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ORIGINAL ARTICLE

Pirarucu larviculture in green water provides heavier fish and modulates locomotor activity

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ABSTRACT

The green water technique uses microalgae in the water of indoor larviculture, providing a darker environment to favor fish growth, welfare and health. We evaluated growth performance and locomotor activity after light exposure of pirarucu (*Arapaima gigas*) larvae reared in green or clear water. During one test, pirarucu larvae (3.6 ± 0.3 cm; 0.36 ± 0.1 g) were reared in 50-L circular tanks (n = 3 per treatment, 50 larvae per tank) in a static system containing green water [microalgae (w3algae; Bernaqua® 10 g m^{-3}) added] or clear water (control). Fish weaning was achieved by co-feeding with *Artemia* nauplii and microdiets for seven days until full microdiet substitution. Larvae were biometrically evaluated on days 10, 17 and 24 to assess growth performance. In a second test, the locomotor activity of the larvae was analyzed before and after light exposure (1400 ± 60 lx) for 48 h according to an ethogram. After 24 days, the larvae reared in the green water were significantly heavier than those from the clear water, and displayed significantly fewer circular swimming movements. Body cortisol increased in both groups after light exposure. The microalgae provided an additional food source for larvae, with positive impact on growth until day 17 of larviculture. Green water can be a strategy to achieve better results in pirarucu larviculture, especially during and up to 10 days after the co-feeding period.

KEYWORDS: *Arapaima gigas*, body cortisol, light exposure, microalgae

Larvicultura de pirarucu em água verde produz peixes mais pesados e modula a atividade locomotora

RESUMO

A técnica de água verde utiliza microalgas na água durante a larvicultura *indoor,* proporcionando um ambiente mais escuro que favorece o crescimento, bem-estar e saúde dos peixes. Avaliamos o crescimento e a atividade locomotora após exposição à luz de larvas de pirarucu (*Arapaima gigas*) criadas em água verde ou clara. Em um teste, larvas de pirarucu (3,6 ± 0,3 cm; 0,36 ± 0,1 g) foram criadas em tanques circulares de 50 L (n = 3 por tratamento; 50 larvas por tanque) em sistema estático contendo água verde [microalgas (w3algae; Bernaqua® 10 g m^{-3}) adicionadas] ou água clara (controle). A transição alimentar dos peixes ocorreu por co-alimentação com náuplios de *Artemia* e microdieta por sete dias até a substituição completa pela microdieta. A biometria das larvas foi avaliada nos dias 10, 17 e 24, para avaliar o crescimento. Um segundo teste avaliou a atividade locomotora das larvas antes e após exposição à luz (1.400 ± 60,47 lx) por 48 horas usando um etograma. Após 24 dias, os peixes criados em água verde pesaram significativamente mais que os da água clara, e apresentaram significativamente menos movimentos circulares de natação. A exposição à luz aumentou o cortisol corporal nos dois grupos depois da exposição à luz. O nível corporal de cortisol aumentou em ambos grupos após exposição à luz. As microalgas forneceram uma fonte adicional de alimento para as larvas, com impacto positivo sobre seu crescimento até o 17º dia de larvicultura. Água verde pode ser uma estratégia para obter melhores resultados na larvicultura de pirarucu, principalmente durante e até 10 dias após o período de co-alimentação.

PALAVRAS-CHAVE: *Arapaima gigas*, cortisol corporal, exposição à luz, microalgas

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INTRODUCTION

The larval period is the fish's life phase that requires most care because it is when the main morpho-physiological transformations occur, such as complete yolk sac depletion, mouth and anus opening, the differentiation and functionality of internal organs, body and eye pigmentation, and fin and scale appearance (Halverson 2013; Portella *et al*. 2014).

Microalgae have been employed as additives in feed formulations to provide highly unsaturated fatty acids, and to enrich water during larviculture, a technique known as green water, which has provided good results in marine fish larviculture (Izquierdo *et al*. 2006; Roy and Pal 2015). The benefits to larvae are related to the nutrients absorbed directly by microalgae ingestion, or indirectly by consumption of filtering microcrustaceans that are enriched with microalgae nutrients (Rocha *et al*. 2008). Microalgae (*Chlorella pyrenoidosa* Starr & Zeikus, 1987 and *Chlorella* spp.) contain vitamins A, C, D3 and E, and highly unsaturated omega-3 fatty acids like eicosapentaenoic and docosahexaenoic acids, EPA and DHA, respectively (Drewery *et al.* 2014). EPA and DHA are essential in fish early larval development (Derner *et al*. 2006) as they participate in the formation of cell membrane phospholipids, and are responsible for maintaining cell integrity, fluidity and permeability (Prieto *et al*. 2006).

