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GROWTH PERFORMANCE AND INCIDENCE OF SKELETAL ANOMALIES IN PACU LARVAE UNDER DIFFERENT WEANING PROTOCOLS*

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ABSTRACT

The inadequate supply of food during a fish's larval stage, in addition to impairing growth and survival, may lead to the emergence of skeletal anomalies, since essential nutrients are involved in the osteogenic process. The aim of this study was to evaluate the effects of two weaning periods on growth, survival and incidence of skeletal anomalies in pacu larvae (*Piaractus mesopotamicus*) during its initial development. The larvae (5 dph) were placed in 100L tanks at a density of 12 larvae L^{-1}, during 42 days. The experiment was conducted in a completely randomized design with four feeding treatments: (A) larvae fed only *Artemia* nauplii; (D) larvae fed only formulated diet; and two treatments with different weaning periods, early at six (W6) and late, at twelve days of feeding (W12). Higher growth indexes and survival rates were observed in A and W12, in comparison to W6 and D. At the end of the experiment, larvae fed *Artemia* nauplii and subjected to late weaning (W12) presented higher mass gain and survival, in comparison to W6 and D, however the incidence of skeletal anomalies was similar among treatments. It was concluded that the emergence of skeletal anomalies in *P. mesopotamicus* larvae was not associated to the weaning protocols used in this study and late weaning, at 12 days of feeding, did not impair growth and survival of these larvae.

Key words: *Piaractus mesopotamicus*; skeletal anomalies; weaning; intensive larviculture.

DESEMPENHO E INCIDÊNCIA DE ANOMALIAS ESQUELÉTICAS EM LARVAS DE PACU SOB DIFERENTES PROTOCOLOS DE TRANSIÇÃO ALIMENTAR

RESUMO

A oferta inadequada do alimento durante a fase larval dos peixes, além de prejudicar o crescimento e a sobrevivência, podem causar anomalias esqueléticas, uma vez que nutrientes essenciais estão envolvidos na osteogênese. O objetivo deste estudo foi avaliar os efeitos de dois períodos de transição alimentar no crescimento, sobrevivência e na incidência de anomalias esqueléticas em larvas de pacu (*Piaractus mesopotamicus*) durante seu desenvolvimento inicial. Larvas com 5 dias pós-eclosão (dph) foram acondicionadas em tanques (100L), em uma densidade de 12 larvas L^{-1}, durante 42 dias. O experimento foi conduzido em delineamento inteiramente casualizado e apresentou quatro tratamentos alimentares: (A) larvas alimentadas apenas com náuplios de artêmia; (D) larvas alimentadas apenas com dieta formulada; e dois tratamentos com diferentes períodos de início da transição entre alimento vivo e formulado, (W6) prematuro, aos seis dias de alimentação, e (W12) tardio, aos 12 dias de alimentação. Ao final do experimento, as larvas dos tratamentos A e W12 apresentaram médias de ganho em massa e sobrevivência maiores que as dos tratamentos W6 e D, no entanto a incidência de anomalias esqueléticas foi similar entre os tratamentos. Foi possível concluir que incidência de anomalias esqueléticas em larvas de *P. mesopotamicus* não foi associada aos protocolos de transição alimentar adotados nesse estudo e a transição alimentar tardia, aos 12 dias de alimentação, pode ser realizada sem comprometer o crescimento e sobrevivência das larvas.

Palavras-chave: *Piaractus mesopotamicus*; anomalias esqueléticas; transição alimentar; larvicultura intensiva.

INTRODUCTION

Skeletal anomalies in reared fish are frequently reported and its occurrence may increase with the intensification of rearing systems (Boglione et al., 2001; Cahu et al., 2003; Izquierdo et al., 2010; Roo et al., 2010a, 2010b; Argüello-Guevara et al., 2014). These anomalies represent a vast problem in aquaculture, as it may cause negative effects under growth, welfare and health of fish (Koumoundouros et al., 2001; Sfakianakis et al., 2006), thus increasing mortality rates and, consequently, production costs.

