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Moments of induced spawning and embryonic development of *Brycon amazonicus* (Teleostei, Characidae)

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Summary

Based on the economic and ecological relevance of *Brycon amazonicus*, the goal of this work was to describe the diameter of oocytes and eggs of this species, as well as the chronological embryonic development. The material was provided by Buriti fish farm, Nova Mutum – MT, Brazil. Samples of both oocytes and eggs were obtained from extrusion to hatching. The material was fixed and measured under stereomicroscope, and the samples were divided for light microscopy or scanning electron microscopy (SEM) analyses. At extrusion, the oocytes were bluish green. The frequency distribution of oocytes revealed that 87.7% of them ranged from 1.11–1.30 mm in diameter. During incubation, the total diameter of the eggs increased from 1.22 ± 0.04 mm to 3.06 ± 0.46 mm in the first 60 min post fertilization (PF), and growth ceased at 180 min PF. Between 10–30 s PF, most eggs were fertilized and fertilization cones were observed from 10 s onwards after gamete activation. The main fertilization events took place asynchronically and spermatozoa were visualized in the micropyle vestibule up to 90 s PF. The first cell was formed in the centre of the blastodisc 20 min PF. The morula stage was identified 2 h PF and, 3 h later, 70% of the yolk was covered by the blastoderm; the blastopore was almost entirely closed at 6 h PF. The cephalic and caudal regions of the embryo could be defined 8 h PF and hatching occurred after 13 h of embryonic development. The larvae hatched with undifferentiated organic systems and with a large yolk sac, free from swimming abilities or visual acuity.

Keywords: *Brycon*, Development, Fertilization, Oocytes, Spawning

Introduction

The distinct reproductive strategies of fish results, among other features, in different sizes of eggs and/or oocytes between species, thereby leading to differential availability of energetic reserves for oogenesis and influencing the duration of embryonic development (Depêche & Billard, 1994).

Differences in the reproductive behaviour and oocyte diameter might be inter-specific or intra-specific, latitudinal, related to spawning period or else to individuals within the same reproductive period (Vazzoler, 1996).

The embryonic process starts with fertilization of the oocyte by the spermatozoon in the micropyle and involves the reorganization of egg components. In order to be understood, topological studies of fertilized eggs should be combined (Depêche & Billard, 1994).

The successful rearing of a fish species depends on knowledge about its early biology, including fertilization and embryonic development traits, which directly affect both the fertilization and the hatching rates. The description of embryonic stages in teleosteans, besides providing support to basic biology features of a given species, can be applied to fish culture and fisheries (Matkovic *et al*., 1985), by gathering useful information for fish mass production in laboratory

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or for systematics and environmental inventory (Reynalte-Tataje *et al*., 2004; Ninhaus-Silveira *et al*., 2006).

The species *Brycon amazonicus* is native from Brazilian Amazon (Lima, 2003) and had been cited as *Brycon cephalus* until recently, because of divergences in the taxonomic classification (Gomes & Urbinati, 2005). The broodstock of *B. amazonicus* in this study are from Guaporé River, Mato Grosso, where the species is locally known as 'jatuarana'. It is a large and humpbacked *Brycon* fish (up to 1 m in length and 8 kg in weight) with a goldish colouration on sides and dark on the back, found mainly in lakes downstream waterfalls (Feitosa, 2004).

Several research groups have studied the viability of fishes of the genus *Brycon* in both fish culture and repopulation of regions where they became threatened by margin deforestation, construction of dams and river pollution (Oliveira *et al*., 2004).

Taking into account the economic and ecological relevance of *Brycon amazonicus*, the goals of the present work were to describe the diameter of eggs and oocytes of this species, as well as the chronology of its embryonic development under controlled conditions in order to provide information about early biological features of this species that might be helpful for management and production programmes.

Material and methods

The samples were collected by Buriti fish farm, in the municipality of Nova Mutum, Mato Grosso, in December 2004.

