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Ostracoda impairs growth and survival of *Arapaima gigas* larvae

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ABSTRACT

The practice of indoor larviculture can be an alternative to enhance the rearing of arapaima larvae, improving their survival and the supply of arapaima juveniles for the grow-out stage. We aimed to evaluate different live microcrustaceans as feed for arapaima larvae to verify groups that can be safely used in laboratory trials, as well as in commercial larviculture. Arapaima larvae (39.45 ± 7.04 mg and 2.09 ± 0.13 cm) were housed in 12-L circular tanks (n = 4 tanks; 30 larvae per tank) in a static water system. The tanks were cleaned twice a day, when 50% of the water was changed. Fish were fed three types of live feed 9 times/day: brine shrimp *Artemia* sp. nauplii (AR), Cladocera-rich zooplankton (CZ), and Ostracoda-rich zooplankton (OZ) for 15 days. Fish fed CZ and AR showed a similar fast growth rate with low mortality rates. The OZ fed Arapaima larvae presented intact Ostracoda in their rectums. This findins, demonstrates their poor digestibility, which resulted in poor growth and low survival. Ostracoda-rich zooplankton must be avoided in arapaima larviculture until 8 days after they begin swimming to the water surface or 17 days after hatching.

1. Introduction

Arapaima gigas (Schinz 1822) (Osteoglossiformes: Arapaimidae) is known as the giant of the Amazon because it can reach sizes of 3 m in total length and up to 200 kg in weight (Ferraris, 2003). Arapaima is one of the main neotropical species with a large interest has been shown by South American aquaculture in its fast growth (around 10 kg per year) (Mattos et al. 2016), its approximate 48% fillet yield (Fogaça et al., 2011), and its boneless flesh and high nutritional quality (Cortegano et al., 2017).

Arapaima farming has grown by at least 800% in Brazil and Peru in the last 7 years (IBGE, 2016; Alvan-Aguilar et al., 2016), and it has already been introduced in the United States (Lawson et al., 2015), China, Cuba, Mexico, the Philippines, Singapore, and Thailand (FAO 2016). Despite growing interest, very few data are available on early stages of arapaima, and fish farms record low survival rates resulting in poor supply and high market value for arapaima juveniles in Brazil and Peru (Chu-Koo et al., 2017, 2017; Lima et al., 2017).

Arapaima reproduction occurs naturally in ponds of fish farms. Fish farmers usually keep arapaima larvae together with their parents until they are seven centimeters in total length (Halverson, 2013; Pereira-Filho and Roubach, 2010). However, this practice results in high mortality rates due to lack of sufficient live feed, changes in water quality, and the presence of predators (aquatic insects, birds, and bats) and pathogens. Collecting of arapaima larvae when they start swim to the water surface with their parents and the practice of indoor larviculture can be alternatives to improve the care of these animals, increasing their survival and the supply of arapaima juveniles for the grow-out stage.

Live feed is indispensable for the successful larviculture of many marine and freshwater fish species (Conceição et al., 2010; Sales, 2011). Arapaima larvae are no exception until weaning size. Natural zooplankton is the usual prey for arapaima larvae and the main feed item used at commercial hatcheries for at least the first 2 weeks, before larvae are weaned into inert feeds. Cladocera, Copepoda and Ostracoda are widely distributed organisms in the world (He et al., 2001; Martens

