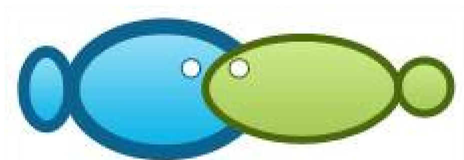

Embryonic development and performance of *Metynnis orinocensis* larvae reared in systems with biofloc technology

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Abstract. Knowledge of the fish reproductive strategy and embryonic development has great importance, which allows the determination of the morphological processes in a progressive way to adjust the management techniques during the induced reproduction, incubation and larval rearing phases. The dollar fish (*Metynnis orinocensis*) is native to the Colombian Orinoquia basin with great ornamental potential for the international market. Their backward fish farming forces them to experiment with intensive modern and sustainable farming systems. In this context, the description of *M. orinocensis* embryonic development was made and the performance of larvae cultured with biofloc at three different carbon/nitrogen (C/N) ratios was evaluated. For the measurement of time and correlation with morphological events, oocyte samples were collected during incubation. *M. orinocensis* larvae were cultured in three Australian tanks (5000 L), biofloc was previously established at experimental C/N ratios: 10/1, 15/1 and 20/1. Each treatment had three experimental units (floating cages with 8.3 L useful), in which the larvae (144 hours post hatch) were stocked at a density of 10 larvae L^{-1}. Embryonic development consisted of 5 phases: blastomeration (0-2.50 hour post fertilization, HPF), blastulation (3-7 HPF), gastrulation (8-14 HPF), organogenesis (15-42 HPF) and finally culminating with hatching (46 HPF). After 40 days of larvae rearing, the growth performance was statistically higher in the CN15 compared to the other treatments, however C/N ratio didn't alter on dollar fish survival. In conclusion, the embrionic stage of *M. orinocensis* lasts 46 hours, and larviculture in biofloc is recommended at a C/N ratio of 15/1, obtaining better water quality condition and growth performance.
Key Words: biofloc, carbon nitrogen ratio, embryology, larviculture, *Metynnis orinocensis*.

Introduction. *Metynnis orinocensis* (Steindachner, 1908), known as dollar fish, is a native fish species belonging to the Serrasalmidae family of the Characiforme order, which is geographically distributed in the Orinoco and Amazon river basins; standing out among the ornamental species for its beauty, its reproductive potential, its high acceptance in the national and international market (Arias-Castellanos 2008). However, the commercialization of ornamental fish in Colombia (more than 90%) is based on individuals extracted from the natural environment, an action that causes a significant reduction of fish resources with economic potential (Landines-Parra et al 2007). Such a panorama can be attributed to the ignorance of the biological aspects of the species for their induced reproduction, incubation management and production in captivity (Moncaleano-Gómez et al 2018; Velasco-Garzón & Gutiérrez-Espinosa 2020). This leads to conducting studies to improve reproductive development and intensify production, guaranteeing growth and survival.

During the embryonic and larval phase in native fish species, alterations in environmental conditions are very critical. The study of embryonic processes in fish facilitates the temporary identification of morphology in the process of formation of a new embryo and organism, which helps in the management of incubation and larvae production (Luz et al 2001; Valbuena-Villarreal et al 2012). The embryonic phase in fish is comprised of morphogenetic processes that occur from fertilization to hatching,

comprising 5 stages: blastomeration, blastulation, gastrulation, organogenesis and hatching (Kimmel et al 1995; Hernández-Cuadrado et al 2016).

Added to the above, the deterioration of water quality and the low survival found in captivity, especially in early stages, generates the need to investigate new intensive farming systems such as biofloc technology, tending to the production of larvae and fingerlings of fish. Biofloc technology (BFT) is basically based on the manipulation of bacterial communities (autotrophs and heterotrophs) in the presence of a carbon source with a high carbon/nitrogen (C/N) ratio in the water, thus degrading organic residues and inorganics, control water quality and the toxic forms of N (NH_3 and NO_2^-) at non-lethal levels to allow the intensive production of aquatic organisms in a sustainable manner (Kubitza 2011; Collazos-Lasso & Arias-Castellanos 2015). Additionally, BFT generates in situ a microbial protein that can be used as food by the cultivated species (Browdy et al 2012; Monroy-Dosta et al 2013; Ekasari et al 2014; Avnimelech 2015).