During the studied period, the following physical–chemical water parameters were evaluated: temperature (mercury-in-glass thermometer), pH (digital potentiometer), dissolved oxygen (digital oxygen meter), alkalinity and ammonium, both determined according to Goltermann *et al*. (1978). The temperature was monitored daily at 6:00 a.m., 1:00 p.m. and 6:00 p.m., while the other variables were analyzed every 3 days early in the morning. To determine ammonium values, samples of water were frozen after collection and transported to the central laboratory of Centro de Aquicultura at UNESP for further analyses. The other analyses were carried out in the fish farm facility.

Induced spawning

Adult reproductively mature fish were induced to spawn, being the selected females and males placed separately in brick-made 2.0 m^2 tanks, with continuous water flow and covered with nets. The spawning was induced using commercial common carp hypophysis extract (dried in acetone). The females received the first hormonal dose at the time they arrived at the laboratory (0.5 mg/kg) and the second one after a 10-h interval (5.0 mg/kg), at the same time as the single application to males (1.0 mg/kg).

When courtship behaviour was noticed, the fish were anesthetized by immersion in benzocaine solution (2 g dissolved in 150 ml of 96% alcohol diluted in 20 l of water), being then submitted to abdominal massage for gamete extrusion. Dry fertilization (Woynarovich & Hórvath, 1983) was carried out by adding semen to oocytes followed by gentle mixing. Afterwards water was added for gamete activation and egg hydration, the eggs were rinsed and placed into 200-l incubators.

Sampling

A randomized experimental design was used. Biometric parameters, such as total length and weight, and reproductive data, such as volume of released oocytes, number of oocytes per ml and hatching rate, were obtained from 10 females and their respective offspring.

A sample comprised of 1 ml of oocytes was taken at the moment of extrusion from the 10 females induced to spawn, then fixed in 10% buffered formaldehyde and transferred to 70% alcohol after 24 h.

Samples that contained 10 oocytes and/or eggs were obtained at the following periods: extrusion; fertilization (when gametes were activated in water); 10 s, 20, 30, 60 and 90 s after fertilization (PF); each minute up to 10 min PF, each 5 min up to 30 min PF, 45 min PF and each hour up to hatching. Samples from five females were fixed in Karnovsky's solution (2.5% glutaraldehyde and 2.5% paraformaldehyde) for 24 h, then washed, transferred into cacodylate buffer, and stored at 4°C; the samples from the other five females were fixed in 10% buffered formaldehyde and transferred into 70% alcohol after 24 h.

Sample processing

The material was transported to the Faculdade de Ciências Agrárias e Veterinárias at Universidade Estadual Paulista (FCAV/UNESP), Jaboticabal Campus, São Paulo, and partly processed in the histology laboratory in the Animal Morphology and Physiology Department and partly in the electron microscopy laboratory.

To estimate the total number of extruded eggs, the number of cells in 1 ml, obtained and fixed when striping, was multiplied by the total volume of oocyte mass released by the 10 females induced to spawn. To calculate the frequency distribution of oocytes in diameter groups, 30 gametes from each female, collected at the moment of extrusion and fixed,

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Table 1 Mean values of physical–chemical water parameters during the sampling period, followed by their respective standard deviation (S), and maximum and minimum values

	O_2 (mg/l)	pH	Alkalinity (mg/l)	Ammonia (mg/l)	Temperature (°C)
Mean	6.70	6.80	77.33	0.061	28.08
S	0.29	0.07	4.80	0.022	0.36
Maximum	7.00	7.0	84.00	0.092	28.37
Minimum	5.91	6.7	72.00	0.038	27.33

Table 2 Reproductive parameters of *Brycon amazonicus* used in the induced spawning, followed by their respective standard deviation (S), minimum and maximum values

Reproductive parameters	Mean ± S	Minimum	Maximum
TL of females (cm)	48.10 ± 2.42	45.00	52.00
Weight of females (kg)	2.10 ± 0.20	1.80	2.45
Volume of extruded oocytes (ml)	183.00 ± 47.62	130.00	270.00
Number of oocytes per ml	1288.30 ± 102.52	1126	1406
Hatching (%)	87.20 ± 13.55	54	100

TL = total length, $n = 10$.

were measured (in mm) using a light stereomicroscope equipped with a micrometer (Leica MZ 8).