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et al., 2008), that are normally found in fish and prawn farm ponds (Shil et al., 2013). Cladocerans are abundant zooplankton found in fresh water ponds, with strong energy transfer and nutrient cycling components in the aquatic ecosystem, and are an extremely common live feed source for larvae in fish culture. With parthenogenetic reproduction, cladocerans present a greater potential for production than copepods that use sexual reproduction (Le Cren & Lowe-McConnell, 1980). Genera like Daphnia, Moina, and Diaphanosoma are largely studied due to their high nutritional value for fish larvae, usually providing 50% of the dry weight in protein and 20–27% as total fat (Rottmann et al., 2003). Additionally, the amino acid content is rich in lysine (10.7%) (Ovie and Ovie, 2006) and shows higher methionine content then Artemia and rotifers, as well as highly unsaturated fatty acids like eicosapentaenoic acid (12.7%) against 2.1% and 1.9% in Artemia and rotifer, respectively (Tong et al., 1988). Brine shrimp, *Artemia* sp., are widely used as live feed for aquaculture due to the standardized cost-effective protocols for its mass production (Conceição et al., 2010). However, Artemia have an incomplete composition for marine larvae, especially in the high unsaturated fatty acid content (Chakraborty et al., 2007). Artemia short life span when used in freshwater species makes it difficult to apply techniques like nauplii enrichment after instar I phase. Copepods are a dominant group of zooplankton and constitute a large part of the fish larval diet in the natural pelagic food chain. The protein content of various copepods varies between 52.4% and 57.6% of dry weight but is higher than Artemia at 41% in newly hatched nauplii and 34% after 24-h enrichment (Evjemo et al., 2003). There are no records of Ostracoda use in aquaculture, however they are on average 10 to 15 times more abundant at fish farms than at nature. (Fernandez-Jover et al., 2016). Ostracods differ from copepods, cladocerans, Artemia, and they cause several fish production issues, such as clogging screens and equipment given their small size and large numbers (Anderson and Tave, 1993). The natural zooplankton used in arapaima hatcheries present low diversity, with mostly Cladocera (78.7%), Copepoda (11.1%) and Ostracoda (10.2%), due to the use of a plankton net with a nominal sieve opening of 200 μm, used to classify zooplankton before offering larvae, relating to the size used for water exchange in the indoor laboratory (Alcantara et al., 2018).

Since arapaima ponds are not ordinarily fertilized, zooplankton is collected from the earth ponds used for other fish farming species such as *Colossoma macropomum*. Fish farmers follow the same methods of pond preparation in all the five countries where *C. macropomum* are primarily raised (Campos-Baca and Kohler 2005), and these methods barely changed with time (Costa et al. 2016). The ponds are cleared and exposed to the sun for approximately 2 days, disinfected using calcium oxide, then fertilized with organic or inorganic fertilizer to stimulate plankton production. Currently, no established protocol for arapaima intensive larviculture exists. Generally, the offspring remain under parental care until they reach sizes larger than 5 cm and then are trained to receive commercial diets in indoor labs, or to a lesser extent, in outdoor earth ponds (Chu-Koo et al. 2017). In this context, the level of relevance of live feed compared to commercial diets for aquaculture is related to their swimming movements in the water column, small size, and high moisture content (> 80%) (Conceição et al., 2010). These characteristics offer greater attractiveness, the capacity of ingestion and palatability to small fish, such as its larvae, and difficulty to replicate in commercial diets.

We aimed to evaluate several live microcrustaceans as feed for arapaima larviculture to verify which groups do not adversely impact the growth or survival of arapaima larvae that can be safely used in both laboratory trials and in commercial larviculture.

2. Material and methods

2.1. Feeding trial conditions

This study was approved by the Ethics Committee of Animal Experimentation and Research of the Instituto Nacional de Pesquisas da Amazônia (INPA), Manaus, Amazonas, Brazil (Protocol Number 016/2016). The terminology adopted in the present study to define larva follows that of Kendall et al. (1984).

Arapaima gigas larvae were obtained from natural spawning in a breeding pond on a commercial fish farm (Piscigranja Boa Esperança, Rondônia, Brazil). Larvae post-hatched were collected once they began swimming to the water surface together with the breeding male, and subsequently were transported to the Fish Farming Station of INPA. Larvae were collected from a broodstock couple in a 1500 m^2 earth pond when they started to swim to the water surface together with the breeding male. A cone-shaped fish net with cloth mesh size of 0.5 mm fixed at a 2 m long metal rod were used to collect the larvae from a single spawn of approximately 12,000 larvae. All the larvae were collected to stimulate breeders in order to initiate new spawning. As far as we know, all records of arapaima oocyte or fertilized egg came from incidental capture. Thus available data are not chronologically accurate and the biological events of larvae surface swimming, total length, and complete absorption of the yolk sac on the collection day correspond to approximately 5–7 days after hatching (Ruiz Tafur et al. 2017). Two days were allowed to elapse between larvae collection and the beginning of the experiment. The wet weight of arapaima larvae was 39.45 ± 7.04 mg and the total length was 2.09 ± 0.13 cm, corresponding to 9 days after hatching (estimate based on growth data from Ruiz Tafur et al. 2017). Larvae were housed in 12-L circular tanks (30 larvae per tank) with a static water system in a completely randomized experimental design (n = 4 tanks). All tanks were cleaned twice a day (6,00, 17:00 h), at which time 50% of the water of each tank was changed. Tanks were wiped with a sponge to remove any material that stuck to the walls. Any material that had fallen to the bottom (feces and uneaten plankton) was siphoned with a plastic pipe and a net to avoid accidentally capturing larvae.