The objective of the present study was to characterize the events of the embryonic development of *M. orinocensis* and to evaluate the performance of larvae cultured with biofloc at different carbon/nitrogen (C/N) ratios.

Material and Method

Description of the study sites. The present study was carried out from July to August 2021 at the biofloc bioassay unit, wich is part of the Aquaculture Institute of the University of the Llanos, located at an altitude of 418 m.a.s.l. in Villavicencio - Colombia.

Induced reproduction of M. orinocensis. For the induced reproduction of *M. orinocensis*, males of 71.9±6.3 g and females of 88.0±11.4 g kept in captivity were selected, which were treated with carp pituitary extract (CPE) to induce final gonadal maturation, ovulation and spermiation. For this purpose, a two-dose protocol was used, in which 7.7 mg kg^{-1} of CPE was administered intramuscularly with an interval of 24 hours: the preliminary dose of 0.7 mg kg^{-1} (10%) and the definitive of 7 mg kg^{-1} (90%), both for females and males. The males were placed in aquariums individually to prevent them from stimulating each other and expelling semen. The females were kept in 250 L plastic tanks, with permanent water aeration and installed thermostats guaranteeing a temperature of 27.0±0.5°C; pH of 6.5±0.5 and dissolved oxygen of 5.5± 0.5 mg L^{-1}.

Spawning was carried out by extrusion 16 h (latency period) after the application of the second dose of CPE, by means of a cephalo-caudal massage of the ventral part of the female (Villamil-Moreno et al 2008). Semen was collected with an insulin syringe after spermiation, immediately spread over the ova, mixed, and activated with the addition of water. Once hydration occurred, the fertilized eggs were transferred to the horizontal flow incubator with constant recirculation. The incubation water was prepared by adding 1 g L^{-1} of NaCl, 1% methylene blue, and the temperature was regulated with the help of thermostats, in order to prevent damage to the fertilized ovules by fungal attack. The incubation of the gametes took place under the following water quality conditions: temperature of 27.95±1.26°C; pH between 6.27-6.61; dissolved oxygen of 5.80±0.58 mg L^{-1}; conductivity of 2077.14±822.79 µS cm^{-1}; salinity of 1.11±0.06 g L^{-1}; alkalinity of 20.71±5.35 mg L^{-1} and total hardness of 18.57±2.44 mg L^{-1}.

Sample collection for embrionic development description. To describe the morphological events of the embryonic development of *M. orinocensis*, which spanned from gamete fertilization to hatching, embryo samples were collected and immediately fixed in 4% buffered formalin with an interval of 15 minutes from the date of hatching. 0 hour post fertilization (HPF) until 2 HPF, then every 30 minutes from 2.50-5 HPF and finally every hour from 6 HPF until hatching (Valbuena-Villarreal et al 2012). The embryonic development of each of the samples was photographically recorded and analyzed in a Nikon SMZ800 stereoscope.

Biofloc preparation. Prior to larviculture process, the biofloc was established and stabilized in three Australian tanks (macrocosm) with a useful volume of 5000 L at

different C/N ratios (10/1, 15/1 and 20/1), adding a N source (fish food, 32% CP) at a total ammoniacal nitrogen (TAN) concentration of 2 mg L^{-1} and an organic carbon source (molasses, 33.65% C) during the first 7 days according to Fauji et al (2018). To maintain alkalinity > 100 mg L^{-1}, sodium bicarbonate ($NaCaCO_3$) was added as an inorganic carbon source. The tanks were protected by a greenhouse with an 80% polyshade mesh and constantly aerated through Aero-TubeTM diffuser hoses powered by a blower (1 HP). The water quality was monitored until biofloc stability was achieved, that is, until the concentrations of TAN and nitrites (NO_2^-) were within permissible ranges for fish larvae (Collazos-Lasso et al 2021b).

Larviculture experiment condition. The treatments consisted of the three C/N ratios: 10/1 (CN10), 15/1 (CN15) and 20/1(CN20), which were evaluated in triplicate. For this, 9 experimental units were distributed (floating cages with a useful volume of 8.3 L, covered with 650 µm SEFAR® mesh and arranged in Australian tanks), where the larvae of *M. orinocensis* were sowed 144 hours after hatching (HPH, length: 8.82±0.21 mm and weight: 4.91±0.25 mg) at a density of 10 larvae L^{-1}, with prior acclimation of two hours to the biofloc condition.

