estudos e Pesquisa em Aquicultura e Genética - NEPAG) at the State University of Londrina (Universidade Estadual de Londrina - UEL). The fish were then acclimated to the water temperature for 20 min and released into 16 aquariums (5 fish per aquarium) in a recirculation system, where they remained for 15 days for acclimatization before the experiment started. After this period, all the fish were anesthetized with benzocaine (0.1 g L^{-1}), weighed individually (average initial weight = 51.51 ± 0.19 g), and randomly distributed in aquariums. The temperature was maintained constant using a space heater and thermostat in the filter of the recirculation system. The laboratory was maintained under a controlled 12 h light/12 h dark photoperiod. Water parameters, such as temperature (28.05 ± 0.88 °C), dissolved oxygen (6.71 ± 0.45 mg L^{-1}) (Hanna Instruments, Barueri, SP, Brazil), and pH (6.72 ± 0.12) (pH meter Akso, São Leopoldo, RS, Brazil), were measured daily. Ammonia levels were measured three times a week using a colorimetric assay (Labcon Test, Camboriú, SC, Brazil) and remained below toxic levels throughout the experiment.

Experimental design

A completely randomized design consisting of four treatments was used to meet the objectives of this study. Each treatment consisted of four repetitions (60 L aquariums), containing 5 juveniles per repetition, 20 juveniles per treatment, and 80 juveniles in total. The fish were fed in a proportion of 1% of the biomass daily, with 2.6 mm commercial feed (12% moisture, 36% protein, 7% lipids, 5% fiber, and 14% minerals) (Supplementary Table 1), divided into one (T1), two (T2), three (T3), or four (T4) feeds daily. The frequency and time of each

feeding session are presented in Table 1. Feeding schedules were defined according to the activity of the fish throughout the day and photoperiod controls in the laboratory. In addition, logistical and work issues in fish farms were considered, since the feeding of most species, including Oscar, is carried out during the day between the times included in this study. The fish were weighed on day 14 of the experiment to adjust the amount of food provided.

Table 1
Feeding schedule of Oscar (*Astronotus ocellatus*) juveniles in the four treatments

Treatments (Food frequency)	08h30am	11h30am	02h30pm	05h30pm
T1 (1 time/day)			√	
T2 (2 times/day)	√			√
T3 (3 times/day)	√		√	√
T4 (4 times/day)	√	√	√	√

Growth performance and hepatosomatic index

The fish were fasted for 12 h at the end of the experimental period (28 days) and anesthetized (benzocaine 0.1 g L^{-1}) to measure growth parameters. The final weight (g), standard length (cm) (from the previous end of the head to the beginning of caudal fin insertion), total length (cm) (from the previous end of the head to the end of the caudal fin), weight gain (g) (final weight – initial weight), and apparent feed conversion (feed intake/weight gain) were determined (Oliveira et al., 2019; G. A. S. Souza et al., 2017). The condition factor (CF, %) and specific growth rate (SGR) were determined using the following formulas: (%) = [weight of fish (g)/total length of fish $(cm)^3$] × 100 and SGR = {Ln (final weight) - Ln (initial weight)/duration (28 days)} × 100 (Dawood et al., 2015).

The hepatosomatic index (HSI) was calculated using the formula HSI (%) = (weight of liver/weight of fish) × 100 (Dawood et al., 2015). Three anesthetized fish from each aquarium (*n* = 12 per treatment) were euthanized through medullary sectioning to collect and weigh their livers.

Liver histology

Liver fragments previously removed for HSI analysis (from euthanized fish) were collected and stored in 10% buffered formaldehyde for 48 h. The fragments were then inserted into Falcon tubes (15 mL) containing 70% alcohol. The samples were dehydrated with increasing serial concentrations of ethanol, diaphonized, and embedded in paraffin to prepare the slides. Sections 5 µm thick were obtained (Leica RM2265 microtome) and inserted into glass slides, which were subsequently stained with PAS (*Periodic Acid-Schiff*) (McManus, 1948).

The number of hepatocyte nuclei in a 10.000 μm^2 field was counted, according to methods adapted from Figueiredo-Fernandes et al. (2007). The images were captured using a digital camera (Moticam 2500. 5.0 Megapixel/USB 2.0) coupled with an optical microscope (Axiophot Zeiss Axiophot). Nuclei were counted in five different histological sections per sample, totaling 30 measurements per treatment, all standardized with a 100 × objective.

Hematological and biochemical parameters

Blood samples (~0.5 mL) were collected from the caudal vein of previously anesthetized fish to measure growth parameters (*n* = 8 samples per treatment, two fish per aquarium) using plastic syringes (3 mL) containing dipotassium ethylenediaminetetraacetic acid (K2EDTA, Hemstab, Lagoa Santa, MG, Brazil) as an anticoagulant. Then, the collected blood was inserted into plastic microtubes, where 5 μL of blood from each microtube was immediately aliquoted to measure plasma glucose (mg dL^{-1}), determined using the FreeStyle Optium Neo glucose meter (Maidenhead, Berkshire, England). Another aliquot (40 μL) was immediately collected and centrifuged at 10 min at 1400 × g for plasma separation. Plasma was then used to measure lactate concentration (mmol L^{-1}). Analysis was performed using an enzymatic colorimetric assay (Interkit, Belo Horizonte, MG, Brazil) at 540 nm using a Coleman 33D digital spectrophotometer.