Water used in the experimental tanks came from a water recirculation system, with phytoremediation. The system consisted of a 160 m^3 pond with a soil bottom and cement walls and phytoremediation with *Eichhornia crassipes* and *Pistia stratiotes*. Well water replaced water lost to evaporation in the pond. One hour prior to the water exchange in the experimental tanks, water was collected by pumping and filtered through a 50 μm mesh before begin aerated until use. The water quality variables that were monitored daily included temperature (27.9 ± 0.02 °C) and dissolved oxygen (5.52 ± 0.87 mg L^{-1}) by a YSI 550A multiparameter (YSI Inc./Xylem Inc.), pH (7.21 ± 0.07) measured using Pro10 pH (YSI Inc./Xylem Inc.), and un-ionized ammonia NH_3 (0.014 ± 0.009 mg L^{-1}) measured with a CD 1570 Colorimetric kit (Alfakit Ltda). Nitrite (0.18 ± 0.01 mg L^{-1}), alkalinity (36.29 ± 7.16 mg L^{-1} $CaCO_3$), and hardness (28.23 ± 8.57 mg L^{-1} de $CaCO_3$) were measured 2 times per week. Water parameters remained within the comfort range for arapaima (Cavero et al., 2003; Chu-Koo et al., 2017). The indoor photoperiod was 12 h light 12 h darkness, simulating the natural conditions of the region.

Fish were fed 9 times per day, every 2 h (8:00, 10:00, 12:00, 14:00, 16:00, 18:00, 20:00, 22:00, and 00:00 h) with 3 types of live feed: shrimp brine *Artemia* sp. nauplii (AR), Cladocera-rich zooplankton (CZ) and Ostracoda-rich zooplankton (OZ), for 15 days. On day 1 of the experiment, fish were fed with a mean proportion of 1:1.350 (1.350 organisms of live feed per meal per larva). On the last day, the fish were fed with a mean proportion of 1:5.853 (Fig. 1).

For counting purpouses, samples were analyzed in 1 mL in the Sedgewick-Rafter chamber for nauplii under a microscope and in 5 mL in acrylic cuvettes for microcrustaceans under a stereo microscope. The term organisms were chosen to represent all individuals, irrespectively

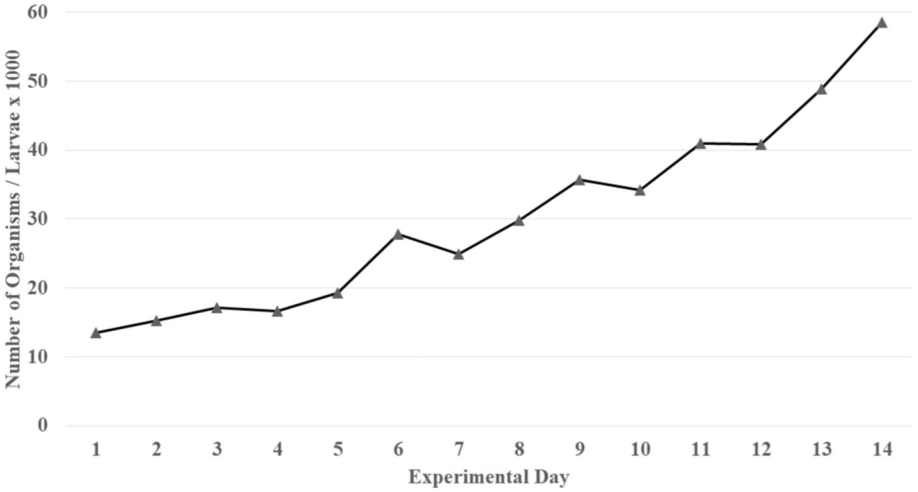

Fig. 1. Daily feeding rate offered to *Arapaima gigas* larvae in each diet.

of taxon, which were counted and determined after each collection. Collection was carried out, the collected plankton concentrated in a bucket with aeration. From that volume density was counted to feed larvae. No qualitative classification took place during the experiment, but later in the laboratory as described in the next section. Brine shrimp *Artemia* sp. nauplii (High5 cysts, INVE) were not enriched as they were offered to the larvae from 2 up to 6 h after hatch still in the instar I phase (without open mouth) from 400 to 500 µm in length. Natural zooplankton was collected using a plankton net (200 µm) from two large earthen ponds. Daily sampling of live feed was performed and fixed (10% formalin) for qualitative analysis of natural zooplankton organisms.

