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Morphophysiological characterization of the embryonic development of *Melanotaenia praecox* (Weber & de Beaufort, 1922)

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Summary

Dwarf rainbowfish (*Melanotaenia praecox*) are an ornamental fish with a high added value and considerable commercial interest. However, little information is known about the organism's reproductive biology, especially the early stages of embryonic development (initial ontogeny). *Melanotaenia praecox* embryos were examined throughout development to describe the dwarf rainbowfish initial ontogeny. Eggs were incubated at 28°C, and observations were recorded at pre-determined times. Development stages were identified and classified according to morphophysiological characteristics. The initial size of the eggs ranged between 0.99 and 1.04 mm. Oil droplets were observed in the eggs, and fixing filaments for adhesion were observed in the corium. Embryonic development was similar to that of other species in the genus *Melanotaenia*, in which hatching begins 119.50 h post-fertilisation or 3405.75 degree-h post-fertilisation. The main features of the newly hatched larvae were excellent swimming activity, a reduced yolk sac, mouth movement and an apparently functional digestive system.

Keywords: *Melanotaenia praecox*, Melanotaeniidae, Ontogeny, Ornamental fish

Introduction

Melanotaenia praecox, popularly known as the 'dwarf rainbowfish', is a species in the Family Melanotaeniidae with an intense, bright blue body and is originally from Australia and New Guinea. This species is sexually dimorphic; males are larger and more colourful with red dorsal, anal and caudal fins, whereas females are yellow-orange. The life expectancy of these animals is approximately 4–5 years (Fishbase, 2011).

The *M. praecox* spawning season is from October until December in their natural habitat, although under controlled laboratory conditions or cultured systems, spawning occurs spontaneously throughout the year, and hormonal induction is not necessary (VIDAL Jr., 2005). Taylor (1999) reported that spawning occurs over thin leafy plants (ceratopteris, cabombas, myriophyllum, ambulias, Java moss) or synthetic fibres. Thus, floating plants are used as a substrate for oviposition, and the eggs adhere to the roots. The eggs are small and usually observed adhering to plants and other substrates in the aquarium because of fixing filaments present in the corium (Reid & Holdway, 1995; Humphrey *et al.* 2003). Hatching occurs 7–8 days after spawning and is temperature dependent. After hatching and in the early stages of development, larvae are carnivorous, and their diet is composed of zooplankton (FishBase, 2011).

Dwarf rainbowfish are highly appreciated in the ornamental fish market and are sold at an average price of US$6.00. The commercial value, which is

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associated with the high capacity for production, has increased interest for this species and other melanotaenids. Therefore, information about dwarf rainbowfish reproductive biology is important to increase production, which can be achieved by studying the initial stages of development and ontogeny. Such studies are critical to increase knowledge about this species and will lead to the identification of proper incubation times. Accordingly, this work aims to describe the sequence of ontogenic processes in the species *M. praecox* during the development of embryos as well as the time required (in degree-h) for morphophysiological events.

Material and methods

Experimental animals

Eggs were obtained from the natural spawning of dwarf rainbowfish breeders (*Melanotaenia praecox*) comprising the aquaculture section breeding stock from the Support Unit for Research in Animal Science of the Laboratory of Animal Science and Animal Nutrition from the Center of Agricultural Science and Technology, State University of Norte Fluminense - Darcy Ribeiro, located in Campos dos Goytacazes – RJ, Brazil.

Breeders were kept in experimental indoor aquariums with recirculation systems. Fish of reproductive age were selected, with 15 males and 30 females divided randomly into three groups of five males and ten females each. Each group was housed in an aquarium with a capacity of 56.1 l.

The continuous and closed recirculation system contained a box for water filtration (by physical and biological processes) and a deposit box where water was held and drained with submersible pumps for return to the experimental aquarium. The water temperature was controlled with immersion heaters (armoured electrical resistance inserted in a glass tube with a thermostat) to maintain the temperature at about 28°C.