Daily, the larvae were fed with pulverized food of 32% protein at a rate of 40% of the initial biomass, distributed in eight rations (08:00, 09:00, 10:00, 11:00, 14:00, 15:00, 16:00 and 17:00 hours) and the C/N ratio was adjusted with molasses taking into account the approaches described by De Schryver et al (2008) and Avnimelech (2015). It should be noted that for the treatments with a C/N ratio of 10/1, an organic carbon source was not added, taking into account that the balanced feed used supplied the necessary C/N ratio.

Water quality. Temperature, pH, and dissolved oxygen (DO) parameters were measured twice daily with a HANNA HI98194 multiparameter in each macrocosm tank. Total ammonia nitrogen (TAN, indophenol method), nitrite (NO_2^-, nitricol method), nitrate (NO_3^-, nitratest method), and alkalinity (single colorimetric method) were monitored weekly with a YSI 9500 photometer. Settleable solids (SS) were analyzed once a week with an Imhoff cone using the method described by APHA (1995).

Growth performance. The performance of the dollar fish larvae was analyzed at the end of the 40 days of culture, quantifying and weighing with an analytical balance (OHAUS DV215CD 210 g / 0.0001 g) all the surviving fish from each experimental unit. Final length (mm), final weight (mg), survival (%), specific growth rate (SGR, % day^{-1}) and final density (fish m^{-3}) were evaluated. For this purpose, the following formulae were used: survival = (final number of fish / initial number of fish) × 100; SGR = [(ln final weight – ln initial weight) / time (days)]; final density = (final number of fish × 1000) / volume of each experimental unit (liters).

Statistical analysis. For statistical analysis, one-way analysis of variance (ANOVA) was applied, followed by Tukey's test (5% significance level) when necessary. Data normality and homoscedasticity were verified using the Shapiro-Wilk and Levene tests (Zar 2010) respectively. Survival data analysis was subject to prior angular transformation. The statistical software used was InfoStat 2018 (Di Rienzo et al 2018).

Results and Discussion

Embryological description of M. orinocensis. The newly fertilized ova of *M. orinocensis* were spherical, non-adherent (demersal), telolecyte type (abundant yolk) and light yellow in color with a mean diameter of 2334±143 µm. Such characteristics were similar to the eggs of Serrasalmid fish such as the red hook, *Myleus rubripinnis* (Villamil-Moreno et al 2008). Díaz-Olarte et al (2010) observed an average diameter of 1680±34 µm in white cachama (*Piaractus brachypomus*) eggs. The size of the ovules can vary between species, which has an important influence on the development of the embryo and the incubation time, that is to say, the larger ovules have a slower development and

reach hatching in a longer period than the smaller eggs (Sargent et al 1987). Once fertilization and hydration occurred, the ovules showed a significant increase in their diameter with a translucent chorion and a differentiation of the pre-vitelline space (Figure 1A). The embryonic development of *M. orinocensis* presented a duration of 46 h, going through the different stages: blastomeration, blastulation, gastrulation, organogenesis and hatching, which is summarized in Table 1.

Table 1

Chronological description of the main morphological events during the embryonic development of dollar fish (*Metynnis orinocensis*)

Stage	*HPF*	*Description*	*Figure*
Blastomeration	0.25	Increase in the diameter of the ovule and formation of the previtelline space.	1A
	0.75	Migration of the cytoplasm and differentiation between the animal pole and the plant pole.	1B
	1.25-2.50	Segmentation or cell division of the animal pole and formation of blastomeres (2, 4, 8, 16, successively).	1C-1F
Blastulation	3.0	Discoblastula elevation.	2A
	7.0	Formation of the blastoderm and periblast, flattened blastomeres, and initiation of epibolic movement.	2B
Gastrulation	9.0	Formation of the germinal ring and epibolism 30%.	2C
	10.0	Beginning of embryo formation and epibolism 50%.	2D
	12.0	Epibolism 70%.	2E
	14.0	Closure of the blastopore.	2F
Organogenesis	18.0	Differentiation of the cephalic-caudal region.	3A
	28.0	Differentiation of somites and embryonic fin.	3B
	34.0-42.0	Emergence of the optic vesicle, otic, heart, otoliths, notochord; and separation of the caudal fin from the yolk sac.	3C-3D
Hatching	46.0	Chorionic membrane rupture.	3E