Several factors relates to the emergence of skeletal anomalies in fish larvae, such as environmental (Koumoundouros et al., 2001; Sfakianakis et al., 2006; Roo et al., 2010a; Cobcroft et al., 2012; Corrales et al., 2014), genetic (Gjerde et al., 2005; Kause et al., 2007) and nutritional factors (Saavedra et al., 2009; Izquierdo et al., 2010; Boglino et al., 2012; Hernandez et al., 2013). These factors may lead to development delays (Zambonino-Infante and Cahu, 2007), as well as skeletal anomalies, since environmental conditions, handling and nutrition (such as the supply of essential nutrients) are involved in osteogenesis (Cahu et al., 2003). Moreover, skeletal anomalies that are caused by genetic factors can be exacerbated if other causative factors are imbalanced (Kause et al., 2007). In this sense, during larviculture, nutritional deficiencies may occur, being related both to the inadequate utilization of live feed and formulated diets, as well as to the process of food transition, leading to the possibility of the emergence of skeletal anomalies.

Despite of the existing studies with formulated diets and its effects on the skeleton development and in the appearance of anomalies in larvae of several commercial fish species (Villeneuve et al., 2005; Sikorska et al., 2012; Argüello-Guevara et al., 2014), the information regarding the osteogenic process in South American fish species are scarce. However, according to Portella et al. (2014), these studies should be performed in order to know the main skeletal anomalies that affects such species.

The pacu, *Piaractus mesopotamicus*, an important species of South American fish farming, had its ossification process previously described (Portella et al., 2014; Barbieri and Bondioli, 2015). The sequence of skeletal ossification begins with the craniofacial structures, followed by the appendicular skeleton. A complete ossification is verified when larvae reaches a standard length of approximately 20 mm (Portella et al., 2014). Another study described, for the first time, the incidence of upper and lower jaw, vertebral column and fin anomalies in pacu, at the first developmental stages, which used larvae deriving from wild-caught broodstock, kept in captivity (Lopes et al., 2014). The authors verified that the described anomalies compromised the growth and development of pacu larvae.

It is known that both diet quality and early weaning from live feed to formulated diets affect the development and growth of pacu larvae, with a consequent increase of mortality and production losses (Jomori et al., 2008; Leitão et al., 2011; Menossi et al., 2012). When studying different weaning periods for *Paralichthys lethostigma* larvae, Faulk and Holt (2009) verified that early weaning at 17 and 23 days post hatching (dph) resulted in high incidences of lordosis, when compared to late weaning (29 dph) and larvae fed with live feed throughout the experiment. According to these results, the authors suggested that nutritional deficiencies may have occurred, which were related not only to the used formulated diets, but also to the incapacity of larvae with less than 6 mm to digest and/or absorb nutrients, when the activity of digestive pancreatic enzymes is low and gastric digestion is absent. Based on the aforementioned, we aimed at evaluating the effects of two weaning periods on the incidence of skeletal anomalies, growth and survival in pacu larvae during its initial development.

MATERIAL AND METHODS

Experimental conditions

The larvae were obtained by hormonally induced spawning from breeders kept in captivity at a governmental institution in São Paulo State and transferred (3 dph) to the Laboratory of Nutrition of Aquatic Organisms (LANOA) of the Aquaculture Center, UNESP, Jaboticabal, SP, where the experiment was conducted during 42 days. Larvae were counted and distributed at the density of 12 larvae.L^{-1} in 20 polyethylene tanks (100 L), supplied with water deriving from an artesian well (open system), in continuous flow, with constant artificial aeration.

The experiment began with 5 dph larvae, initiating exogenous feeding (0.6 ± 0.1 mg wet weight and 5.72 ± 0.33 mm total length). The temperature throughout the experiment was 29.5 ± 0.6 °C, daily measured at 8h and 18h, while the water quality parameters were weekly measured: 5.8 ± 0.5 mg.L^{-1} of dissolved oxygen (YSI 550A), pH 7.7 ± 0.4 (Corning pH30), 209.0 ± 44.0 µS.cm^{-1} of electrical conductivity (Corning PS 17) and 35.9 ± 19.7 µg.L^{-1} of total ammonia (Koroleff method). Solids were manually removed from the bottom of the tanks by siphon vacuuming after the first feeding period.