All the fish were weighed and measured on days 4, and 8, and on the last day of experimental period. The performance parameters were evaluated according to the following formulas (NRC, 2011): survival rate (SR) = (final number of fish × 100)/initial number of fish; weight gain (WG) = final weight (g) – initial weight (g); relative growth rate (RGR) = (eg^{-1}) × 100, e: Euler's number, where: g = (ln(final weight) –ln(initial weight))/(total length of the assay period). The mortality rate (MR) was obtained by taking the mean of the experimental units of each treatment within each sampling interval, where: MR = total dead fish until each sampling / number of fish at the beginning of the experiment.

After performing the biometric analysis of each sampling, six larvae (2 larvae per tank) per treatment were euthanized using a lethal dose of eugenol (100 mg.L^{-1}, Biodinâmica Química e Farmacêutica LTDA, Paraná, Brazil) and fixed in 10% formaldehyde to further identify zooplankton. Nine larvae (3 larvae per tank) per treatment were euthanized in eugenol solution and fixed in Karnovsky's solution for the histological analysis to evaluate the digestion of the consumed food. This sampling was conducted 2 h after a meal.

2.2. Qualitative analysis of zooplankton organisms

The natural zooplankton samples were placed in a 250 mL volumetric flask coupled to a Stempel pipette. The sample was homogenized and an aliquot of 2.5 mL was withdrawn. This aliquot was placed in a mm Petri dish and microcrustaceans were quantified per class (Ostracoda, Copepoda, and Cladocera) (Ruppert and Barnes, 1996) under a stereomicroscope (Wild M3C) and by consulting the specialized bibliography for Cladocera (Elmoor-Loureiro, 1997) and Copepoda (Santos-Silva, 2000). The percentage of each class in the sample was calculated and the natural zooplankton treatments were named according to the most abundant group of its composition (Fig. 2).

2.3. Fish histological analysis

The histological analysis was performed in the Thematic Laboratory of Optical and Electronic Microscopy of the INPA. Larvae were infiltrated in paraffin. Longitudinal sections of 3 µm were obtained by a semi-automatic microtome (LEICA RM 2245), then mounted on permanent slides, and stained with Hematoxylin and Eosin (HE). The larvae rectums were evaluated under a microscope (Axio Lab A1-Zeiss), which was coupled to a scientific camera (AxioCam ERc5s) to verify the micro-crustacean's integrity and the digestive capacity of arapaima larvae using the image analyzer software Zen 2 lite.

2.4. Statistical analysis

The final total length, wet weight, final wet weight, wet weight gain, and relative growth rate were analyzed by a one-way ANOVA. The treatments that presented differences ($p < .05$) were compared by Tukey's Unequal N HSD test ($p < .05$). Mortality frequency was analyzed by the Kruskal-Wallis and Student-Newman-Keuls tests ($p < .05$).

3. Results

Arapaima larvae fed AR were longer in length (4.60 ± 0.18 cm) than the CZ and OZ fed larvae, which is 1 cm shorter on average (Table 1). AR and CZ had a similar final weight and weight gain, increasing by > 11-fold over a 15 days period (Fig. 3). However, OZ showed lower values (221.13 ± 35.91 mg and 176.14 ± 35.91 mg, respectively). The RGR was similar for all treatments, which could be due to the wide variation seen in the standard deviation values.

On days 4 and 8, the distal intestines of the larvae fed OZ were full of intact Ostracoda microcrustaceans, which suggests a poor digestion capacity of OZ organisms (Fig. 4G and H). However, on day 15, the distal intestine of the arapaima larvae fed OZ showed a crumbled mass (Fig. 4I), as did the fish fed AR and CZ on days 4, 8, and 15. Between the first and second biometric evaluations, several cases of intestinal evisceration were observed only in the OZ treatment (Fig. 5).