The health condition of larvae is crucial for their development and some behavioral characteristics are indicators of animal quality (Dias *et al*. 2004). The environment's color is one of the factors that can increase or depress behavioral patterns (Fanta 1995; Papoutsoglou *et al*. 2000; Merighe *et al*. 2004). For example, black, white, yellow and red should be avoided in tilapia (*Oreochromis niloticus* Linnaeus, 1758) farming as they cause stress or significant changes in fish behavior (Merighe *et al*. 2004). Green, however, is similar to the color of natural environments and does not interfere with animal behavior (Merighe *et al*. 2004). Accordingly, we hypothesized that the green water technique would reduce the physiological response resulting from a stressor (e.g. light intensity) and increase fish growth.

In outdoor ponds, the presence of suspended inorganic (clay, silt and carbonate) and organic (plankton and small organisms) particles affects turbidity and light penetration in the underwater environment (Yi *et al*. 2003; Villamizar *et al*. 2011). Optimal light conditions during fish larviculture increase growth and survival, and promote normal development (Villamizar *et al*. 2011). However, under outdoor conditions, fish larvae are more susceptible to the action of predators (piscivorous birds, aquatic insects, bats) (Gonçalves *et al*. 2019). Dominant larvae stand out in food competition, making access to food difficult for submissive larvae, which can become smaller and eventually die (Lima *et al*. 2017).

In current pirarucu (*Arapaima gigas* Schinz, 1823) larviculture, larvae remain in outdoor ponds and are cared for by parents until they reach 7-10 cm in size, when they are collected and allocated to indoor tanks to be fed commercial diets (Pereira-Filho *et al*. 2010; Halverson 2013). During the period under parental care, larval mortality rates can reach around 90% (Ono *et al*. 2004; Pereira-Filho *et al*. 2010; Gonçalves *et al*. 2019).

An alternative to achieve higher zootechnical performance and survival rates of pirarucu larvae is the capture of larvae when they have an inflated vesicle and swim close to the breeder's head, transfer the larvae to the laboratory and train them to receive formulated feed by weaning, which can increase larval survival up to 95% (Araújo da Silva *et al*. 2018; Gonçalves *et al*. 2019). Weaning consists of gradual feed transition, also known as co-feeding, and is characterized by progressively reducing live food concomitantly with increasing inert feed supply until the fish have adapted to exclusive inert food ingestion (Azevedo *et al*. 2016). The co-feeding strategy is used not only to promote the intake of formulated feed, but also to stimulate the development of the digestive system (Engrola *et al*. 2009).

Rearing fish in the laboratory allows greater management control and easier observation of behavioral indicators of health, stress and hunger (Johansen *et al*. 2006). For example, swimming speed is inversely proportional to the amount of food present in the rearing environment, so that the observation of the swimming behavior can be related to the food satiety level (Nunn *et al*. 2011). We evaluated zootechnical performance, locomotor activity and response to stress factors of pirarucu larvae reared in green water and in conventional environment (clear water system) during the weaning period.

MATERIAL AND METHODS

This study was approved by the ethics committee on animal experimentation and research of Instituto Nacional de Pesquisas da Amazônia (INPA), Manaus, Amazonas, Brazil (protocol # 016/2016 CEUA/INPA). The experiments were carried out at the INPA's aquaculture experimental station at the Coordination of Technology and Innovation (COTEI). The pirarucu larvae were obtained from natural spawning in a fish pond at Santo Antônio farm (AM-240 highway, km 48, Amazonas, Brazil), and were collected when their gas bladder inflated and they began to swim next to the breeder's head and immeadiately transported to INPA.

Two experiments were performed, referred as Test I and Test II. In Test I, we evaluated the zootechnical performance of larvae reared in green or clear water during the weaning period. In Test II, the behavior and body cortisol of the Test-I larvae were evaluated in relation to light exposure as a stress factor.