The experiment was conducted in completely randomized design. Four treatments were defined by feed type and by the beginning of the weaning process: (A) larvae fed exclusively *Artemia* nauplii; (D) larvae fed exclusively with formulated diet (NRD 1.2/2.0 - INVE, 57%); and two treatments with different weaning periods from live to formulated diet: (W6) or "early weaning", larvae fed with *Artemia* nauplii for five days and at the 6^{th} day post first feeding (dpff) were weaned to the formulated diet, with a co-feeding period of six days; and (W12) or "late weaning", larvae received *Artemia* nauplii for 11 days and at the 12^{th} dpff were weaned to the formulated diet, with a co-feeding period of six days.

Larvae fed with *Artemia* nauplii received increasing quantities of nauplii, adjusted every three days (addition of 500 nauplii.$larvae^{-1}$) up to the 15^{th} day, followed by a 1000 nauplii.$larvae^{-1}$ increase every seven days until the end of the experiment, as suggested by Jomori et al. (2003, 2008). Regarding treatments in which co-feeding was applied for weaning (W6 and W12), during six days larvae received nauplii in decreasing quantities, with increasing quantities of the formulated diet till total suppression of the live feed. Feed (live or formulated) was offered six times a day (7:00, 10:00, 13:00, 16:00, 19:00 and 22:00 h) and the formulated diet was supplied in all treatments, i.e. in sufficient quantities.

This study is in accordance with the ethical principles of animal experimentation, adopted by the Brazilian College of Experimentation (COBEA) and was approved by the Ethics Committee on Animal Use (CEUA) of the Faculty of Agrarian and Veterinary Sciences, FCAV/UNESP, protocol nº 001218/12.

Analysis of larvae growth and survival

For the evaluation of total length (Lt) and weight (W), 20 larvae were sampled from each replicate at 0, 6, 12, 18, 24, 30, 36 and 42 dpff. The animals were euthanized in a benzocaine solution (0.2 g.L^{-1})

and fixed in formalin 10% with phosphate buffer. With the mean values of initial (W_i) and final weight (W_f) of each replicate, the Specific Growth Rate was calculated, be the expression **SGR = 100. (*ln* W_f – *ln* W_i)/Δ*t***, considering Δ*t* as the interval in days. The Fulton's condition factor was calculated by means of the expression **K = 100000 x (W_f/Lt_f^3)**. Weight gain was calculated by the expression **WG = W_f – W_i**.

Skeletal anomalies analysis

Skeletal anomalies were verified and quantified in larvae sampled at 6, 12, 18, 24, 30, 36 and 42 dpff, which were previously weighted and measured. Then, larvae underwent a bone and cartilage staining process (Potthoff, 1984; Darias et al., 2010), which involves several stages, including the differential staining of bone (Alizarin Red) and cartilage (Alcian Blue), thus allowing a detailed view of the skeletal structures. These evaluations were performed with the aid of a stereoscope Olympus SZX7, equipped with a digital photographic camera Olympus DP26 and an image analyzer CellSens 1.8.

The incidence of skeletal anomalies was evaluated as suggested by Boglione et al. (2001). Ossified structures were registered as the presence (1) or absence (0) of anomalies in the upper and lower jaws, vertebral column (kyphosis, scoliosis, lordosis and abnormal vertebrae) and fins (caudal, dorsal and anal). With the exception of the first sampling (6^{th} dpff), the evaluation of bone structures occurred when these were partially or completely ossified. In this sense, fins and abnormal vertebrae were assessed only in samples from 24 and 30 dpff, respectively.

Statistical analysis

Growth results (weight and length), survival and total incidence of skeletal anomalies were submitted to a variance analysis (ANOVA), considering the mean values of the replicates of each treatment. All data were tested for error normality (Cramer-von Mises) and variance homoscedasticity (Brown and Forsythe) and means were compared by the Tukey test, at 5% probability. The data for the SGR and survival were arcsine transformed before ANOVA analysis, but only the percentage data are presented. These univariate statistical analyses were performed by means of the Statistical Analysis System - SAS (SAS Institute Inc, version 9.0).