The remaining blood was used for hemoglobin determination, erythrocyte count, globular volume, and the calculation of hematimetric indices. The hemoglobin concentration (Hb; g dL^{-1}) was determined using the hemoglobin cyanide method (Collier, 1944) using the commercial Labtest kit (Lagoa Santa, MG, Brazil). Hematocrit (Ht; %) was measured using the microhematocrit method (Ranzani-Paiva et al., 2013), and the red blood cell (RBC; 10^6 μL^{-1}) count was performed using a Neubauer camera after dilution (1:200) of the blood in Dacie's solution (Blaxhall & Daisley, 1973). Subsequently, the following hematimetric indices were calculated: mean corpuscular volume (MCV; fL) and mean corpuscular hemoglobin concentration (MCHC; g dL^{-1}) (Ranzani-Paiva et al., 2013).

Serum samples (3 per aquarium, *n* = 12 per treatment) were obtained after blood collection in 3 mL syringes without anticoagulant and centrifuged for 10 min at 1400 × g for serum separation. Enzymatic colorimetric assays (Analisa, Belo Horizonte, MG, Brazil) were used to measure the albumin (g dL^{-1}), triglyceride (mg dL^{-1}), and cholesterol (mg dL^{-1}) concentrations. Absorbance was determined at 630 nm for albumin, 505 nm for triglycerides, and 500 nm for cholesterol using a Coleman 33D digital spectrophotometer.

Intestinal microbiota

Intestinal content samples were collected from nine fish from treatments T1 and T4 that were euthanized for liver collection. The intestinal contents were expelled after the ventral surface of the abdomen had been opened. Three pools

were prepared per treatment, and each pool consisted of samples from three fish. The samples were removed aseptically from the entire intestinal tract and stored at −80 °C. A commercial QIAamp DNA Stool Mini Kit (QIAGEN, Hilden, Germany) was used to extract bacterial DNA and DNA integrity was evaluated on 1% agarose gels.

The DNA samples were sent to NGS Soluções Genômicas (Piracicaba, SP, Brazil) for sequencing (paired-end library) on an Illumina MiSeq platform. PCR amplification of 16S rRNA was performed using primers for the V3-V4 regions containing adapters for Illumina MiSeq. For this purpose, two PCRs were performed. The first was performed under the following conditions: 95 °C for 3 min; followed by 25 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s; and a final extension at 72 °C for 5 min, to amplify the 16S rRNA V3-V4. A second PCR was subsequently performed with the index sequences under the following conditions: 95 °C for 3 min; followed by 12 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s; and a final extension at 72 °C for 5 min. PCR Bio Ultra Mix (PCR Biosystems, London, United Kingdom) was used for both PCR reactions, and AMPure XP beads (Beckman Coulter, Brea, CA, USA) were used for purification. The samples were grouped into sequencing libraries and sequenced using a MiSeq system and the standard Illumina primers provided in the kit V3 with 250 cycles at each end.

For bioinformatics analyses, Mothur software (v.1.43.0) was used, following the method described by Kozich et al. (2013) and Schloss et al. (2009), with some modifications. First, contigs were assembled based on the 'fastq' files of the 'read1' (forward) and 'read2' (reverse) produced for each sample. Sequences containing ambiguous base pairs were removed and aligned using the SILVA 16S rRNA reference database (Quast et al., 2013). Homopolymers, redundancies, and nonspecific amplicons were removed. The VSEARCH algorithm was used to remove the chimeras (Rognes et al., 2016). The sequences were classified into operational taxonomic units (OTUs) for taxonomic comparison, and a subsample was used to normalize the sequence numbers. Therefore, a rarefaction curve was generated, and the number of observed OTUs (Sobs), Chao richness, and Shannon and Simpson indices were calculated. Analysis of molecular variance (AMOVA) was used to determine the significance of clustering between the groups.

Statistical analysis

Statistical analyses were performed using R statistical software (R Core Team [R], 2017). For the analysis of growth performance, hematological and biochemical parameters, and liver histology, analysis of variance (ANOVA) was performed, followed by Tukey's test if significant differences ($p < 0.05$) were found. Analyses that did not meet the assumptions of residual normality or homogeneity were subjected to nonparametric Kruskal–Wallis analysis followed by Dunn's test. An independent samples t-test was performed for the metagenomic data (Sobs, Chao richness, Shannon, and Simpson indices).