The size of eggs during incubation was analyzed using the offspring of six females, and defined by two measurements taken between fertilization and hatching, as follows: total diameter of eggs with perivitelline space (distance between yolk mass and chorion; CEP) and yolk diameter, disregarding the perivitelline space (SEP), both in millimetres.

To analyze the fertilization events based on scanning electron microscopy (SEM), four offspring were fixed in Karnovsky's solution, transferred to sodium cacodylate buffer, post-fixed in 2% osmium tetroxide for 2 h, washed again in buffer and dehydrated in a graded series of ethanol at 30, 50, 70, 80, 90, 95 and 100%. Afterwards, the samples were dried to the critical point in a liquid CO_2 drier (BAL-TEC), mounted on a copper grid, metalized with gold, and electron micrographed under a scanning electron microscope (JEOL-JSM 5410).

The alterations observed from oocyte extrusion up to larvae hatching were recorded by a light camera attached to the stereomicroscope (Leica MZ 95).

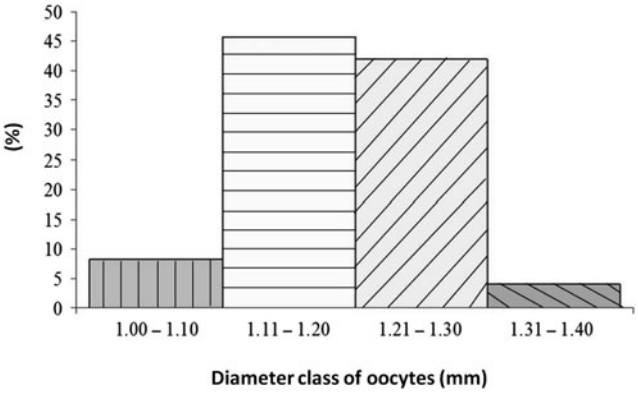

Figure 1 Frequency of oocytes per diameter class immediately after extrusion in *Brycon amazonicus.*

Results

The mean values of physical–chemical water parameters during the sampling period are shown in Table 1.

The biometric and reproductive data obtained during the induced spawning of *B. amazonicus* are presented in Table 2. At the moment of extrusion, the eggs were bluish green, spherical, translucent, semi-dense and presented a mean diameter among repetitions ($n = 30$) of 1.21 ± 0.06 mm.

The frequency distribution of oocytes released by *B. amazonicus* females, in length classes (Fig. 1), showed that nearly half of them (45.70%) had a diameter between 1.11–1.20 mm, with the great majority of female gametes (87.7%) being between 1.11–1.30 mm at the moment of extrusion.

From fertilization onwards, the total diameter of eggs of *B. amazonicus* increased quickly up to 60 min PF, changing from 1.22 ± 0.04 mm to 3.06 ± 0.46 mm within this period. This enlargement was progressive, but less accentuated, between 60 and 120 min PF, and reached 3.65 ± 0.42 mm, and continued slowly up to 180 min PF when it became stable (Fig. 2).

As the yolk (SEP) and total (CEP) diameter of eggs at 180 min PF remained the same as that observed

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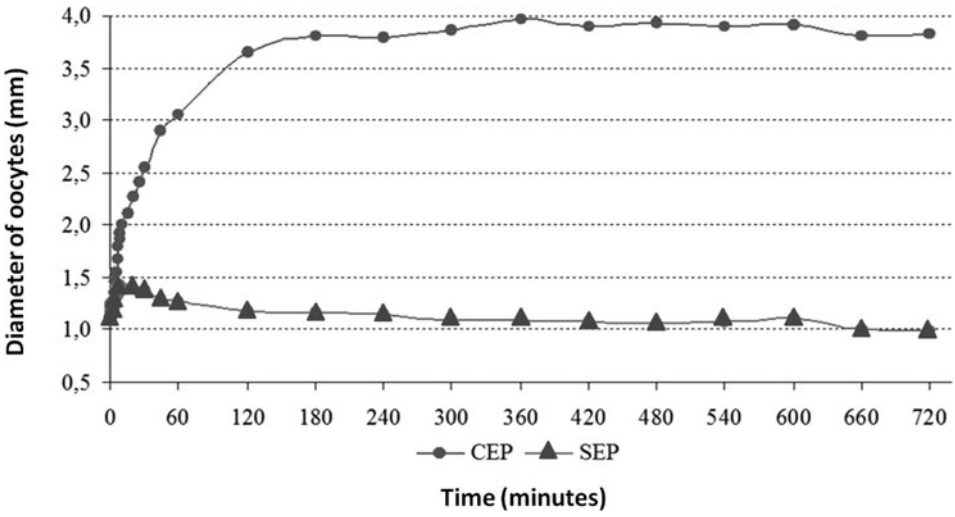