Complementarily, a numeric base of categorical data of presence and absence was elaborated, in order to calculate the incidence of anomalies and evaluate in a descriptively way the kinds of anomalies present in the bone structures, according to the feeding treatments which larvae were submitted. Zootechnical and categorical data were also analyzed by the exploratory multivariate techniques of Clustering (by Euclidian distances) and Simple Correspondence (SCA), aiming to seek existing associations between treatments and types of skeletal anomalies, at a 5% significance level. The exploratory analysis were performed with the aid of the software STATISTICA 7.0.

RESULTS

Growth and survival

The larvae fed with *Artemia* nauplii (treatment A) presented the greatest growth results throughout the experiment. However, total length mean values at the end of the experiment and mean weight from 36 dpff did not differ ($P < 0.0001$) from the ones of W12 treatment. Early weaned larvae (W6) presented lower length and weight when comparing to treatments A and W12, considering all biometric evaluations after the substitution of live feed to formulated diet (Table 1).

Larvae fed only with the formulated diet (D) were sampled at 6, 12 and 42 dpff due to low survival rates. Only a few larvae of the D treatment remained alive by the end of the experiment (1.8 ± 0.1%), while the highest survival rates were found in treatments A (76.5 ± 6.5%) and W12 (82.8 ± 12.7%), which were statistically similar (Table 1). The effect of the beginning of weaning can be observed by the decrease of the specific growth rate, as SGR was lower than treatment A within the interval 6-12 dpff in treatment W6 and 12-18 dpff in treatment W12 (Table 1).

At the end of the experiment, A and W12 fish presented mean values of weight gain similar and higher than the ones from treatments W6 and D. However, a statistical difference of the condition factor ($P < 0.0001$) was verified between treatments A and W12 (Table 1).

Incidence of skeletal anomalies

Throughout the experimental period, 2109 larvae were analyzed and 262 presented some kind of skeletal anomaly. Furthermore, a few larvae presented more than one single anomaly, which increased the total incidence of anomalies to 361. All treatments displayed the occurrence of skeletal deformities in pacu larvae, and the most common anomalies were found in the vertebral column: lordosis (7.4%) and scoliosis (4.7%). These two kinds of anomalies combined were presented in 78 larvae. Differently, the lowest incidences were found in the fins (only 7 larvae or 0.3% of the total) (Table 2).

The frequency of deformed fish in each sampling day ranged from 4.9 to 34.0% throughout the experiment (Table 3). At the 6^{th} dpff, larvae still did not present signs of bone mineralization (Figure 1A), process which was observed at the 12^{th} dpff (Figure 1B). At the 18^{th} dpff, severe anomalies were registered in the vertebral column (Figure 1C) and, at the 24^{th} dpff, the first lower jaw anomaly was verified, at the same time of the beginning of fin ossification (Figure 1D). At 30, 36 and 42 dpff, several anomalies were registered, which visually affected swimming performance and growth (Figures 1E and 1F).

At the end of the experiment, the total incidences of larvae with skeletal anomalies were not statistically different among treatments, characterized for 84 (12.3%) deformed larvae in the A treatment, 78 (11.0%) in W12 and 100 (14.0%) in W6 (Table 3). In addition, the high initial mortality and low final survival rates (less than 2%) presented by the larvae subjected to treatment D, did not allow a sufficient sample size in order to perform skeletal analysis, thus it was suppressed.

Table 1. Mean values of the total length, weight and specific growth rate (SGR) of pacu larvae after the experiment beginning, condition factor, survival and mass gain at the end of the experiment (42 dpff). Values are presented as Mean ± SD.