Experimental aquariums were equipped with independent input and output water systems at constant flux (approximately 2 l/min) of renewal to maintain a high level of oxygen and eliminate faeces, preventing plankton formation and possible sudden changes in water quality.

Stimulation of breeding and incubation of eggs

After a 7-day period for acclimation of the female breeders, two water hyacinths (*Eichhornia crassipes*) were placed in each aquarium to stimulate breeding and to serve as substrate for the eggs. Water hyacinths were observed every half-hour with the naked eye until eggs were observed adhering to the roots.

Eggs were removed from the roots of water hyacinths with scissors and tweezers, placed on glass slides and observed under an optical microscope at a magnification of ×25 or ×100 to characterize and identify the embryonic stage according to the number of cells. The diameter and height of the egg samples were measured. Height was defined as the distance from the animal pole to the diametrically opposed end, and the diameter was defined as the longitudinal axis of the egg. These two measurements were made with ocular glasses equipped with a micrometre scale.

Observation by optical microscopy

After initial observation, eggs were taken to the incubators, where a heater coupled to an automatic thermostat maintained the temperature at about 28°C with minimal variation. Aeration was provided in the incubators with porous stone coupled to the aeration pump.

After acclimatisation, the eggs were placed in floating sieves during the incubation period. The embryos were observed every half-hour during the initial period of 0–24 h, once per hour between 24–48 h, and every 2 h from 48 h until hatching. At each observation, some eggs were removed from the sieve, placed on glass slides, and observed under optical microscope (×25 and ×100). Digital images were subsequently taken with a DSC P-200 digital camera (SONY®) coupled to a light microscope. At the end of the experiment, the eggs were returned to their respective sieves. Each observation was accompanied with a notation of the corresponding degree-hour, and non-fertilised and/or mouldy eggs were noted and discarded to control the quantity.

Characterization of development and differentiation

The developmental stages and the main events in the differentiation of the embryo were identified and characterized at the moment that most eggs reached each event or stage. The classification of events was based on morphological characteristics according to the methodology used by Ferreira (2007) for the red rainbowfish (*Glossolepis incisus*) and Fujimoto *et al.* (2006) for the pond loach (*Misgurnus anguillicaudatus*). The stages occurring during the early developmental periods were classified according to the nomenclature proposed by Fujimoto *et al.* (2004), with cleavage characterized as the period between 2 and 64 blastomeres and the blastula period beginning at 128 blastomeres. Definitions for the blastula stages were adapted from Fujimoto *et al.* (2006). After hatching, the newborn larvae were visually assessed for swimming ability.

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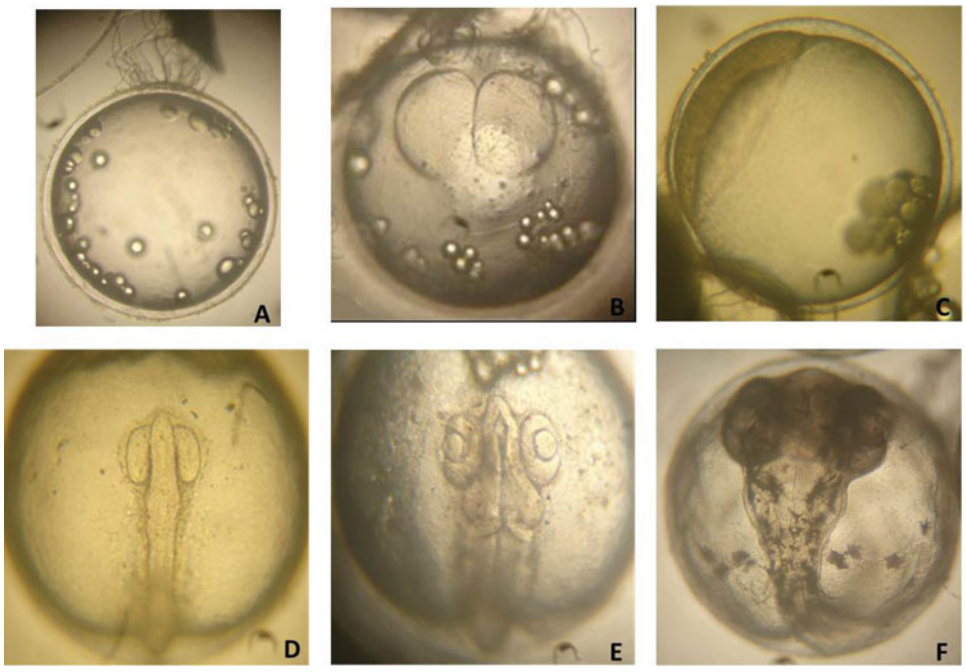