Figure 2 Mean diameter of eggs of *Brycon amazonicus* during incubation. (●) CEP: with perivitelline space; (▲) SEP: without perivitelline space.

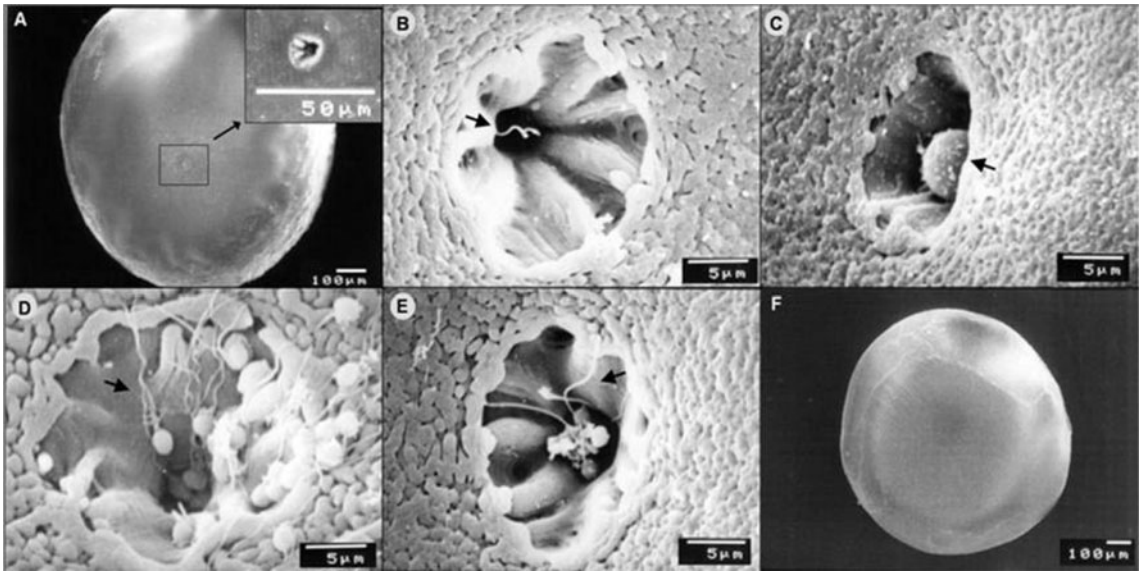

Figure 3 Electron micrograph of fertilization in *Brycon amazonicus*. (*A*) Morphology of micropyle (arrow). (*B*) Spermatozoon tail (arrow) penetrating the oocyte 10 s post fertilization (PF). (*C*) Formation of fertilization cone (arrow) at 10 s PF. (*D*) Spermatozoa (arrow) migrating towards the micropyle at 30 s PF. (*E*) Several spermatozoa (arrow) in the micropyle vestibule at 90 s PF. (*F*) Fertilized egg.

at fertilization (1.16 ± 0.13 mm and 3.81 ± 0.40 mm, respectively), the increase in the total diameter indicated a period of progressive hydration of eggs, leading to a larger perivitelline space after 3 h of incubation (Fig. 2).

The description of the events related to fertilization of *B. amazonicus* under scanning electron microscope revealed a single micropyle in each oocyte (Fig. 3*A*), characterized by a depression of chorion in the animal pole of female gametes, and resembled a funnel-like canal, with longitudinal fringes along the walls. At 10 s PF the penetration by male gametes had already taken place in several oocytes, as visualized in Fig. 3*B*, in which the spermatozoon tail can be seen at micropyle vestibule.