Test I

The test was carried out in a completely randomized design with two environments: clear water (CW) (no microalgae were added) and green water (GW), with the inclusion of microalgae (w3algae; Bernaqua®), with *Chlorella pyrenoidosa* and *Chlorella* spp. at a concentration of 10 g m^{-3}, with three replicates for each treatment. For each replicate, pirarucu larvae (3.6 ± 0.32 cm; 0.36 ± 0.06 g) were transferred to circular polyethylene tanks with a useful volume of 50 L and 50 larvae per tank, in a static system with 50% water cleaning and renewal twice daily, during 24 days. The stocking density followed Santana *et al.* (2020). The water quality parameters observed during the 24-day experiment were: temperature = 26.52 ± 2.57 ºC; pH = 6.50 ± 0.28; dissolved oxygen = 6.46 ± 0.31 mg L^{-1}; nitrite = 0.29 ± 0.15 mg L^{-1}; carbon dioxide = 15.66 ± 8.77 mg L^{-1}; ammonia = 2.28 ± 0.40 mg L^{-1}. All the parameters fell within the comfort range for pirarucu (Chu Koo *et al.* 2017). Regarding ammonia, pirarucu tolerates high levels of ammonia, as in a similar study in a static system, pirarucu juveniles tolerated an ammonia concentration of 25 mg L^{-1} (Cavero *et al.* 2004). The ammonia levels in our study did not interfere with larval growth.

During the first seven days, weaning was performed by co-feeding with *Artemia* nauplii (3000 nauplii larvae day^{-1}) and microdiet pellets (Sparos®) with 200-400 µm size, which were offered until day 10. From day 11 to day 24, larvae were fed microdiet pellets of 400-600 µm. Both pellet types contained 63.51% crude protein, 15.34% fat, 1.70% crude fiber and 8.10% ash, according to the manufacturer's information. Larvae were fed 10 times a day, and the daily feeding rate was planned according to the daily growth rate and feed conversion in previous trials.

The following zootechnical performance parameters of larvae were determined on days 10, 17 and 24: survival (S, %) = number of fish alive/initial number of fish*100; weight gain (WG, g) = present weight - initial weight; daily weight gain (DWG, g day^{-1}) = weight gain/number of days since start of experiment; relative growth rate (RGR, % day-1) = (E^g-1)*100, where e = 2.718 and g = (ln (final weight) - ln (starting weight))/(final time - initial time); condition factor (K) = total weight/total $length^3$. All larvae were measured on all occasions. On day 24, all the larvae were classified into size classes as small (< 6.1 cm length), medium (6.2 - 7.3 cm) or large (> 7.4 cm).

Test II

After Test I ended, the larvae (6.70 ± 0.49 cm; 1.75 ± 0.47 g) were placed into aquaria (to make filming possible) maintaining treatment and replicate identity. Each aquarium had a useful volume of 8 L (six aquaria with CW and six aquaria with GW larvae; eight larvae per aquarium). Larvae remained in the same treatments as in Test 1 in a 2x2 factorial scheme in two rearing environments (CW and GW), with and without light exposure (three aquaria per treatment). The same management procedures as in the previous test were applied. The experiment began after a 48-hour period for larvae to acclimatize to their new environment. Three aquaria of each water treatment (CW and GW) were exposed to a light intensity of 1400 ± 60 lx for 48 h, which is considered a stressor for fish larvae (Lopes *et al.* 2018). The other three aquaria of each water treatment were not exposed to the light. Larvae behavior was recorded (Intelbras® camera VHD 1010 B G3) for a 1-hour period before and after light exposure. The locomotor activity of three larvae from each tank was quantified for a 10-minute period (Lopes *et al.* 2018) (from minutes 30 to 40) by quantifying the frequency of movements (rotational, vertical, horizontal), static moments (larva stopped) and random contacts (Olla *et al.* 1978; Sabate *et al.* 2008). Total movements corresponded to the sum of the frequencies of all movements (rotational, vertical and horizontal), described in an ethogram applied to matrinxã (*Brycon amazonicus* Agassiz, 1829) (Souza *et al.* 2014) and adapted to pirarucu larvae (Table 1).

Table 1. Ethogram of locomotor activity for pirarucu (*Arapaima gigas*) larvae (adapted from Souza *et al.* 2014).