Figure 1 Stages of embryonic development. (*A*) Fertilised eggs with lipid drops; (*B*) 2 blastomere stage; (*C*) beginning of head and tail differentiation; (*D*) optical primordium; (*E*) chondrocranium and optic vesicle; and (*F*) melanophores and embryo's pigmentation.

Hours post-fertilisation (hpf) and degree-h post-fertilisation (hgpf) were used to measure time and to correlate morphophysiological events. Hpf corresponds to the time spent after fertilisation at hour 0 (H0 – initial), whereas hgpf corresponds to the sum of the temperature at every hour after the fertilisation of recently spawned eggs. The dissolved oxygen level and water temperature were measured using a digital oxymeter and thermometer, respectively, at each observation. The water pH was measured with a pH meter four times daily, at 6, 12, 18 and 24 h.

Results

The mean values observed for the physical and chemical parameters of water from the incubators during the trial period were 28.06 ± 0.49°C for temperature, 6.98 ± 0.15 for pH and 8.78 ± 0.85 mg/l for dissolved oxygen. The diameter of the newly fertilised eggs varied from 0.99 mm to 1.04 mm, with an average value of 1.02 ± 0.01 mm. The fertilised eggs of *M. praecox* (Fig. 1*A*) had a spherical shape, with a translucent corium and yolk sac. The eggs had adhesive filaments in a small area of the corium close to the animal pole that are used for adhesion to the spawning substrate. Eggs that were not fertilised appeared opaque. A meroblastic cleavage pattern that was similar to that of other teleost fishes was observed. Initially, the blastomere divided an average of every 30 min. The main stages observed during embryonic development are displayed in Table 1.

The cleavage period encompassed 0.50 hpf/14.0 hgpf at two cells (Fig. 1*B*) until 3.50 hpf/101.5 hgpf when 64 cells could be visualised. At this timepoint, oil droplets were also observed grouped at the periphery of the vegetative pole at the region opposite of the blastodisc.

The blastula stage began at approximately 4.00 hpf and 114.00 hgpf and consisted initially of divisions causing deformation of the blastodisc. Later, organisation of the blastodisc and migration of its edges onto the yolk sac was observed.

The oval stage was observed at 5.00 hpf/140.00 hgpf. At this stage, the movement of the blastodisc edge on the yolk sac together with the organisation of the blastomere generated the ellipsoidal shape of the embryo. However, at this stage, the observed syncytial layer was flat.

The equivalent to the spherical phase was observed at 5.50 hpf/154 hgpf, when the boundaries between the blastoderm and the yolk sac became continuous. At this point, the embryo had a spherical shape, and the syncytial layer was curved.

The gastrula stage, at 08.50 hpf and 242.25 hgpf, began with epiboly, which is the envelopment of the yolk sac by the blastoderm. The blastoderm was initially formed from a dome-shaped structure that covered 15% of the yolk sac. At 10.00 hpf/285 hgpf, the blastoderm covered 30% of the yolk sac, and

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Table 1 Main stages, embryonic events and hatching time of *Melanotaenia praecox* embryos