Results and Discussion

Growth performance, hepatosomatic index, and liver histology

The mean values of final weight, weight gain, total and standard length, and SGR were numerically higher in T2 but without significant differences among the treatments ($p > 0.05$) (Table 2). This treatment resulted in the lowest feed conversion values, with no significant differences ($p > 0.05$). The CF ranged from 2.28 (T1 and T2) to 2.34 (T4) ($p > 0.05$; Table 2).

Table 2
Growth performance and hepatosomatic index of Oscar juveniles (*Astronotus ocellatus*) subjected to different feeding frequencies

Treatment	T1	T2	T3	T4
Final Weight (g)	56.24±1.04	58.20±1.49	57.69±1.24	56.55±1.00
Weight Gain (g)	4.64±0.33	6.67±0.42	6.45±0.71	4.88±0.46
Total Length (cm)	13.53±0.07	13.61±0.10	13.52±0.09	13.41±0.08
Standard Length (cm)	10.87±0.07	10.96±0.08	10.86±0.07	10.83±0.06
Feed Conversion (g / g)	1.81±0.11	1.26±0.09	1.44±0.16	1.85±0.20
SGR	0.31±0.02	0.43±0.03	0.41±0.04	0.32±0.03
CF (%)	2.28±0.02	2.28±0.02	2.31±0.02	2.34±0.03
HSI (%)	2.98±0.08^{a}	2.91±0.04^{ab}	2.54±0.05^{ab}	2.48b±0.07^{b}

SGR: specific growth rate; CF: condition factor; HSI: hepatosomatic index. Means in the same row with different lowercase superscript letters are significantly different, as determined by Tukey's test ($P < 0.05$).

Studies have shown that feeding frequency can affect fish growth parameters depending on the stage of life, size, species, environmental conditions, and type of feeding. Higher final weight and SGR were found in fish that received two or four meals daily compared to those that received a daily meal in a study evaluating the feeding frequencies of juvenile angelfish (*Pterophyllum scalare*) (0.87 g) fed a commercial diet for 90 days (Kasiri et al., 2011). Larvae of severum cichlid (*Heros severus*) (4.50 mg) that received four daily artemia feeds for 15 days showed greater length, final weight, length gain, and SGR ($p < 0.05$) than those who received two feeds (Abe et al., 2016), whereas larvae of large yellow croaker (*Pseudosciaena crocea*) (4.08 mg) who received eight and twelve daily feeds (formulated diet supplied for 30 days) had a higher SGR ($p < 0.05$) than those that received two or four daily feeds (Xie et al., 2011). When evaluating six feeding frequencies in Dolly Varden char juveniles (*Salvelinus malma*) (9.40 g) for 56 days, Guo et al. (2018) observed greater weight gain ($p < 0.05$) in animals that received commercial feed in four or five meals daily, followed by those that received two, three, or six meals, and less weight gain in fish that received a daily meal. Dwyer et al. (2002) evaluated

four feeding frequencies (once, twice, and four times daily, and two meals every other day) in juvenile yellowtail flounder (*Limanda ferrugínea*) (6.80 g) using a commercial feed for 70 days, and found that, based on food conversion and weight gain, two daily meals is the most ideal for this stage of life. In the last two studies, the highest food frequencies were not the most indicated. According to Liu and Liao (1999), when meal intervals are short, food passes more quickly through the digestive tract, resulting in decreased food efficiency. However, fish fed once daily may not have sufficient nutritional requirements for good somatic development (Biswas et al., 2010) and may show an increase in social dominance behavior (Ribeiro et al., 2012).

Although no significant differences ($p > 0.05$) were found in the growth parameters in the present study, it is important to note that even numerically, treatments with intermediate food frequencies (T2 and T3) resulted in higher values for final weight, weight gain, and SGR than T1 and T4, in addition to better feed conversion. It is also important to highlight that the effects found in juveniles in the aforementioned studies (Dwyer et al., 2002; Guo et al., 2018; Kasiri et al., 2011) were obtained using experimental periods longer than those used in this study. This demonstrates that the experimental period in the present study may have influenced the lack of effects on the growth parameters. Therefore, future studies with longer experimental periods may accentuate the differences in these parameters in juvenile Oscar. In addition, it is important to highlight the advanced life stage of the fish in the present study (juveniles with an average initial weight of 51.51 ± 0.19 g). Normally, fish in more advanced stages of life, as in the present case, require less daily feeding than fish in early stages (larval stage or fry, for example). This was demonstrated in a study with juveniles of blunt snout bream (*Megalobrama amblycephala*) (8.87 g), which showed greater weight gain with three daily meals than with six (Tian et al., 2015) and with yellowtail flounder juveniles (6.80 g) with two daily meals (Dwyer et al., 2002). According to Okomoda et al. (2019), younger fish need to feed more frequently than older fish to achieve maximum growth because they have smaller stomachs. Thus, it is possible that higher feeding frequencies are more suitable for this species in the early stages, such as larvae or fry.