Behavioral unit	Description
Circular swimming movement (CSM)	Fish move from one place to another in a circle
Vertical movement (VM)	Fish move to the surface to breathe
Horizontal movement (HM)	Fish move from one place to the other in a straight line
Static moment (SM)	Fish do not perform locomotion movement
Contact with chance (CC)	Fish occasionally touch other animals while moving

Immediately after the end of Test II, three larvae from each aquarium were euthanized by physical methods (CONCEA 2018) and macerated individually in a porcelain crucible), dissolved in diethyl ether, centrifuged (3500 rpm, 5 min), dried in nitrogen vapor and stored at -20 ºC. For body cortisol reading procedure, samples (total weight after freezing: 0.9 - 1.2 g) were suspended in 1 mL of PBS and plate assembly followed the manufacturer's directions. The reading was done by ELISA (Cortisol ELISA kit - DRG Diagnostics), which has been tested and validated for fish by Santamaría and Casallas (2007) and by Canavello *et al.* (2011).

Statistical analysis

All response variables of both tests had normal distribution (Shapiro-Wilk test) and variance homogeneity (Levene test). The zootechnical performance variables (on days 10, 17 and 24) were submitted to a one-way ANOVA (N = 3 tanks per treatment). The frequency distribution of size classes was compared between treatments with a Chi-square test.

Behavioral variables and cortisol were compared among treatments with a two-way ANOVA, considering each larva as an observation unit (nine larvae per treatment, three larvae per tank). When interaction between factors (water type and light exposure) occurred, a pairwise comparison was performed using the Tukey test . When the interaction were non-significant, the factors were evaluated individually. The significance level was 5% in all analyses.

RESULTS

After 10 and 17 trial days, the larvae reared in GW had significantly higher weight and length than CW larvae (Figure 1). On day 24, however, no statistical difference was observed for total length and growth performance, but GW larvae (1.97 ± 0.10 g) remained significantly heavier than CW larvae (1.79 ± 0.16 g) (Figure 1 and Table 2). Rearing water did not interfere with the frequency distribution of larvae in size classes, with over 75% of GW and CW larvae classified as medium size after 24 days (Figure 2).

In Test II, there was an interaction effect between water type and light exposure for vertical movement ($F = 4.4053$; df = 32; $p = 0.044$), horizontal movement ($F = 8.2228$; df = 32; $p = 0.007$) and total movements ($F = 12.4940$; df = 32; $p = 0.001$). CW larvae showed significantly higher movement rates than GW larvae when exposed to light.

Larvae exposed to light showed more circular swimming movement ($F = 4.9079$; df = 32; $p = 0.034$) and less static

Table 2. Survival and zootechnical performance of pirarucu (*Arapaima gigas*) larvae after 24 days reared in clear water and green water. Values are the mean ± standard deviation of three replicates (50-L tanks with initial population of 50 larvae).

Parameter	Clear water	Green water	p-value
L (cm)	6.7 ± 0.6	6.8 ± 0.5	3.00
W (g)	1.8 ± 0.2 a	2.0 ± 0.1 b	0.04
WG (g)	1.4 ± 0.2	1.6 ± 0.2	0.25
DWG (g day^{-1})	0.1 ± 0.0	0.1 ± 0.0	0.25
RGR (% day-1)	6.9 ± 0.9	7.4 ± 0.5	0.34
K	0.6 ± 0.0	0.6 ± 0.0	0.89
S (%)	76.0 ± 7.2	78.7 ± 3.1	0.59

L = total length; W = weight; WG = weight gain; DWG = daily weight gain; RGR = relative growth rate; K = condition factor; S = survival. P-values correspond to one-way ANOVA (p ≤ 0.05)