At the end of the experiment, the lowest survival rate and its widest variation were observed in OZ (40 ± 5.63%) (Table1). However, the mortality rate in OZ was marked, until day 8 when it reached 55%. The mortality rate of fish fed with CZ and AR remained below 5% throughout the experimental period (Fig. 6).

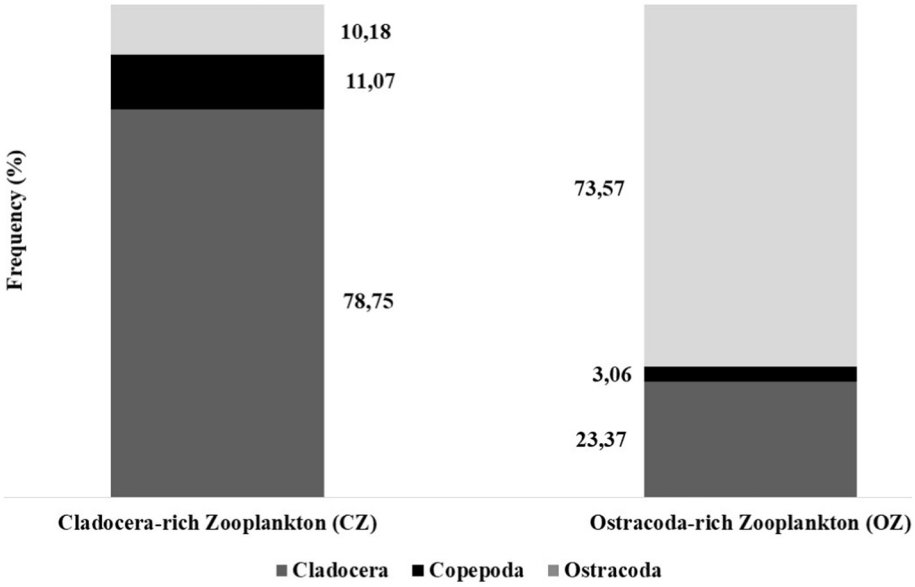

Fig. 2. Qualitative composition of wild zooplankton as live feed for *Arapaima gigas* larvae.

4. Discussion

CZ and AR were able to meet the nutritional requirements of early arapaima larvae, with a similar fast growth rate in 15 days with low mortality rates. OZ were well ingested by arapaima larvae, although these fish presented poor growth and low survival. The presence of intact Ostracoda in arapaima rectum demonstrated the poor digestibility of these microcrustaceans due to the calcified carapace surrounding Ostracoda's body (Martens et al., 2008). Their rigid carapace impairs digestion, by preventing crushing and access to the arapaima's digestive enzyme to dissolve the Ostracoda's soft body, at least until day 8 of the experiment, which corresponds to 17 days after hatching (this estimate was based on the growth data of Chu-Koo et al., 2017). Mack and Andraso (2015) have also described problems with ostracod digestion in round goby (*Neogobius melanostomus*), such as the hard-bodied ostracod survived passage through the gut with 16.6% alive in the feces.

The poor digestibility of OZ contributed to the high mortality rate of arapaima larvae and the presence of intestinal evisceration after herniation-like injuries especially during the first days of the experiment. Ostracoda feeds mainly on organic and dead material, acts as predator in some cases (Vannier et al., 1998). This provides them with an advantage of surviving and multiplying in ponds of fish farms, especially in the rainfall season when there is an impairment in the phytoplankton production and consequently in the other groups of zooplankton that require microalgae as feed. The rainfall season coincides with the season of neotropical species larviculture, and high mortality rates have been reported by fish farmers when high Ostracoda concentration are noted in Brazilian.

We recently observed (Alcântara et al., 2018) no significant changes in the morphology of digestive tract of the arapaima larvae from 60 to 500 mg except for the gastric glands. Following fish development, gastric glands increased in concentration (histochemical assay), as did the thickness of the stomach muscular layer. However, from day 8 to day 15, the mortality rate decreased in the larvae fed with OZ. With this the presence of whole ostracods in the intestine suggests that prior to 8 DAH, arapaima larvae have neither the mechanical ability to crush Ostracoda carapaces nor a set of enzymes to digest them. At this time larvae presented a mature gastrointestinal tract with digestive enzyme activity, capable of digesting the crushed Ostracoda carapace, which is observed as a digested mass inside distal intestine of arapaima larvae.