Treatments	A	W12	W6	D
Weight (mg)				
6 dpff	3.6 ± 0.5 [a]	4.5 ± 0.8 [a]	4.0 ± 0.7 [a]	0.6 ± 0.1 [b]
12 dpff	21.1 ± 1.5 [a]	20.5 ± 1.7 [a]	10.0 ± 0.8 [b]	0.5 ± 0.1 [c]
18 dpff	54.5 ± 6.4 [a]	28.3 ± 5.1 [b]	14.9 ± 6.6 [c]	-
24 dpff	122.5 ± 14.3 [a]	56.9 ± 10.9 [b]	32.3 ± 4.6 [c]	-
30 dpff	219.7 ± 32.6 [a]	164.5 ± 19.0 [b]	60.5 ± 24.0 [c]	-
36 dpff	394.5 ± 26.5 [a]	364.0 ± 42.0 [a]	194.6 ± 24.2 [b]	-
42 dpff	586.4 ± 48.6 [a]	541.9 ± 62.9 [a]	323.9 ± 71.5 [b]	1.3 ± 0.6 [c]
Length (mm)				
6 dpff	9.4 ± 0.2 [a]	9.6 ± 0.2 [a]	9.4 ± 0.1 [a]	5.4 ± 0.3 [b]
12 dpff	13.3 ± 0.2 [a]	13.0 ± 0.3 [a]	11.5 ± 0.4 [b]	5.9 ± 0.4 [c]
18 dpff	17.2 ± 0.5 [a]	14.4 ± 0.4 [b]	12.0 ± 0.6 [c]	-
24 dpff	21.0 ± 0.3 [a]	18.0 ± 0.9 [b]	15.5 ± 0.4 [c]	-
30 dpff	24.9 ± 0.5 [a]	22.5 ± 0.6 [b]	17.7 ± 1.4 [c]	-
36 dpff	28.1 ± 0.7 [a]	26.4 ± 1.1[b]	22.0 ± 0.8 [b]	-
42 dpff	31.8 ± 0.9 [a]	29.8 ± 0.9 [a]	25.2 ± 1.4 [b]	7.4 ± 0.4 [c]
SGR (%.day^{-1})				
0-6 dpff	30.1 ± 1.7 [a]	32.2 ± 3.0 [a]	31.3 ± 1.6 [a]	-0.8 ± 2.6 [b]
6-12 dpff	28.9 ± 1.2 [a]	25.8 ± 2.0 [a]	14.1 ± 3.8 [b]	-2.5 ± 4.4 [d]
12-18 dpff	15.3 ± 2.3 [a]	6.2 ± 3.0 [b]	8.7 ± 3.3 [b]	-
18-24 dpff	13.5 ± 2.5	11.6 ± 3.2	11.2 ± 4.9	-
24-30 dpff	9.7 ± 3.6 [b]	17.9 ± 1.8 [a]	11.7 ± 3.2 [b]	-
30-36 dpff	9.9 ± 3.1 [b]	13.2 ± 2.0 [ab]	16.9 ± 2.2 [a]	-
36-42 dpff	6.6 ± 1.2 [a]	5.3 ± 2.4 [a]	8.3 ± 1.7 [a]	1.7 ± 0.5 [b]
Condition Factor	18.2 ± 0.4 [b]	20.4 ± 1.4 [a]	19.9 ± 1.2 [ab]	-
Survival (%)	76.5 ± 6.5 [a]	82.8 ± 12.7 [a]	25.7 ± 8.5 [b]	1.8 ± 0.1 [c]
Weight Gain (mg)	585.8 ± 48.6 [a]	505.1 ± 97.7 [a]	323.3 ± 71.5 [b]	0.7 ± 0.2 [c]

Mean values in the same line followed by the same letter did not statistically differ according to the Tukey's test (P > 0.05). (A) Larvae fed with *Artemia* nauplii; (W12) larvae submitted to late weaning starting at 12 dpff; and (W6) early weaning starting at 6 dpff; (D) larvae fed only commercial diet; (dpff = days post first feeding); (SD) standard deviation" to the footnotes.

Table 2. Incidence of different anomalies (%) found in pacu larvae evaluated throughout 42 days post first feeding, total incidence (%) of anomalies, total incidence (%) of larvae with anomalies and number of evaluated larvae (n) in each treatment.

Treatments	Typologies of skeletal anomalies							Total		
	UJ	LJ	K	S	L	AV	F	Anomalies	Larvae with anomalies	Evaluated larvae
A	0.1	0.6	1.3	5.4	7.9	1.5	0.1	16.9	12.3	685
W12	0.7	0.7	2.1	4.7	6.1	1.7	0.4	16.4	11.0	708
W6	0.4	0.3	2.5	4.2	8.4	1.8	0.4	18.0	14.0	716
Total	0.4	0.5	2.0	4.7	7.4	1.7	0.3	17.1	12.4	2109

(A) Larvae fed with *Artemia* nauplii; (W12) larvae submitted to late and (W6) early weaning starting at 12 and 6 dpff, respectively. UJ = Upper Jaw; LJ = Lower Jaw; K = Kyphosis; S = Scoliosis; L = Lordosis; AV = Abnormal Vertebrae; F = Fins.