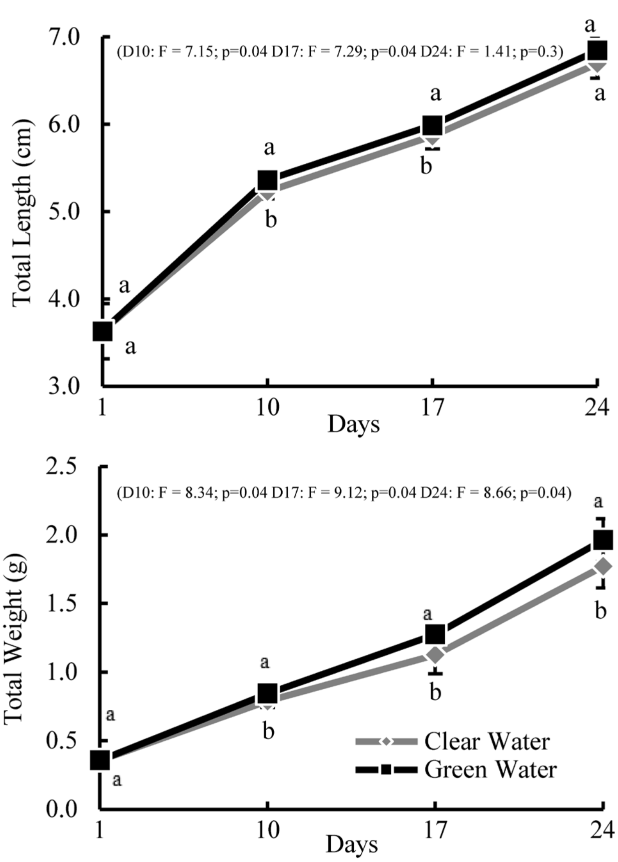

Figure 1. Evolution of the total length (A) and total weight (B) of pirarucu (*Arapaima gigas*) larvae reared during 24 days in clear water (CW) and green water (GW). Points indicate the mean and bars the standard deviation of three replicates (50-L tanks with initial population of 50 larvae). Letters at each time-point indicate whether the means differed significantly according to an ANOVA F-test.

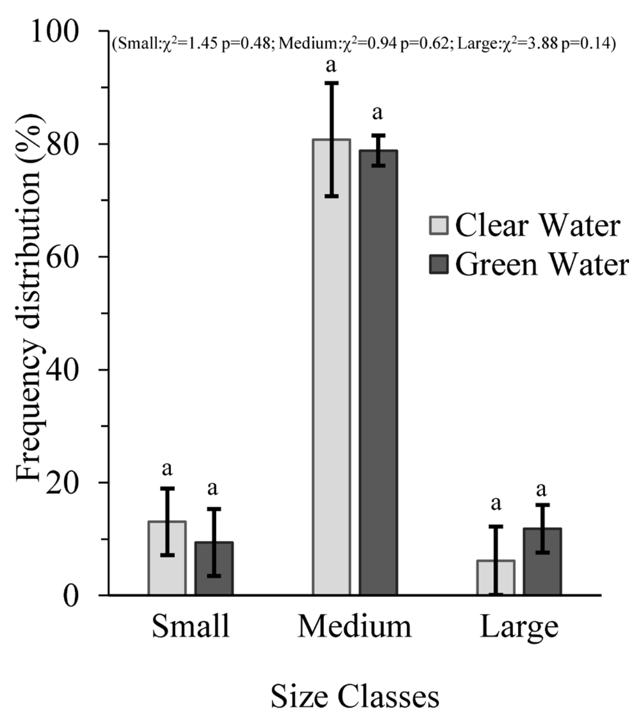

Figure 2. Frequency distribution of pirarucu (*Arapaima gigas*) larvae in size classes after 24 days reared in clear (CW) and green water (GW). Columns are the mean and bars the standard deviation of three replicates.

moment than those not exposed to light (F = 48.933; df = 32; p <0.0001). Circular swimming movements and contact by chance were significantly higher in CW compared to GW (Table 3). The body cortisol level was significantly higher with light exposure independently of the rearing water (Figure 3).

DISCUSSION

The higher final weight of GW larvae was probably related to the intake of vitamins and fatty acids provided by the microalgae, unlike the CW larvae that did not receive this nutritional support. Pirarucu larvae can filter microalgae through gill traces, which they use as a feed source (Ono *et al.* 2004) until the juvenile phase when the weight is around 300 g (Lima *et al.* 2018). The application of lyophilized or inoculated microalgae to marine fish larviculture environment helps to stabilize water quality (Navarro and Sarasquete 1998), to feed larvae, and to maintain both the nutritional value of live food and larval development during weaning (Ferreira 2009).