The HSI differed significantly between T1 and T4 ($p < 0.05$), with a higher mean for T1. The HSI in the T2 and T3 treatments did not differ significantly ($p > 0.05$) from each other and in relation to the T1 or T4 treatments (Table 2).

According to Chellapa et al. (1995), HSI is an indirect index of energy reserves in which surplus energy from food is stored in the liver. The increase in HSI in T1 compared with that in T4 ($p < 0.05$) may be related to the greater supply of food in a single period, leading to greater energy deposition that was not consumed or directed to growth. Some species such as clupeids, which are seasonal feeders, can store energy reserves for periods when food is scarce, with feeding intensity being the main factor determining these reserves (Blaxter & Holliday, 1963). In a study by Aderolu et al. (2017), African catfish (*Clarias gariepinus*) fed to satisfaction only for four days and starved for three days subsequently presented a higher HSI ($p < 0.05$) than fish fed 4% of body weight twice daily. De Boeck et al. (2013) showed that

there was a reduction in HSI by almost half of the original level (1.9 to 0.9) in Oscar fish subjected to fasting for 10 to 14 days. This demonstrates that, in this species, liver reserves are highly used in situations of feed restriction. Therefore, in the present study, the accumulation of hepatic energy reserves in T1 (increase in HSI) seems to be related to the physiological characteristics specific to *A. ocellatus*, which can be used as a strategy to overcome adversities such as possible dietary restrictions.

The hepatocyte nuclei count demonstrated higher mean values in the T2 and T4 (30.5 ± 1.02 and 30.07 ± 0.89 per 10^4 µm², respectively) and lower in T1 (25.76 ± 0.79 per 10^4 µm²) treatments (Figure 1). That of treatments T2 and T4 differed significantly from that of T1 ($p < 0.05$), and T3 did not present significant differences compared with that of the other treatments ($p > 0.05$) (Figure 1). Examples of histological sections with varying numbers of hepatocyte nuclei are shown in Figure 2.

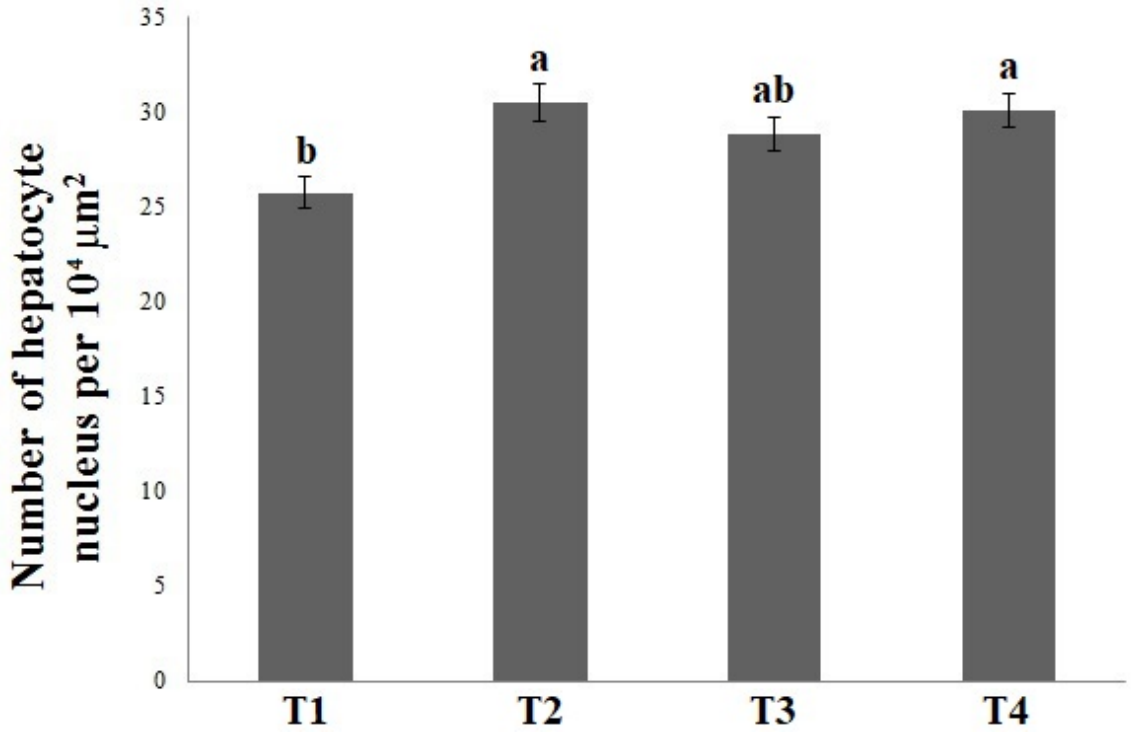

Figure 1. Number of hepatocyte nuclei measured in the hepatic tissue of Oscar (*Astronotus ocellatus*) fed once (T1), two (T2), three (T3), or four (T4) times daily.