Also at 10 s PF, some eggs had already been fertilized and protected from polyspermy by the formation of a fertilization cone through the micropyle canal (Fig. 3*C*). At 30 s PF, many spermatozoa could be seen in the micropyle walls of some oocytes or moving towards this region (Fig. 3*D*), although the masculine gamete had already penetrated the cell and the fertilization cone was present earlier in most of sampled gametes.

Even though most analyzed eggs had been fertilized between 10 and 30 s PF, the fertilization events in

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B. amazonicus occurred asynchronically; at 90 s PF (Fig. 3*E*) some oocytes with spermatozoa in the micropyle vestibule were detected, but the tails of these masculine gametes were extended and detached from the oocyte surface, differing from the pattern observed in the first 30 s PF, and their viability was unknown. In Fig. 3*F*, a fertilized egg can be observed (10 min PF) displaying a flattened shape because of the cell movement towards the animal pole, giving rise to the blastodisc.

In Fig. 4, a few morphological alterations are observed in the female gametes between extrusion (Fig. 4*A*) and fertilization (Fig. 4*B*). A remarkable flattening of eggs was observed 10 min PF (Fig. 4*C*). The differentiation of blastodisc that typifies the animal pole, arranged over a large amount of yolk from the vegetative pole was confirmed 20 min PF (Fig. 4*D*).

At 30 min PF (Fig. 4*E*), the first division of blastodisc had already occurred, showing two blastomeres of similar sizes, followed by the formation of four and eight blastomeres at 45 min (Fig. 4*F*) and at 1 h PF (Fig. 4*G*), respectively. Successive cell divisions resulted in overlapping layers of blastomeres arranged in a 'half-berry' shape, characterizing the morula stage 2 h PF (Fig. 4*H*). At 3 h PF (Fig. 4*I*), a cell proliferation was detected with divergent movements between animal and vegetative poles and indicated the beginning of the epiboly and gastrula stages, covering about 30% of the yolk at this moment and reaching nearly 70% 2 h later (Fig. 4*J*).

The formation of the germ ring and closure of the blastopore were almost complete 6 h PF (Fig. 4*K*) and the development of embryonic axis and the neural tube was reported 7 h PF (Fig. 4*L*), being the cephalic and caudal regions defined 1 h later (Fig. 4*M*), when 8 –12 somite pairs were detected. The otic and optic vesicles, as well as the Küpffer's vesicle, could be identified 10 h PF, simultaneously to the detachment of tail from the yolk sac (Fig. 4*N*). The chorion rupture took place at 13 h of embryonic development, when non-pigmented larvae were released with undifferentiated organic systems (Fig. 4*O*).

Discussion

No remarkable variation in the physical–chemical parameters of water was observed during the studied period, and the mean values were close to optimal for development of tropical fish (Sipaúba-Tavares, 1995).

The number of released oocytes of females of *B. amazonicus* after induced spawning was comparable with that reported in previous studies for *B. cephalus* (Gomes, 1998), in which the oocyte mass corresponds to nearly 10% of body weight. The quantity of oocytes per gram is inversely correlated to their size (Vazzoler, 1996).

The overall aspect of *B. amazonicus* oocytes at the moment of extrusion was similar to that observed in fish of this genus. Spherical, translucent, semi-dense and greenish eggs were also detected in *B. cephalus* (Lopes *et al*., 1995; Romagosa *et al*., 2001; Vasques, 2003), *B. insignis* (Andrade-Talmelli *et al*., 2001b) and *B. orbignyanus* (Ganeco, 2003; Reynalte-Tataje *et al*., 2004).

The small diameter of oocytes at extrusion is another common feature as, in this study, the mean diameter was equal to 1.21 ± 0.06 mm, with nearly 90% of gametes ranging from 1.11–1.20 mm, close to the values reported for the same species by Pardo-Carrasco *et al*. (2006a), in which a diameter varying from 1.00–1.45 mm was detected, with modal values between 1.09–1.18 mm, in females induced with carp pituitary extract. These diameter values were close to those observed for *B. cephalus*, with a mean diameter of 1.010 mm (Romagosa *et al*., 2001) and 0.91 ± 0.015 mm (Vasques, 2003); *B. insignis* in which the most frequent diameter was equal to 1.45 mm (Andrade-Talmelli *et al*., 2002); and *B. orbignyanus*, with a mean value of 1.59 ± 0.15 mm (Landinez *et al*., 2004) with slight variation among species.