As Cladocera, Ostracoda, and Artemia are filtering microcrustaceans, that can be enriched with amino acids, fatty acids, and vitamins to guarantee nutritional value. Artemia nauplii can be used as live feed in arapaima larviculture without harming arapaima's growth or survival, and the use of Artemia nauplii can be practical thanks to standardized protocols existing for their mass production. Arapaima larvae start exogenous feeding at 20 mm total length (Ruiz-Tafur et al., 2017), 40 mg wet weight, and grow 0.25 mm^{-day} (Alcantara et al., 2018). The biomass gain obtained in this experiment is twice that obtained with cobia, *Rachycentron canadum*, a species that is also carnivorous, in a recirculating raceway system (Faulk et al., 2007). This means a large amount of live food for the maintenance of these individuals during the larval period, showing the relevance of these food

Table 1
Growth performance of *Arapaima gigas* larvae fed different live feed for 15 days of culture.

Variables	Live feed			*P* value
	AR	CZ	OZ	
FL (cm)	4.60 ± 0.18[a]	4.44 ± 0.10[b]	3.40 ± 0.18[c]	0.0001
FW (mg)	527.12 ± 68.39[a]	530.41 ± 64.25[a]	221.13 ± 35.91[b]	0.0005
WG (mg)	482.13 ± 68.39[a]	485.42 ± 64.25[a]	176.14 ± 35.91[b]	0.0006
RGR (%/day)	36.39 ± 11.92[a]	35.96 ± 10.31[a]	21.02 ± 8.50[a]	0.1669
SR (%)	95.83 ± 1.67[a]	96.67 ± 4.71[a]	40.00 ± 15.63[b]	0.0190

Initial weight: 39.45 ± 7.04 mg; Initial length: 2.09 ± 0.13 cm; AR: Artemia sp. nauplii; CZ: Cladocera-rich Zooplankton; OZ: Ostracoda-rich Zooplankton;FL:final length; FW:final weight; WG:weight gain; RGR: relative growth rate; SR: survival rate; ANOVA and Tukey's Unequal N HSD Test; Kruskal-Wallis and Median Test. Different letters in the same row indicate differences ($P < .05$) between groups.

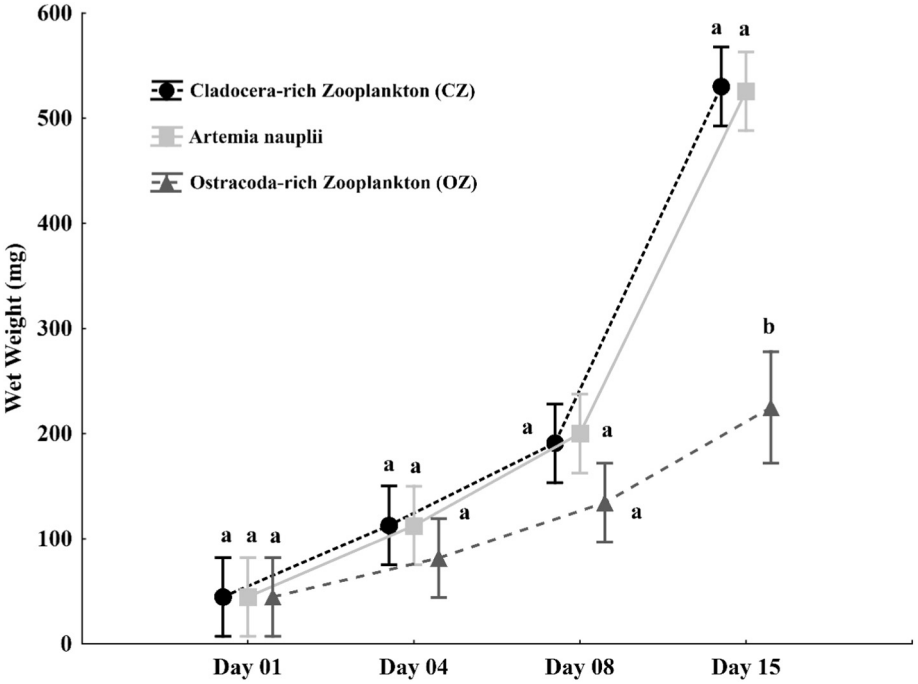

Fig. 3. Wet weight of *Arapaima gigas* larvae fed with different live feed during 15 days of culture. Error bars: 95% confidence intervals.