After weaning (on day 10), the total length of larvae had increased by almost 49% in GW and by more than 45% in CW. Fish larvae performance depends on the quantity and nutritional quality of live food (Portella *et al.* 2012). Pirarucu larvae showed a similar length increase of 47.9%

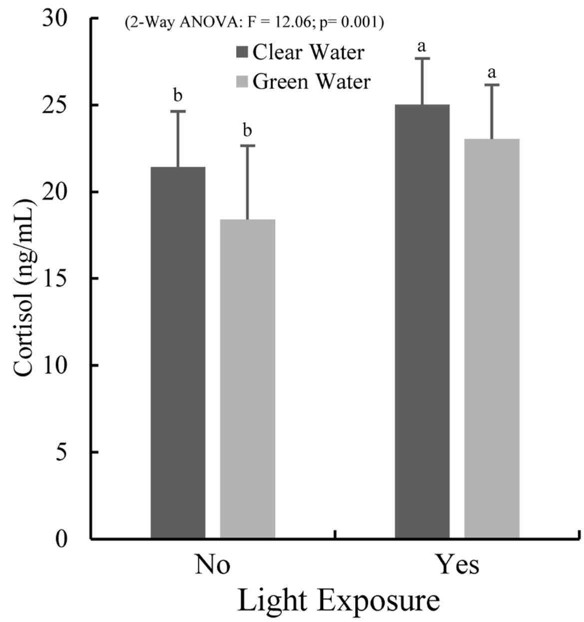

Figure 3. Mean ± standard deviation of body cortisol of pirarucu (*Arapaima gigas*) larvae reared for 24 days in clear water (CW) or green water (GW) after 48 hours of light exposure or non exposure. Different letters indicate a significant difference between presence or absence of light exposure (two-way ANOVA, F = 12.06; p = 0.0014).

Table 3. Number of behavior units of pirarucu (*Arapaima gigas*) larvae reared in clear (CW) and green water (GW) with or without light exposure. Values are the mean ± standard deviation of nine replicates.

Behavior	Water	Light exposure (LE)			p value		
		Yes	No	Overall	L	W	L x W
CSM	CW	6.78 ± 0.97	6.22 ± 2.59	6.50 ± 1.92 A	0.0342	0.0003	0.2058
	GW	5.11 ± 1.27	2.89 ± 1.76	4.00 ± 1.88 B			
	Overall	5.94 ± 1.39 a	4.56 ± 2.75 b				
VM	CW	1.11 ± 0.78 Bb	2.33 ± 1.00 Aa	1.72 ± 1.07	0.1658	0.4415	0.0440
	GW	2.11 ± 0.93 Aa	1.89 ± 1.27 Bb	2.00 ± 1.08			
	Overall	1.61 ± 0.98	2.11 ± 1.13				
HM	CW	1.11 ± 1.05 Bb	4.89 ± 2.15 Aa	3.00 ± 2.54	0.0004	0.8913	0.0073
	GW	2.78 ± 1.39 Ab	3.44 ± 1.67 Ba	3.11 ± 1.53			
	Overall	1.94 ± 1.47	4.17 ± 2.01				
TM	CW	9.00 ± 0.71 Bb	13.44 ± 2.88 Aa	11.22 ± 3.06	0.1343	0.0229	0.0013
	GW	10.00 ± 1.94 Aa	8.22 ± 3.67 Bb	9.11 ± 2.99			
	Overall	9.50 ± 1.50	10.83 ± 4.18				
SM	CW	1.44 ± 1.42	5.33 ± 1.66	3.39 ± 2.50 A	0.0000	0.3455	0.2663
	GW	2.44 ± 1.01	5.44 ± 1.51	3.94 ± 1.98 A			
	Overall	1.94 ± 1.30 b	5.39 ± 1.54 a				
CC	CW	2.33 ± 0.87	1.78 ± 1.39	2.06 ± 1.16 A	0.5790	0.0210	0.0805
	GW	0.44 ± 0.73	1.44 ± 1.88	0.94 ± 1.47 B			
	Overall	1.39 ± 1.24 a	1.61 ± 1.61 a				