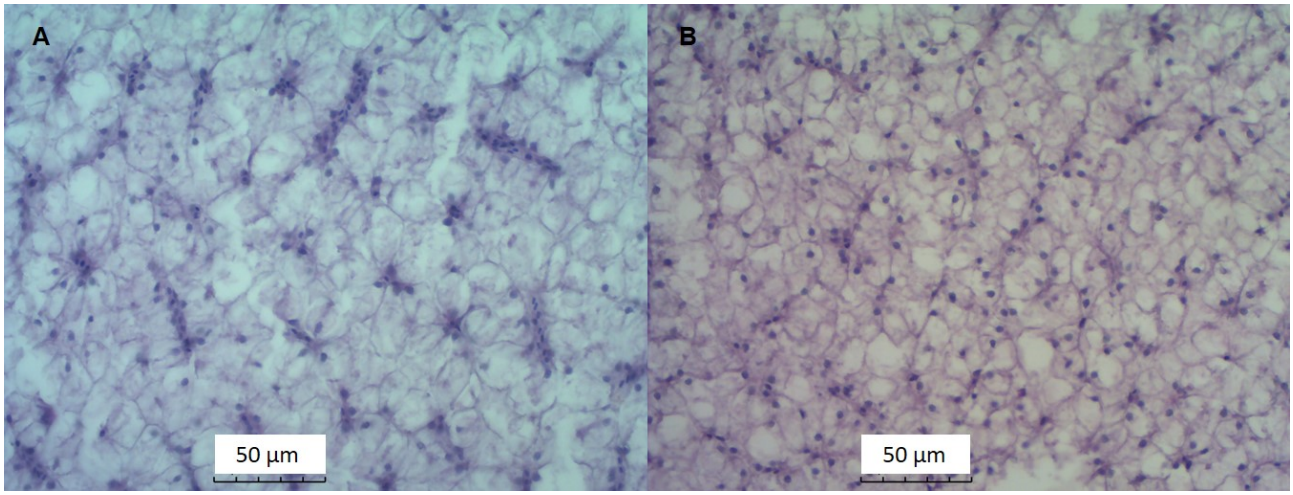

Figure 2. Histological sections of the liver of Oscar (*Astronotus ocellatus*) subjected to different feeding frequencies, demonstrating smaller (T1) (A) and greater number of nuclei per area (T4) (B).

In a study with Nile tilapia analyzed at 17 or 27 °C, Figueiredo-Fernandes et al. (2006) found lower HSI in tilapia subjected to higher temperatures. According to the authors, the lower volume (and mass) of the liver possibly occurred owing to the increased mobilization of energy reserves (glycogen and lipids) in fish at 27 °C. Because the feeding interval was shorter and the food was supplied in smaller quantities in T4, the rapid passage of food through the digestive tract probably resulted in less deposition of energy reserves in the liver, decreasing the HSI. These results were corroborated by the hepatic histology analysis, which demonstrated a lower count of hepatocyte nuclei in T1 than in T4 ($p < 0.05$), indicating an increase in the volume of hepatocytes in the first treatment. This occurred because of the consumption of daily feed in a single meal. This greater accumulation of energy can be used in eventual energy-restricted situations. As already mentioned, the results found by De Boeck et al. (2013) showed that hepatic nutrient reserves in A. ocellatus are highly used in fasted fish. This demonstrates that the accumulation of hepatic reserves is an important mechanism. Thus, as with HSI, the accumulation of hepatic reserves in T1, observed in the liver histological analysis, appears to be related to the characteristics of this species.

Hematological and biochemical parameters

Hematological parameters, including RBC count, Hb, Ht, MCV, and MCHC, did not differ significantly between treatments ($p > 0.05$) (Table 3). Regarding blood biochemical parameters, the plasma glucose, plasma lactate, serum triglycerides, and cholesterol concentrations did not show significant differences between treatments ($p > 0.05$) (Table 3). However, serum albumin levels were affected by different feeding frequencies, showing significantly lower mean values ($p < 0.05$) in T1 than in T2 and T4 (Table 3).

Table 3
Hematological and blood biochemical parameters of Oscar juveniles (*Astronotus ocellatus*) subjected to different feeding frequencies

Treatment	T1	T2	T3	T4
RBC ($\times 10^6$ μL^{-1})	1.70±0.03	1.80±0.04	1.59±0.02	1.60±0.02
Hb (g dL^{-1})	6.37±0.10	6.27±0.10	7.10±0.12	6.68±0.14
Ht (%)	28.12±2.53	25.87±0.51	25.87±0.74	26.37±0.53
MCV (fL)	152.76±13.17	147.88±3.81	167.77±4.30	167.09±3.33
MCHC (g dL^{-1})	23.69±0.92	24.33±0.53	28.07±0.63	25.28±0.36
Albumin (g dL^{-1})	0.64±0.02^{b}	0.80±0.02^{a}	0.75±0.02^{ab}	0.82±0.02^{a}
Glicose (mg dL^{-1})	62.50±2.34	60.38±2.64	59.38±2.16	52.43±0.86
Lactate (mmol L^{-1})	2.40±0.22	2.28±0.26	2.83±0.24	2.72±0.22
Triglycerides (mg dL^{-1})	272.51±11.41	254.91±11.02	304.30±13.62	270.03±13.23
Cholesterol (mg dL^{-1})	96.63±3.19	94.31±1.95	106.39±3.28	109.72±1.99

RBC: red blood cell; Hb: hemoglobin; Ht: hematocrit; MCV: mean corpuscular volume; MCHC: mean corpuscular hemoglobin concentration. Means in the same row with different lowercase superscript letters are significantly different, as determined by Tukey's test ($P < 0.05$).