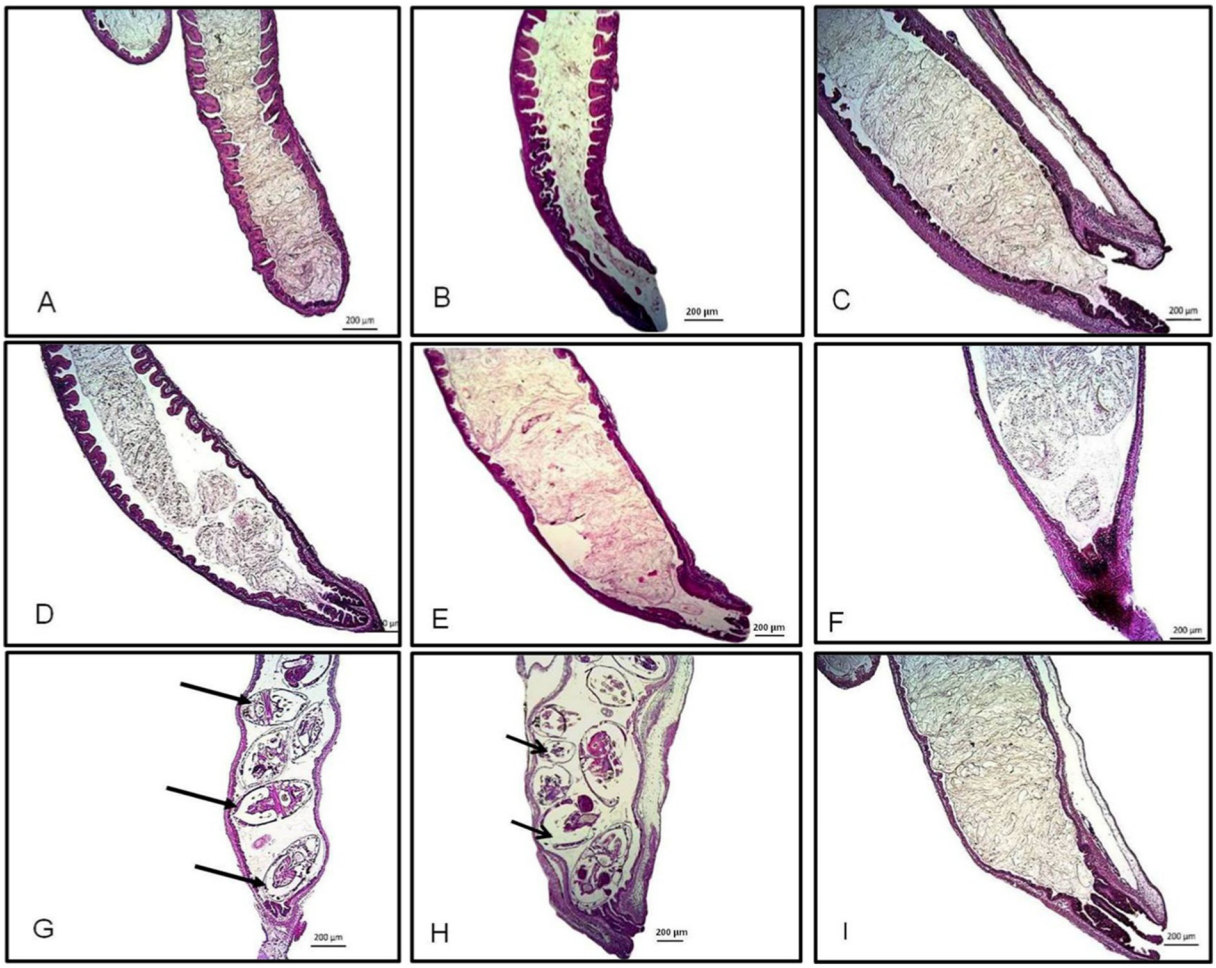

Fig. 4. A. B. C. Rectum of arapaima larva fed artemia nauplii at day 4, 8 and 15; D. E. F. Rectum of arapaima larva fed cladocera-rich zooplankton at day 4, 8 and 15. G. H. Rectum of arapaima larvae fed ostracoda-rich zooplankton with intact ostracoda (arrows) at day 4 and 8, respectively; I. Rectum of arapaima larvae fed with ostracoda-rich zooplankton at day 15, with a digested mass.