The oocyte size is related to the reproductive behavior, and both fecundity and diameter of mature oocytes are labile strategies, characterized by latitudinal, season, inter-specific and intra-specific variation, including differences among individuals of the same size within the spawning period. Migratory species with full spawning, external fertilization and no parental care, as the studied species, produce a larger number of small eggs (Vazzoler, 1996).

The fast hydration and development of a relatively large perivitelline space in the eggs of *B. amazonicus* after fertilization is another trait associated with the reproductive behaviour of *Brycon* fish, characterized by external fertilization and development without parental care, being the offspring left in the water column. The increased perivitelline space of eggs directly after fertilization indicates that the embryos are likely to be protected from environmental injuries (Ribeiro *et al*., 1995). The perivitelline space is formed even in non-fertilized eggs but activated by the contact between female gametes and water, which triggers out the cortical reaction that separates the chorion from the yolk once cortical alveoli are ruptured (Kunz, 2004).

Eggs with large perivitelline spaces after hydration were also reported in *B. cephalus* (Romagosa *et al*., 2001), *B. orbignyanus* (Reynalte-Tataje *et al*., 2004), and *B. insignis* (Andrade-Talmelli *et al*., 2002), as well as in other species from distinct genera of Characiformes with similar reproductive strategies, such as *Prochilodus lineatus* (Castellani *et al*., 1994) and *Leporinus piau* (Borçato *et al*., 2004). Those species that undergo reproductive migration in a specific