Stages	hpf	hgpf °C
Lipid drops	0.00	0.00
2 blastomere	0.50	14.00
4 blastomere	1.00	28.00
8 blastomere	1.50	42.00
16 blastomere	2.00	57.00
32 blastomere	2.67	77.33
64 blastomere	3.50	101.50
128 blastomere	4.00	114.00
256 blastomere	4.50	128.25
Oval phase	5.00	140.00
Spherical phase	5.50	154.00
Gastrula 15%	8.50	242.25
Gastrula 30%; embryonic axis differentiation	10.00	285.00
Gastrula 50%	10.50	299.25
Gastrula 80%	13.00	370.50
Gastrula 95%	14.00	392.00
Blastopore closure	15.75	448.88
Beginning of head and tail differentiation	16.00	464.00
Optical primordium	17.00	476.00
Somites (5), beginning of Kupffer vesicle formation	17.50	490.00
Beginning of chondrocranium and optic vesicle formation	23.50	669.75
Otic vesicle	31.00	883.50
Heartbeat	32.82	963.78
Melanophores	36.00	1026.00
Blood circulation along the embryo	39.00	1111.50
Totally formed heart	47.00	1316.00
Muscular contraction and movement	48.00	1344.00
Beginning of blood pigmentation and release of tail	49.00	1421.00
Embryo and pigmented optic vesicle	57.00	1567.50
Pigmented blood circulation	58.92	1649.67
Visible fin	68.00	1870.00
Pigmented hear	70.00	1925.00
Intense and pigmented blood circulation	83.00	2324.00
Mouth opening	10.00	2884.00
Pectoral fins movement	105.00	2887.50
Reduction of perivitelline space; embryo movement	115.00	3220.00
Beginning of hatching	119.50	3286.25
End of hatching	126.53	3542.93

the establishment of the embryonic axis could be visualised. At 10.50 hpf/299.25 hgpf, half of the yolk sac was covered with the blastoderm. The closure of blastopore occurred at 15.75 hpf/448.88 hgpf, when the edges of blastoderm came into contact and merged, covering 100% of the yolk sac.

As gastrulation ended, organogenesis began and was observed until the moment before hatching. During organogenesis, tissues and organs became differentiated. Differentiation of the head and tail (Fig. 1*C*) was observed at 16.00 hpf/464.00 hgpf, with specification beginning at the anterior region of the embryonic body, generating the cephalic region of the embryo.

The optical primordium was observed at 17.00 hpf/476.00 hgpf, and at 17.50 hpf/490.00 hgpf, five pairs of rudimentary somites in an elliptical shape were identified in the medial region of the embryo. The development of the optic vesicle progressed in the head, and opposite of the head, the Kupffer vesicle was observed, which according to Alves & Moura (1991) has excretory functions. Formation of the chondrocranium and the optic vesicle (Fig. 1*D*) was observed beginning at 23.50 hpf/669.75 hgpf. In addition, retinal differentiation of the optic primordium, early specification of the optic vesicle and the generation of the chondrocranium (Fig. 1*E*) in the cephalic region were visualised at this timepoint.

The embryonic heart was initially observed as a portion of the axial vein in the mesoderm that began peristaltic movement, which slowly began

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blood circulation. At the start of heart development at 32.82 hpf/963.78 hgpf, the average heart rate was 92 beats per minute.

Melanophores (Fig. 1*F*) appeared randomly along the embryonic axis at 36.00 hpf/1026.00 hgpf and were observed shortly thereafter in the yolk sac. Initially, these cells were dendritic, with several branches.

Blood circulation, previously verified along the embryonic axis, was observed to have a higher flow speed at 39.00 hpf/1111.50 hgpf, and blood vessel branches on the yolk sac promoted irrigation of the peripheral region of the yolk.

The heart of the embryo at 47.00 hpf/1316 hgpf had two distinct chambers beating in antagonistic systolic and diastolic movement, representing the atrium and the ventricle.

Muscle contractions were observed with a low frequency of spasm repetition at 48.00 hpf/1344.00 hgpf. At this point, the somites had formed a 'V'.

Blood began the process of pigmentation, going from colourless to red at 49.00 hpf/1421.00 hgpf. At this time, the caudal button was also released.

Differentiation of the optic capsule and the beginning of crystalline pigmentation were verified at 57.00 hpf/1567.50 hgpf.