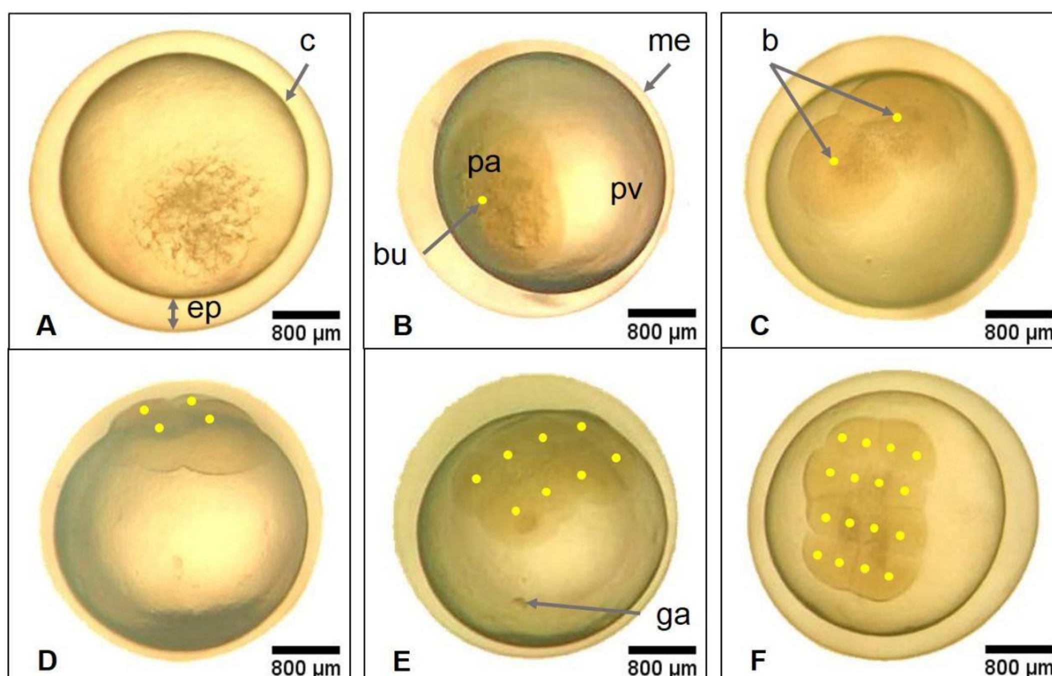

Figure 1. Blastomeration stage of *Metynnis orinocensis* (20x). A) Fertilized and hydrated oocyte, 0.25 HPF; B) unicellular blastodisc, 0.75 HPF; C) first cleavage: 2 blastomeres, 1.25 HPF; D) second cleavage: 4 blastomeres, 1.50 HPF; E) third cleavage: 8 blastomeres, 1.75 HPF; F) fourth cleavage: 16 blastomeres, 2 HPF. Chorion (c), outer membrane (me), previtelline space (ep), animal pole (pa), vegetal pole (pv), unicellular blastodisc (bu), blastomeres (b), oil droplets (ga).

The blastomeration was of the discoidal meroblastic type, observing at 0.75 HPF the migration of the cytoplasm, the unicellular blastodisc and the differentiation of the animal and plant poles (Figure 1B). The first cell divisions or cleavage in *M. orinocensis* followed a synchronous pattern of meridional and equatorial segmentation, evidencing the presence of oil droplets. These divisions began at the animal pole from 1.25 HPF, with the formation of 2 blastomeres; in the case of *Myleus rubripinnis*, the first cleavage was evidenced at 1.00 HPF (Villamil-Moreno et al 2008), being similar to those reported in the present study. The segmentation continued with the formation of 4, 8 and 16 blastomeres and finally making it difficult to observe and count more blastomeres at 2.50 HPF (Figure 1C-1F).

Blastulation covered a period of 4 h, from 3 HPF with the elevation of the discoblastula (Figure 2A) to 7 HPF with the formation of the blastoderm and periblast, presenting flattened blastomeres and initiating gastrulation (Figure 2B). This time varied among other species of the order Characiformes: while in *P. brachypomus* it lasted 2 h (Díaz-Olarte et al (2010), in the bocachico (*Prochilodus lineatus*) it was 3 h (Botta et al 2010), and in *M. rubripinnis* it was 4 h (Villamil-Moreno et al 2008).