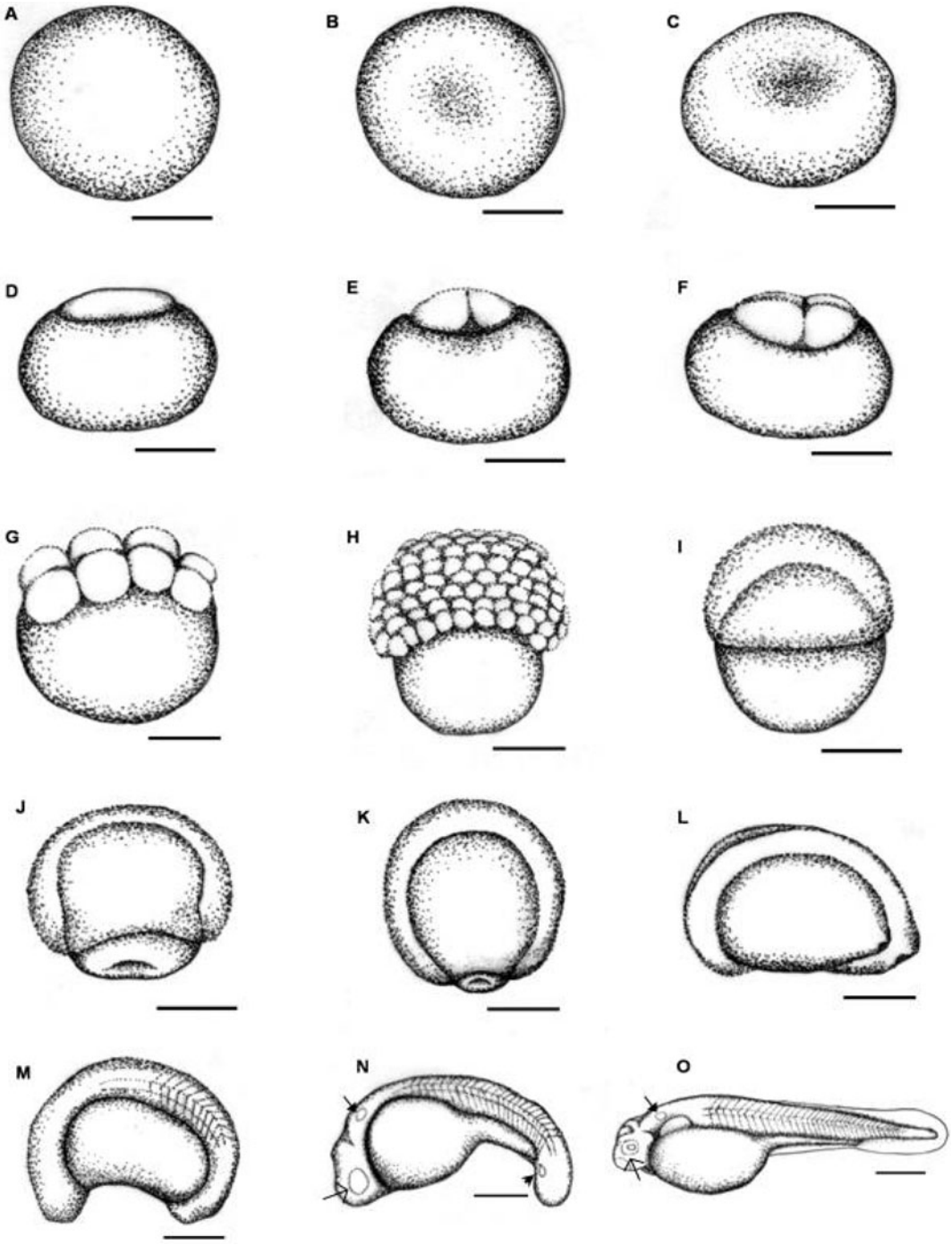

Figure 4 Embryonic development of *Brycon amazonicus*. (A) Oocyte (extrusion). (B) Fertilized egg. (C) 10 min post fertilization (PF). (D) 20 min PF. (E) 30 min PF. (F) 45 min PF. (G) 1 h PF. (H) 2 h PF. (I) 3 h PF. (J) 5 h PF. (K) 6 h PF. (L) 7 h PF. (M) 8 h PF. (N) 10 h PF. (O) 13 h PF (hatching). Otic vesicle (➞), optic vesicle (→) and Küpffer's vesicle (➤). Scale bar = 0.5 mm.

season and produce free eggs of fast hatching without parental care should be carefully monitored once any modification of hydrographic systems might affect their biological diversity (Andrade-Talmelli *et al*., 2002).

In teleosteans, the eggs are characterized as telolecithal because of the presence of animal and vegetative poles, being also referred to as polylecithal, once large amounts of yolk are detected, undergoing partial or meroblastic segmentation, restricted to the animal poles (Ribeiro *et al*., 1995). These features were observed in eggs of *B. amazonicus* in this study.

The asynchrony in the fertilization events observed in *B. amazonicus* has also reported in *B. orbignyanus*

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(Ganeco & Nakaghi, 2003) and *B. orthotaenia* (Sampaio, 2006). This behaviour along with micropyle morphology, the presence of a funnel-like micropyle vestibule and several straight-arranged grooves, are common reproductive features for these *Brycon* species. Funnel-like micropyles are observed in most teleosteans and they allow the passage of a single spermatozoon through the internal opening (Rizzo & Bazzoli, 1993). The micropyle microstructure is noteworthy as useful for both identification and phylogenetic studies of fishes, once differences can be found among species in the same genus or family, putatively acting as mechanisms to prevent interspecific hybridization (Chen *et al*., 1999).

The non-adhesive smooth chorion with regular pores reported in *B. orbignyanus* and *B. amazonicus* in this work is associated with the reproductive strategy of these species, i.e. migratory fish with free eggs (Rizzo *et al*., 2002). The micropyle morphology varies in freshwater teleosteans and these differences are related to variation in the morphology of spermatozoa, although a similar pattern can be observed within certain systematic groups (Ricardo *et al*., 1996).

As observed in *B. amazonicus*, the first event after gamete activation in *Prochilodus lineatus* was the entrance of a spermatozoon through micropyle, followed by the formation of the fertilization cone (Brasil *et al*., 2002). However, these events occurred asynchronically in the egg mass, i.e. different events were observed within the same sample as has been reported in *B. amazonicus*, in which spermatozoa were detected close to the micropyle vestibule in some oocytes at 30 s PF, while most eggs had already been fertilized and presented the fertilization cone.