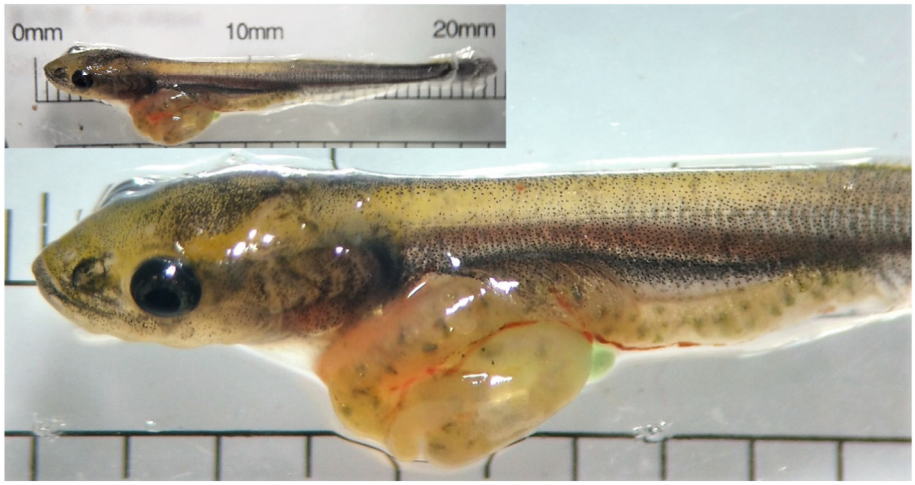

Fig. 5. Intestinal evisceration injury in *Arapaima gigas* larvae fed Ostracoda-rich zooplankton diet (73.57%) for 4 days.

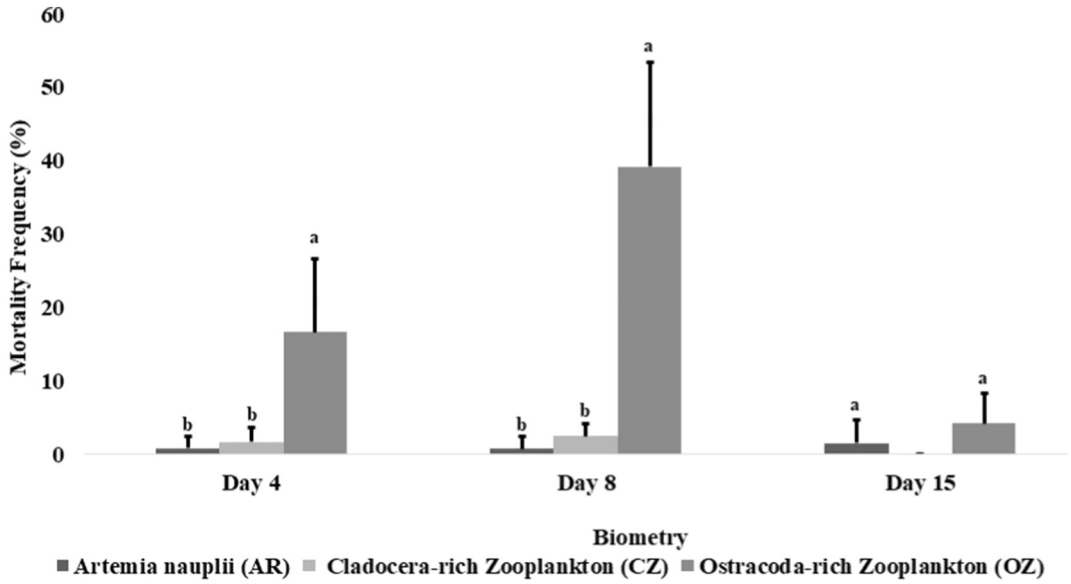

Fig. 6. Mortality rate of *Arapaima gigas* larvae fed with different sources of live feed.

sources. This means that, the volume of live food required for the technical and economic viability of arapaima larvae could be challenging and still need to be evaluated.

Our results support further studies conducted on arapaima early weaning during the co-feeding period. Although Artemia nauplii and Cladocera rich zooplankton are suitable for feeding arapaima larvae, it is necessary to evaluate larvae at the earliest age to be weaned to commercial diet.

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