Gastrulation began with the emergence of the germinal ring and the epibolic movement from 7 HPF until the closure of the blastopore at 14 HPF (Figure 2C-2F). The closure of the blastopore is one of the most important events in fish embryogenesis, which indicates the fertility of the eggs that will result in the formation of an embryo. In the case of *P. brachypomus*, this event occurred at 8 HPF (Díaz-Olarte et al 2010), in the black cachama (*Colossoma macropomum*) it was observed at 4 HPF (Leite et al 2013).

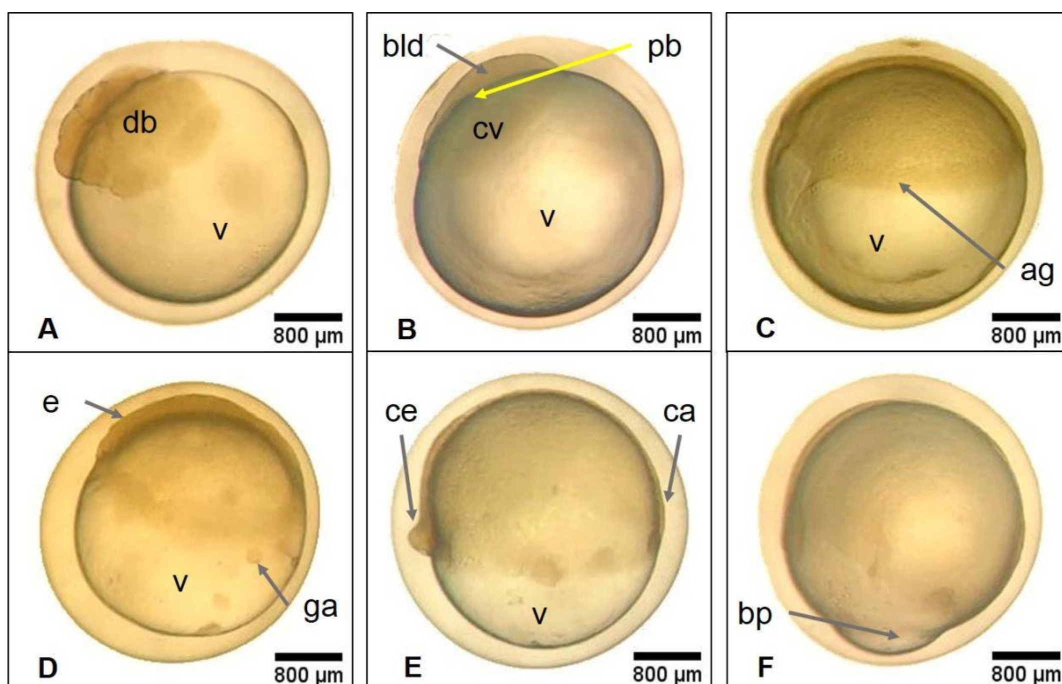

Figure 2. Blastulation and gastrulation stage of *Metynnis orinocensis* (20x). A) Discoblastula formation, 3 HPF; B) formation of blastoderm and periblast, 7 HPF; C) epibolism ≈ 30%, 9 HPF; D) epibolism ≈ 50%, 10 HPF; E) epibolism ≈ 70%, 12 HPF; F) closure of the blastopore, 14 HPF. Yolk (v), discoblastula (db), blastoderm (bld), periblast (pb), yolk cells (cv), germ ring (ag), embryo (e), oil drops (ga), cephalic region (ce), caudal region (ca), blastopore (bp).

The organogenesis phase occurred from 18 HPF to 42 HPF, characterized by the appreciation of the cephalic and caudal region, the emergence of organs such as somites, the embryonic fin, the optic and otic vesicle, the heart, the otoliths, the notochord and separation of the caudal fin from the yolk sac (3A-3D). Finally, embryonic development culminated at 46 HPF at a mean incubation temperature of 27.95±1.26°C, with the rupture of the chorionic membrane called hatching (Figure 3E).