Proteins are multifunctional in blood plasma, with their main functions related to host defense, blood coagulation, cell metabolism, metabolite transport, nitrogen balance provision for nutrition, and maintenance of oncotic pressure. The latter three functions are associated with albumin (Kaneko et al., 2008). This protein is produced in the cytoplasm of hepatocytes and has the highest serum concentration of all serum proteins (Kaneko et al., 2008; Rothschild et al., 1988). According to Rothschild et al. (1988), albumin is synthesized during periods of adequate nutrition, and decreases in its concentration may be related to malabsorption or decreased protein intake, in addition to other factors such as liver disease or changes in oncotic pressure.

In the present study, the lowest feeding frequency treatment (T1) produced lower serum albumin levels than the other treatments. Protein-calorie malnutrition is a potential cause of decreased albumin synthesis, with a consequent decrease in blood albumin levels (Kaysen et al., 1995). Based on this, fish fed more times daily may have better amino acid and peptide absorption. Consequently, they have a greater substrate supply for albumin synthesis by hepatocytes, increasing their serum values. Other blood parameters evaluated showed that feeding frequency did not significantly influence ($p > 0.05$) erythrocyte responses or glucose and blood lactate plasma concentrations. In addition, the mean values of cholesterol and triglycerides indicated that the number of daily treatments did not influence these parameters at the serum level.

Intestinal microbiota

The metagenomic data generated 818,821 contigs. After quality filtering, 680,422 unique contigs were generated and aligned using SILVA database. In total, 108,458 sequences were used to generate a subsample to normalize the number of sequences per sample. A coverage greater than 99.9% in all samples indicated a high representativeness of the total microbial population. The number of strings (after the subsample) relative to the number of OTUs in each sample is represented by the rarefaction curve shown in Figure 3.

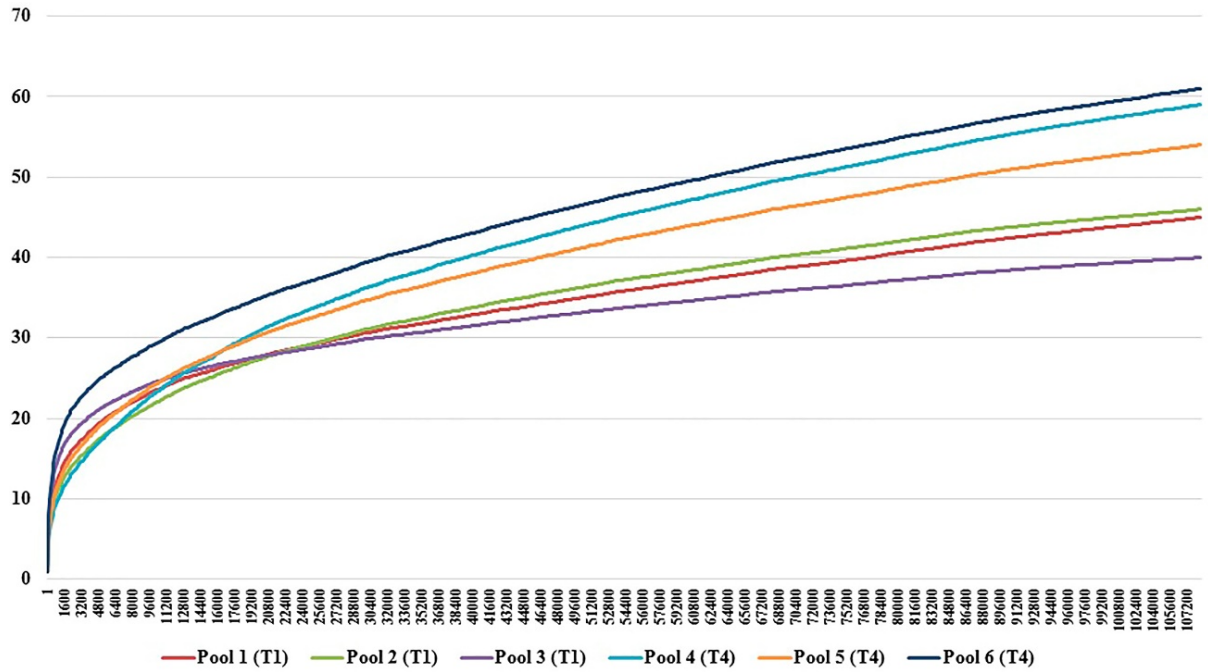

Figure 3. Rarefaction curve of each pool sample showing the number of reads (x-axis) in relation to the number of OTUs (y-axis). T1 group: Pools 1, 2, and 3; T4 group: Pools 4, 5, and 6.