The spermatozoa observed in the micropyle vestibule at 90 s after fertilization, displaying extended tail and detached from oocyte surface (a different disposition in relation to earlier moments) were probably unviable. Motility studies in spermatozoa of *B. amazonicus* revealed a short activity period of these cells, ranging from 26 to 50 s (Pardo-Carrasco *et al*., 2006b). Other studies on male gametes in fish of the genus *Brycon* have also reported a short activity period, being nearly 40 s in *B. insignis* (Andrade-Talmelli *et al*., 2001a) and 41 ± 7 s in *B. siebenthalae* (Cruz-Casallas *et al*., 2005). Analysis of seminal traits in *Brycon cephalus* classified the spermatozoa of this species as aquasperm, characterized by a small round head without acrosomal vesicle (Ninhaus-Silveira *et al*., 2006).

The formation of the fertilization cone right after the penetration of spermatozoon in the micropyle canal, as observed in *B. amazonicus*, occurs by cytoplasm movements that lead to cytoplasm projection into the micropyle canal, giving rise to a bubble-like structure (Kudo, 1980). When the cytoplasm content is projected into the micropyle canal, the spermatozoon nucleus stays in the base of expanding cone, being inserted in the oocyte cytoplasm (Kudo, 1980). The formation of this cone reinforces the evolution of strategies to avoid the access of extra spermatozoa that might potentially cross the micropyle canal even after the entrance of the fertilizing spermatozoon (Brasil *et al*., 2002).

The time of embryonic development in *B. amazonicus* described in this work was similar to that observed for the same species in a previous study (Mira-López *et al*., 2007), which reported an embryogenesis period of 13.5 h at 28.2 ± 1.5°C. The sequence of embryogenesis events in the species analyzed in the present work is also similar to that observed in other species of this genus, such as *B. insignis* (Andrade-Talmelli *et al*., 2001b), *B. cephalus* (Lopes *et al*., 1995; Romagosa *et al*., 2001; Vasques, 2003), *B. orbignyanus* (Ganeco, 2003; Landinez *et al*., 2004; Reynalte-Tataje *et al*., 2004) *Brycon orthotaenia* (Sampaio, 2006) and *Brycon gouldingi* (Faustino *et al*., 2010), as well as in other Characiformes, like *Piaractus mesopotamicus*, *Colossoma macropomum* (Ribeiro *et al*., 1995), *Prochilodus lineatus* (Castellani *et al*., 1994; Ninhaus-Silveira *et al*., 2006), *Leporinus piau* (Borçato *et al*., 2004), *Hoplias unitaeniatus*, *H. malabaricus* and *H. lacerdae* (Gomes *et al*., 2007). On the other hand, differences in the chronology of events were observed, even within *Brycon* species, what can be related to inter-specific variation or else to different water temperature during incubation.

In *B. amazonicus*, the beginning of somitogenesis took place after epiboly and closure of the blastopore, as has been reported in other Characiformes (Castellani *et al*., 1994; Lopes *et al*., 1995; Ribeiro *et al*., 1995; Andrade-Talmelli *et al*., 2001b; Ganeco, 2003; Borçato *et al*., 2004; Sampaio, 2006; Ninhaus-Silveira *et al*., 2006; Gomes *et al*., 2007; Faustino *et al*., 2010). In fact, epiboly movements in small eggs finish before the formation of somites (Ribeiro *et al*., 1995). In *Oreochromis niloticus*, the two first somite pairs appear before the end of gastrulation, and the tail portion of the embryo did not reach the vegetative pole, which according to the authors would be related to the large size of eggs in this species, typical of fish with parental care as cichlids (Perciformes; Morrison *et al*., 2001).

Conclusion

The results of this study showed that *B. amazonicus* presents a reproductive strategy that is common to most rheophilic species that perform spawning migration. They release a large number of small eggs in the water column and are susceptible to predators and adverse environmental conditions that fast develop a large perivitelline space that putatively acts as

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a mechanical protective mechanism for embryos that undergo a short embryonic period and thereby enhances their survival chances. The larvae hatch with undifferentiated organic systems, lack fins and present rudimentary eyes and large yolk sacs; they are unable to face environmental risks such as escape from predators and searching for food.

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Declaration of Interest

The authors declare that they have no competing interests.

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