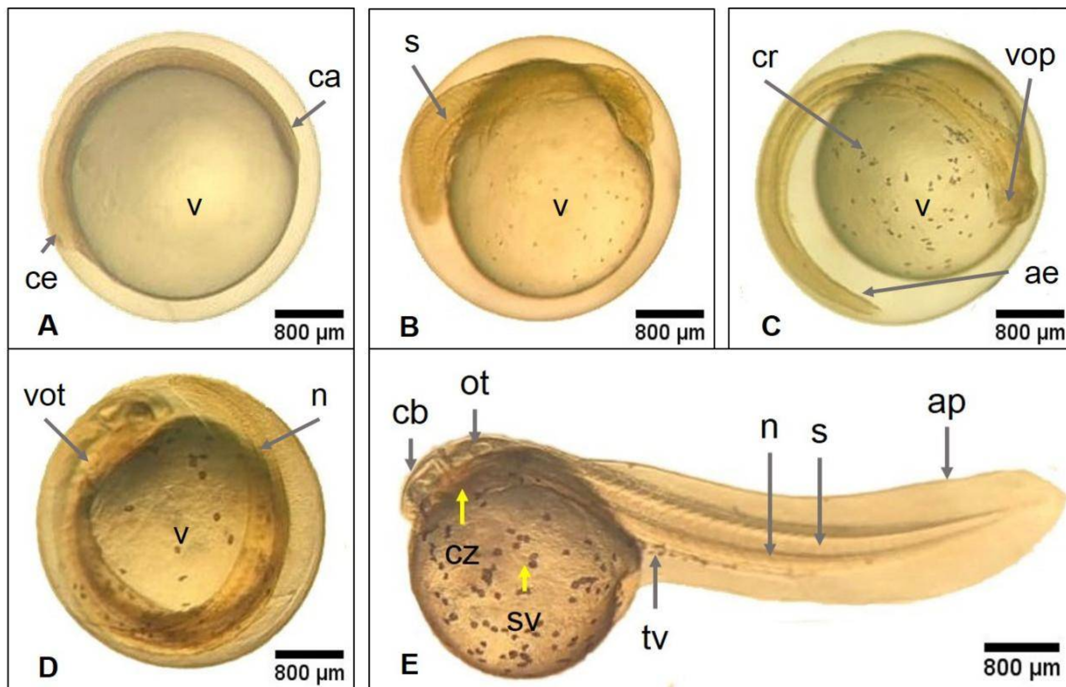

Figure 3. Organogenesis and hatching stage of *Metynnis orinocensis* (20x). A) 18 HPF; B) 28 HPF; C) 34 HPF; D) 42 HPF; E) Hatched larva of *Metynnis orinocensis*, 46 HPF. Yolk (v), cephalic region (ce), caudal region (ca), somites (s), chromatophores (cr), optic vesicle (vop), otic vesicle (vot), embryonic fin (ae), notochord (n), head (cb), yolk sac (sv), heart (cz), otoliths (ot), yolk tube (tv), primordial fin (ap).

The embryonic development time is largely related to the qualities of each family in question, the size of the oocytes and water quality conditions such as temperature during incubation, although this may vary. Ninhaus-Silveira et al (2006) indicates that, at higher temperatures, it has a shorter incubation time; while at lower temperatures this time is increased. Botta et al (2010) observed that hatching in *P. lineatus* occurred at 18 HPF at a temperature of 26°C. While Díaz-Olarte et al (2010) and Leite et al (2013) reported the end of embryonic development in *P. brachypomus* and *C. macropomum* at 13 HPF at a mean temperature of 26.9 and 27.5°C, respectively.