CSM: circular swimming movement; VM: vertical movement; HM: horizontal movement; TM: total movements; SM: static moment; CC: chance contact; L: light exposure; W: rearing water. Means followed by different lower case letters indicate significant differences for this behavior between larvae exposed or not to light. Different upper case letters in columns for each behavior and light treatment indicate significant differences between water treatments (two-way ANOVA and Tukey test; p ≤ 0.05) after being fed *Artemia* nauplii for 15 days (Araújo da Silva *et al*. 2018), and 93% after being fed *Artemia* nauplii and zooplankton for 11 days (Alcântara *et al*. 2018). Pirarucu larvae fed only Ostracoda-rich zooplankton displayed an increased length of 62.7%, but lower survival (40%) than larvae fed zooplankton rich in cladocerans, copepods and rotifers (Gonçalves *et al*. 2019). The zooplankton from natural environments (cladocera, copepoda, rotifera) is the main food item during the larval and juvenile periods of carnivorous fish like pirarucu (Lima *et al.* 2018). Abundant microcrustaceans with predominance of Cladocera were found in stomach contents of pirarucu juveniles weighing up to 500g reared in earth ponds (Lima *et al.* 2018). Wild freshwater zooplankton ia an excellent nutritional source for fish larvae and juveniles, but its abundance is very much dependent on climatic conditions, it is potential vector of diseases, and no adequate technology is available to allow its low-cost mass production in freshwater (Vega-Orellana *et al*. 2006). *Artemia* sp. in nauplii stage offer good nutritional value and can be produced on a large scale within 24 hours, and their cysts are easily accessible on the market (Samat *et al*. 2020). However, *Artemia* nauplii represent most of the production cost in fish larviculture (Jomori *et al*. 2005), which is why the feed transition by co-feeding makes economic sense, in addition to the benefits of stimulating inert food intake, digestive system development, and improving growth and survival (Engrola *et al*. 2009; Portella *et al*. 2012).

The type of larviculture water did not affect larvae survival rates, which were around 76-78%, but were lower than those observed in other studies on pirarucu larviculture (93 – 99%) (Alcântara *et al*. 2018; Araújo da Silva *et al*. 2018; Gonçalves *et al*. 2019). This could be due to the initial weaning period and the live food type supplied before the feed transition period, as pirarucu larvae fed *Artemia* nauplii and zooplankton until day 11 showed 99% survival after 21 larviculture days (Alcântara *et al*. 2018) Survival in the initial pirarucu hatchery is related to many factors such as available food, size at the beginning of weaning, density in the tank, sanitary and feeding management (Gonçalves *et al*. 2019). The use of microalgae in the larviculture rearing environment has been reported to shorten the feed-transition period without altering production rates, allowing to achieve higher survival rates, lower cost per larva and higher production yields than the traditional larviculture system (Jomori *et al*. 2005). We observed no difference in larvae survival between treatments, but the weight and length of larvae after feed transition (at 11 and 17 days) were higher in GW than in CW. In *Paralichthys dentatus* Linnaeus, 1766, no difference in growth performance of larvae reared in GW and CWwas observed after 42 days, but survival was higher in GW (76.1%) than in CW (27.8%) (Bengtson *et al*. 1999).

Pirarucu larvae are commercialized by length, and fish farmers are more interested in larvae that have already been trained to consume commercial feed. After weaning, fish are more morphologically and physiologically developed and, consequently, are more resistant (Rebelatto Junior *et al*. 2015; Lima *et al*. 2017). On day 17 of larviculture, the average total length of our larvae reared in GW was 6 cm, and they were already being fed only commercial feed, which eases management and reduces feed costs and production time.

Pirarucu larvae form schools that move in synchrony to feed (Harvelson 2013), and larvae housed in circular tanks show circular swimming movements, which are fast when fish are hungry, and slow down after feeding, indicating satiety (the authors, pers. obs.). Thus the lower swimming frequency in circular movements in GW in our study likely indicates that larvae were less hungry due to the presence of microalgae as a food source, which is further evidenced by the higher weight of the GW larvae. In addition, greater locomotor activity of CW larvae implies more energy expenditure (Gerry and Ellerby 2014), which may also have contributed to the lower weight of larvae in this treatment. GW larvae moved less and presented less chance encounters with each other than CW larvae, which potentially reduces injury rates due to interaction among larvae, which serves as a gateway to pathogens that can increase mortality rates (Huntingford *et al.* 2006).

CONCLUSIONS

Our results indicate that the inclusion of microalgae in water provides an additional food source for pirarucu larvae, with positive impact on larva growth until day 17 of indoor larviculture. The green water technique can be a strategy to achieve better results in pirarucu larviculture, especially during and until 10 days after the co-feeding period.

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