Table 3. Total incidence (%) of anomalies in pacu larvae evaluated in each sampling day. according to the respective feeding protocols.

	Days post first feeding						
Treatments	6	12	18	24	30	36	42
A	8.5	4.9	24.7	11.2	15.3	24.3	25.5
W12	7.6	6.3	13.7	19.6	34.0	23.1	9.3
W6	12.5	13.1	17.6	14.7	22.4	16.8	26.4
Total	9.5	8.4	18.7	15.2	24.1	21.3	20.4

(A) Larvae fed with *Artemia* nauplii; (W12) larvae submitted to late and (W6) early weaning starting at 12 and 6 dpff, respectively.

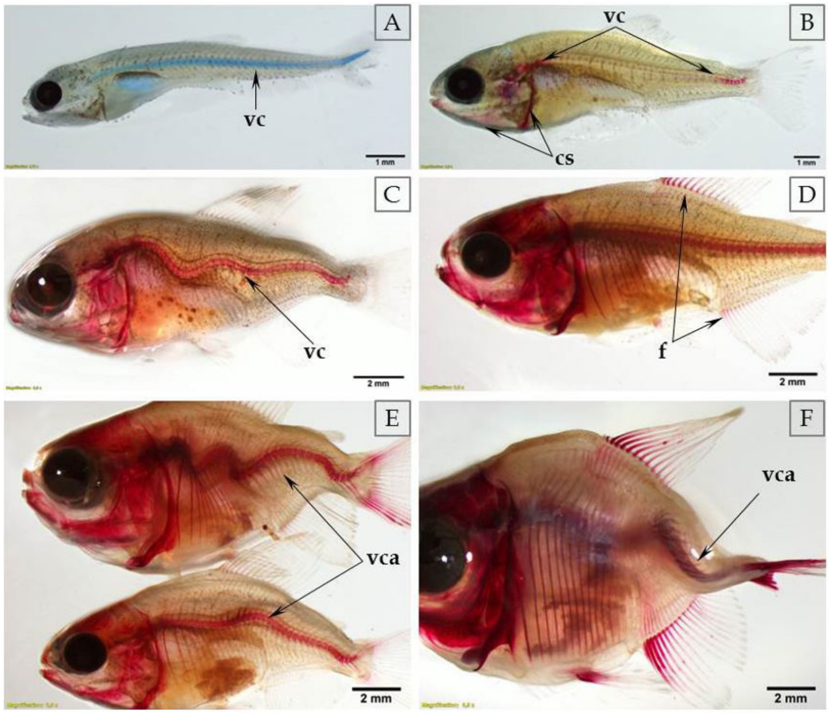

Figure 1. (A) Overview of pacu larvae (*P. mesopotamicus*) at 6dpff (total length mean values for the larvae of each treatment: (A) 9.4 ± 0.2 mm; W12 = 9.6 ± 0.2 mm; W6 = 9.4 ± 0.1 mm), blue area = cartilage, vertebral column (vc); (B) Ossification process (red area = ossification) of cranial structures (cs) and vertebral column (vc) in larvae at 12th dpff (A = 13.3 ± 0.2 mm; W12 = 13.0 ± 0.3 mm; W6 = 11.5 ± 0.4 mm); (C) Skeletal abnormality in the vertebral column (vc) in larvae at 18th dpff (A = 17.2 ± 0.5 mm; W12 = 14.4 ± 0.4 mm; W6 = 12.0 ± 0.6 mm); (D) Ossification process of fins (f) displayed in larvae at 24th dpff (A = 21.0 ± 0.3 mm; W12 = 18.0 ± 0.9 mm; W6 = 15.5 ± 0.4 mm); (E and F) Severe vertebral column abnormally (vca) observed in pacu larvae submitted to early weaning (W6 = 25.2 ± 1.4 mm) and larvae fed with *Artemia* nauplii (A = 31.8 ± 0.9 mm), respectively, at 42th dpff.