Dollar fish larviculture in biofloc system at different C/N ratios. During the larviculture stage, the average temperature of 26.88±1.1°C), dissolved oxygen (6.96±0.48 mg L^{-1}), and pH (7.63-8.43) were maintained at comfort levels for the production of fish larvae and fingerlings with biofloc in all treatments, as recommended by Poli et al (2015) and Emerenciano et al (2017). The levels of TAN, NO_2^- and NO_3^- were higher in CN10 compared to the other treatments and these levels decreased as the C/N ratio increased (Table 2). This panorama was also evidenced in the work of Panigrahi et al (2018) during the culture of marine shrimp (*Litopenaeus vannamei*) in a system with BFT at different C/N ratios. The alkalinity was different between the treatments ($p < 0.05$), increasing the concentration in proportion to the C/N ratio (Table 2). The fact that the alkalinity was lower in CN10 compared to the the other treatments which had carbohydrate contributions (molasses), could be due to a greater dominance of autotrophic nitrifying organisms. Because nitrification consumes alkalinity (in the form of $CaCO_3$) to oxidize nitrogenous waste, thus transforming TAN into NO_2^- and then NO_2^- into NO_3^- (Ebeling et al 2006); that is, a lower contribution of organic carbon or a lower C/N ratio could favor the activities of nitrifying bacteria, resulting in a greater consumption of alkalinity and higher levels of NO_3^- (Xu et al 2018). SS, the final product of heterotrophy, were statistically similar ($p>0.05$) between treatments (Table 2). However, the SS levels showed a raise as the C/N ratio increased, thus corroborating the greater heterotrophic activity in the treatments with higher contributions of organic carbon (Ebeling et al 2006; Xu et al 2018).

Table 2
Physicochemical variables of the water from the production of dollar fish seed (*Metynnis orinocensis*) reared in a biofloc system with different C/N ratios for 40 days

Parameter	Treatments			p-value ANOVA
	CN10	*CN15*	*CN20*	
TAN (mg L^{-1})	1.09±0.60^{a}	0.28±0.15^{b}	0.22±0.14^{b}	0.005
NO_2^- (mg L^{-1})	0.37±0.18^{a}	0.18±0.13^{ab}	0.09±0.08^{b}	0.021
NO_3^- (mg L^{-1})	62.21±14.53^{a}	25.15±9.34^{b}	20.55±10.59^{b}	< 0.001
Alkalinity (mg L^{-1})	84±10.84^{a}	145±11.73^{b}	170±18.37^{c}	< 0.001
SS (ml L^{-1})	2.68±4.72	5.20±9.44	9.82±7.09	ns

Note: Data are presented as the mean±standard deviation. Different letters in the same row indicate statistical differences ($p < 0.05$). TAN: total ammoniacal nitrogen, SS: settleable solids, ns: not significant.

Table 3 summarizes the performance of dollar fish obtained after 40 days of culture with biofloc at three C/N ratios. The survival and final density of *M. orinoquensis* larvae were not affected by the addition of organic carbon source (molasses). However, survival in the present study was below that reported in *P. orinoquensis* larvae by Collazos-Lasso et al (2021b), probably due to the low availability of planktonic communities in the biofloc. On the other hand, the dollar fish growth in terms of final length, final weight and specific growth rate (SGR) was statistically better ($p < 0.05$) in the CN15 treatment compared to the other treatments; these results being similar for the culture of altricial larvae in a biofloc system, as reported by Collazos-Lasso et al (2021a) and Collazos-Lasso et al (2021b). This could be attributed to two conditions: the water quality and the proximal composition of the biofloc (Ueno-Fukura et al 2020), presenting a favorable condition for a better performance of *M. orinoquensis* larvae when they are cultured with biofloc at C/N ratio of 15/1.

Table 3
Zootechnical parameters of *Metynnis orinocensis* grown in a biofloc system with different C/N ratios for 40 days

Parameter	Treatments			p-value ANOVA
	CN10	*CN15*	*CN20*	
Final length (mm)	14.12±0.36^{b}	17.42±0.62^{a}	15.08±1.31^{b}	<0.001
Final weight (mg)	112.17±6.98^{b}	148.86±24.90^{a}	75.19±19.22^{c}	<0.001
Survival (%)	42.97±4.56	34.14±12.66	25.70±2.51	ns
SGR (% d^{-1})	7.56±0.12^{b}	8.34±0.52^{a}	6.47±0.41^{c}	<0.001
Final density (fish m^{-3})	4297±456	3414±1266	2570±251	ns

Note: Data are presented as the mean±standard deviation. Different letters in the same row indicate statistical differences ($p < 0.05$). SGR: specific growth rate, ns: not significant.

Conclusions. This study has successfully documented reference information for induced reproduction with a detailed description of embryonic development and intensive seed production of *M. orinoquensis* in captivity with biofloc technology, which may be useful for the productive sector and future research. The C/N ratio has an influence on the performance of dollar fish larvae in a culture with biofloc system, suggesting better growth in the C/N ratio of 15/1, as long as adequate water quality and live food availability are maintained.

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Conflict of interest. The authors declare that there is no conflict of interest.

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