In total, 127 OTUs were identified, which were distributed across 12 phyla (7 in T1 and 12 in T4), 24 classes (16 in T1 and 23 in T4), 43 orders (26 in T1 and 41 in T4), and 83 families (48 in T1 and 74 in T4). The relative abundances at the phylum and genus levels in both treatments are shown in Figure 4. The phylum Fusobacteria was the most abundant in both treatments, followed by Proteobacteria and Firmicutes (Figure 4A). At the genus level, *Cetobacterium, Aeromonas, Romboutsia,* and *Clostridium_sensu_stricto,* in that order, were the most abundant genera in T1. In T4, *Cetobacterium* and *Aeromonas* were the most abundant, followed by *Clostridium_sensu_stricto* and *Romboutsia,* in that order (Figure 4B).

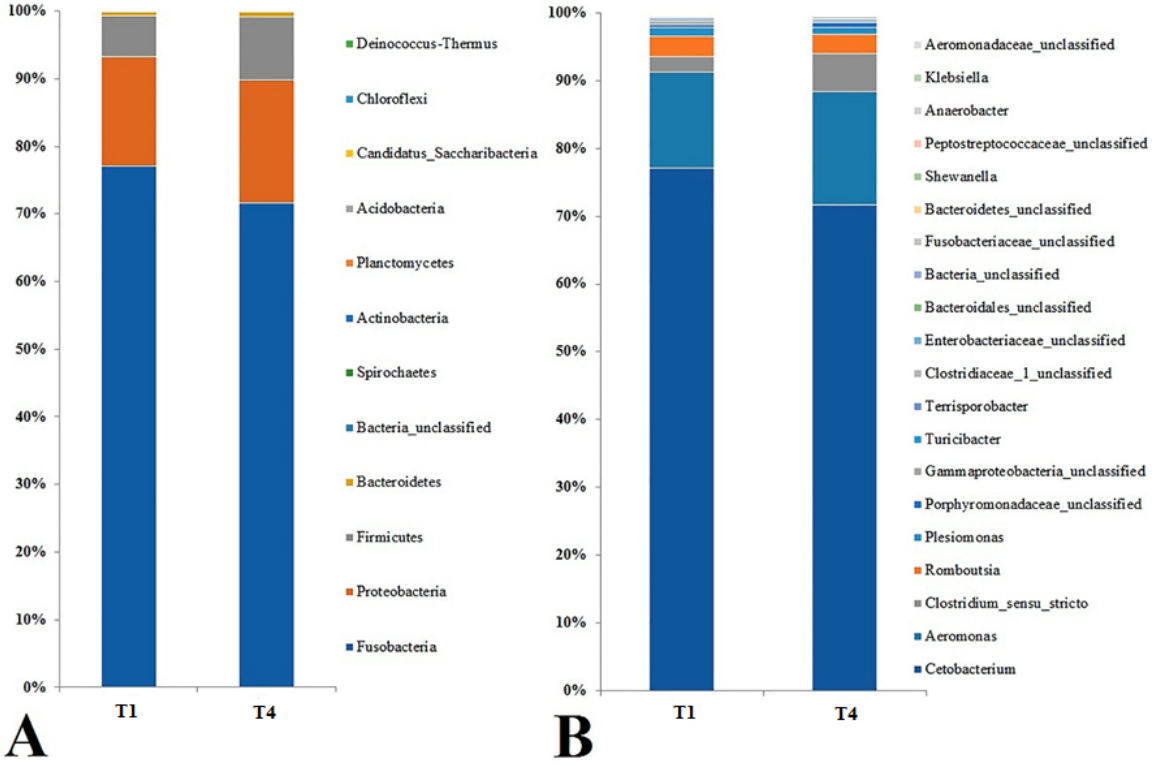

Figure 4. Mean relative abundance of different OTUs comprising the gut bacterial communities of Oscar. The phyla (A) and 20 most abundant genera (B) are shown.

The number of observed OTUs (Sobs), Chao richness, and Shannon and Simpson indices (alpha diversity) are shown in Table 4. A significant difference ($p < 0.05$) was observed in the average Sobs, with a higher value in T4 (58.00 ± 2.08). In addition, there was an increasing trend in the mean Chao richness values in T4 ($p = 0.10$). The means of the Shannon and Simpson indices were not significantly different between treatments ($p > 0.05$) (Table 4).

Table 4
Number of observed OTUs (Sobs), Chao Richness, Shannon, and Simpson diversity indices (mean ± standard error) of the gut microbiota of Oscars fed once (T1) and four times (T4) daily

Index	T1	T4
Sobs	43.67±1.86[b]	58.00±2.08[a]
Chao Richness	58.63±8.74	83.50±8.41
Shannon	0.84±0.09	0.83±0.28
Simpson	0.62±0.06	0.64±0.15

Different letters indicate significant differences ($P < 0.05$) between treatments.