Exploratory analysis

With the skeletal anomalies incidence, length and weight data, the cluster analysis revealed a proximity between treatment A and W12, beyond distancing both from the W6 treatment (Figure 2). These observations corroborate with the results obtained by the univariate analysis and the mean multiple comparisons tests.

Differently, the exploratory simple correspondence analysis did not reveal any significant associations among different feeding protocols and the incidence of skeletal anomalies, as observed in the univariate analysis. The general Chi-square value was 21.139 (P = 0.173), rejecting the possibility that feeding protocols are significantly associated to the development of skeletal anomalies.

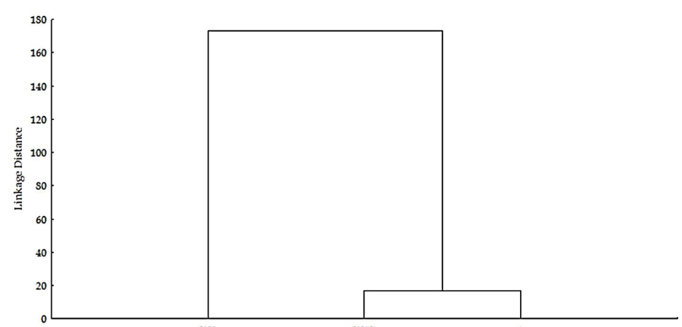

Figure 2. Dendrogram obtained with the cluster analysis displaying proximity between treatments A and W12, distancing both from treatment W6. (A) Larvae fed with *Artemia* nauplii; (W12) late weaned larvae starting at 12 dpff and (W6) early-weaned larvae starting at 6 dpff.

DISCUSSION

Several studies demonstrated the inefficiency of using formulated diets as first feed for fish larvae (Tesser et al., 2005; Jomori et al., 2008; Leitão et al., 2011; Menossi et al., 2012), as verified in this study (D treatment). When considering intensive production systems, evaluating the minimum time needed to substitute live to formulated feed is mandatory, in order to increase larvae's zootechnical performance and reduce production costs (Jomori et al., 2005; Portella et al., 2014). In the present study, the larvae subjected to an early weaning protocol (W6) presented lower growth performance in comparison to larvae fed exclusively live feed (A) and larvae that underwent late weaning (W12). At the beginning of weaning, these larvae presented an average of 4 mg, which was inferior to 10 mg indicated by Jomori et al. (2008) as the ideal weight to start the weaning process. Additionally, these organisms were considerably young, which might indicate that their digestive system was not completely developed, and with a rapid supply of the formulated diet, both digestion and nutrient assimilation were hindered, as suggested by Menossi et al. (2012). Jomori et al. (2005) compared the performance of two commercial diets and two experimental diets applied during the weaning process of pacu larvae and observed the influence of diets quality, showing that the best quality diet promoted similar growth both in larvae that initiated weaning at 12 and 21 dph. This high quality diet has also resulted in larvae about twice as heavier than the ones that received live feed (*Artemia* nauplii). In this study, the high quality formulated diet was adequate for larvae growth, in comparison to larvae fed only *Artemia* nauplii, however, when offered from the beginning of feeding, larvae were unable to grow (treatment D).

Besides the lower growth performance, W6 larvae also presented low survival rates (25.7 ± 8.5%), which indicates that these organisms were not able to stop being fed by live feed at an early stage. Different authors suggest that fish larvae in its early life stages are incapable of digesting complex proteins, of high molecular weight (Carvalho et al., 2003), and therefore the formulated diet must contain high amounts of easily digestive amino acids, in order to meet their nutritional needs (Aragão et al., 2004). In a similar experiment, Freitas (2015) observed that pacu larvae with about 3.2 mg presented incipient differentiation of the stomach and absence of pyloric cecum, despite the presence of the first gastric glands. The author has also verified that larvae of this size presented insignificant activity of acid proteases, which increased significantly in larvae with 11.2 mg. In this sense, at the beginning of the early weaning, larvae from the treatment W6 probably still did not have the digestive apparatus completely formed and were not capable to digest the formulated diet, compromising its homeostasis and reflecting in lower growth and survival rates. On the other hand, the larvae of the W12 treatment showed compensatory growth after weaning, evidenced by SGR (17.9% day^{-1}) during the period of 24-30 days of experiment, and at the end they displayed similar growth in comparison to the larvae fed exclusively *Artemia* nauplii. A similar growth condition was observed in a weaning study with pacu larvae after *Artemia* nauplii suppression, in which the authors observed growth of 46% in weight in five days (Tesser et al., 2005).

Nutritional deficiencies are also related to the emergence of skeletal anomalies, especially in young organisms, such as fish larvae (Cahu et al., 2003; Lall and Lewis-McCrea, 2007), as essential nutrients are directly involved in osteogenesis (Cahu et al., 2003; Roo et al., 2009). However, despite the differences observed concerning growth performance and survival rates, the incidence of skeletal anomalies was similar among treatments, so this hypothesis alone does not explain the appearance of such anomalies. Additionally, none of the feeding protocols (A, W6 and W12) was significantly associated with any of the skeletal anomalies found, as revealed by the exploratory data analysis. As the appearance of skeletal anomalies also relates to genetic factors (Boglione et al., 2013b) and its incidence may increase in cases of poor nutrition - a factor that may trigger differential expressions of genes related to anomalies (Kause et al., 2007), we hypothesized that the malformations found in all larvae used in this study may be related to genetic and/or other unknown epigenetic factors.

Any study up to now has investigated the possible effects of weaning on the occurrence of skeletal anomalies throughout the first days of development, and the anomalies found in this study were similar to the ones previously registered by Lopes et al. (2014) in pacu larvae obtained from a wild-caught broodstock. Similarly, these larvae were also fed *Artemia* nauplii throughout 30 days post-hatch and the authors verified that the occurrence of anomalies was not concentrated in any specific developmental stages. In our study, anomalies were found since the first samples (6 dpff, or 11 dph), and the first occurrence of cranial anomalies (lower jaw) occurred at 24th dpff (29 dph). The most common anomalies observed were found in the vertebral column, represented by lordosis (7.4%), scoliosis (4.7%) and kyphosis (2%), as verified by Lanes et al. (2012) in *Gadus morhua* larvae. Differently, Lopes et al. (2014) observed a greater incidence of anomalies in the upper (7.8%) and lower (3.1%) jaws in pacu larvae obtained from wild breeders, while in the present study, lower incidences of these anomalies were found: 0.5% and 0.4%, respectively. Additionally, Lopes et al. (2014) suggested that the incidence of upper jaw or vertebral column anomalies negatively influenced growth and development of pacu larvae, by directly affecting feeding and swimming performance. Likewise, Wittenrich et al. (2009) clearly showed that such anomalies affected growth by hampering swimming performance and makes it difficult to catch feed, in larvae of *Centropomus undecimalis*. Our results refuted the hypothesis that weaning protocols might affect skeletal development by displaying more anomalies, however the zootechnical performance of the larvae was directly affected by such protocols, as growth was hampered in early-weaned larvae.

In general, although apparently of greater severity, the occurrence of the anomalies in pacu larvae registered in this study was similar to the one observed by Lopes et al. (2014) and do not seem to be significant when compared to the high frequencies observed in other freshwater or marine species (Boglione et al., 2001; Izquierdo et al., 2010; Boglino et al., 2012; Lopes et al., 2018). Nevertheless, even in low incidence, skeletal anomalies are considered a problem regarding economic losses to fish farmers, since deformed fish tend to be discarded. Moreover, these anomalies may occur more frequently with the intensification of rearing systems, caused by the incapability of homeostatic mechanisms to compensate induced stress by the production environment and/or altered genetic factors (Boglione et al., 2014).

CONCLUSION

The register of the incidence of skeletal anomalies serve as a base to future studies on the occurrence of skeletal malformations and its possible causes in this species. Both early and late weaning protocols did not associate with the incidence of skeletal anomalies in pacu larvae; however, the adoption of late weaning, at 12 days of feeding, did not impair growth and survival of these organisms.

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