When comparing the phyla and bacterial genera of the intestinal microbiota of Oscar with another cichlid species, the Nile tilapia (*Oreochromis niloticus*), the maintenance and dominance of the phyla Fusobacteria, Proteobacteria, and Firmicutes occurred in both fish species (Fan et al., 2017; F. P. Souza et al., 2020a,b, Zhang et al., 2016). The most abundant genera, such as *Cetobacterium, Romboutsia, Pseudomonas* (F. P. Souza et al., 2020a; Suphoronski et al., 2019), *Clostridium* (Fan et al., 2017), *Aeromonas,* and members of the *Porphyromonadaceae* family (Zhang et al., 2016), were also abundantly identified in the intestinal microbiota of tilapia. The dominance of these taxa indicates that part of the "core" microbiota is maintained among these cichlids. It is important to note that the individuals in the studies cited, as well as in the present study, were fed artificial diets (commercial rations); however, Oscars in natural habitats are generally carnivores, feeding on live prey such as small fish, insects, crayfish, and earthworms (Froese & Pauly, 2019).

The results of the microbiota analysis indicated that adaptation to commercial diets possibly provides the microbiota the capacity to capture nutrients from artificial food, resulting in a similar bacterial community to species highly adapted to commercial diets, such as tilapia. The influence of habitat has already been demonstrated in a study that evaluated the intestinal microbiota of Mozambique tilapia (*Oreochromis mossambicus*) in a natural lake in India. Gaikwad et al. (2017) observed the dominance of the phyla Actinobacteria, Cyanobacteria, and Planctomycetes, whereas tilapia captured in the Mula River (India) showed a dominance of Fusobacteria and Proteobacteria. The authors showed that the divergent microbiota resulted from environmental and dietary factors, suggesting the ability to acquire bacteria from different habitats, which could help the host survive in diverse environments. Similar to in tilapia, the plasticity of intestinal microbiota in Oscar could give the host the capacity to use nutrients from various food sources. This is an important characteristic for aquaculture and aquarists. Future studies on Oscars in natural habitats may provide new information on the intestinal microbiota of this species.

Although the Shannon and Simpson indices did not show significant differences between treatments ($p > 0.05$), an increase in food frequency promoted an increase in Sobs ($p < 0.05$) and an increasing trend ($p = 0.10$) in Chao richness. The last parameter gives more weight to low-abundance species, considering singletons and doubletons (species that appear once or twice in the samples, respectively) (Kim et al., 2017). In addition, the rarefaction curve, which represents species richness against the number of sequences (species density) (Dias & Bonaldo, 2012), reinforced these results by demonstrating the different formats of the T1 and T4 sample curves (Figure 3).

Microbial competition for metabolic energy access is intense and constant within the intestine; for this, bacteria have several specialized enzymes, such as glycoside hydrolases and polysaccharide lyases, to break complex bonds between carbohydrates and make them consumable (Martens et al., 2011; Sonnenburg & Sonnenburg, 2014).

In addition, non-digestible fibers can be fermented to produce short-chain fatty acids, which are then captured and sent to the liver to modulate lipid production (Bell et al., 1999). Thus, the composition, richness, and genetic characteristics of bacterial communities influence various metabolic pathways in the host, which can affect the productive performance, immune responses, and responses to stress. For example, weight gain can benefit from an increase in glucose absorption in the intestine through the extraction of energy from non-digestible sources by the host and the lipogenic substrate supply (Cani & Delzenne, 2009).

It is possible that the more frequent food supply (T4) in the present study favored the maintenance of certain bacterial taxa and, consequently, increased richness by providing substrates (energy sources) for the bacteria constantly throughout the day. However, we could not relate such changes in microbiota richness to differences in biochemical or physiological parameters (HSI) between these treatments. However, richer and more diverse bacterial microflora can competitively exclude pathogenic bacteria by searching for nutrients or by occupying bacterial-binding receptors in the gut (Jung-Schroers et al., 2016). Investigations of the functional microbiota through transcriptomic analyses and assessments of the metabolic processes produced by these bacteria, could clarify detailed mechanisms of energy use by different bacterial genera and demonstrate more precisely how microbiota could influence the blood parameters and physiological factors evaluated in the present study.

Conclusions

We concluded that feeding frequencies of two, three, and four times daily were the most suitable for juvenile Oscars since the frequency of feeding once daily resulted in a higher hepatosomatic index compared to feeding four times daily. Feeding once daily also resulted in lower albumin levels and greater deposition of energy reserves in the liver tissue (indicated by the hepatocyte nuclei count) compared to feeding two and four times daily. Furthermore, feeding frequency significantly influenced microbiota richness, presenting higher values at four times daily than once daily.

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