The pectoral fins began to develop during embryogenesis. They were highlighted on the embryonic axis as a clear bilateral oval region and were symmetric with respect to the end of the cephalic region. In the present study, the exact onset of fin formation could not be observed; however, at 68.00 hpf/1870 hgpf, the fins were evident. Formation of the mouth was observed at 103.00 hpf/2884.00 hgpf. When opened, the movement of the mouth was slow and well spaced, and a single molariform tooth was also identified in the lower jaw on the symphysis region.

At the end of pectoral fin differentiation (105.00 hpf and 2887.50 hgpf), the embryos began moving the fins with fast and well spaced beats. Embryo hatching began at 119.50 hpf and 3286.25 hgpf and occurred until 126.53 hpf and 3542.93 hgpf. The emergence of larvae by disruption of the corium occurred very quickly. The newly hatched larvae were very active and possessed great swimming ability, sometimes rendering collection difficult.

Recently hatched larvae rapidly moved the opercula, mouth and digestive tract, and prominent otoliths were identified.

The newly hatched larvae still contained yolk residue, and within the residue, oil droplets were observed. Filling of the swim bladder was not observed in the newly hatched *M. praecox* larvae, but sinking did not occur when swimming stopped.

The average heartbeat frequency of the newly hatched larvae of *M. praecox* was 186.40 ± 26.95 per min.

Discussion

In this study, the eggs of *M. praecox* had an average diameter (1.02) similar to that of other Melanotaeniidae, such as *Glossolepis incisus* at 0.90 mm to 1.10 mm (Ferreira, 2007), *Melanotaenia splendida australis* at 1.07 mm (Ivantsoff *et al.*, 1988), *Melanotaenia nigrans* at 1.05 mm and *Melanotaenia splendida inornata* at 0.88 mm (Reid & Holdway, 1995).

The embryonic development of *M. praecox*, despite some significant differences when compared to other teleost species, has an obvious similarity to previous studies conducted with animals of the same family, such as *M. fluviatilis*, *M. s. inornata*, *M. s. australis*, *M. nigrans* and *M. s. splendida* (Crowley & Ivantsoff, 1982; Ivantsoff *et al.* 1988; Reid & Holdway, 1995, Humphrey *et al.*, 2003), indicating that the type of embryonic development observed and the genus to which the fish belongs are related.

The cleavage period at the 64 cell stage of the embryo was verified in this work at 3.50 hpf and 101.5 hgpf. Duarte (2009) noted this stage at 4.00 hpf and 112.00 hgpf for *Betta splendens* embryos, whereas Ferreira (2007) observed the sixth cleavage at 3.00 hpf and 84.00 hgpf for *G. incisus*.

The oval stage was observed at 5.00 hpf/140.00 hgpf and was close to the value described by Ferreira (2007), 5.83 hpf and 149.32 hgpf, for *G. incisus*, probably because they belong to the same family, whereas Duarte (2009) observed this stage at 7.00 hpf and 196.00 hgpf for *B. splendens*. According to Fujimoto *et al.* (2006), the embryos of *Misgurnus anguillicaudatus* had over 2,000 blastomeres at this stage.

The gastrula phase was visualised in 15% of embryos at 08.50 hpf and 242.25 hgpf. Reid & Holdway (1995) observed the beginning of gastrulation at approximately 13.00 hpf for *M. fluviatilis*, and Humphrey *et al.* (2003) reported that for *M. s. splendida*, the beginning of the gastrula phase was about 10.0 hpf. Ferreira *et al.* (2006) observed an early gastrula phase at 2.0 hpf that lasted approximately 3.50 hpf at 28°C for *Astyanax cf bimaculatus*. Nakatani (2001) observed that the gastrula phase in cultivated Brazilian rheophilic fishes occurred earlier than that observed for *M. praecox*.

Blastopore closure, when the edges of the blastoderm came together and merged, covering 100% of the yolk sac, occurred at 15.75 hpf/448.88 hgpf. Reid & Holdway (1995) reported blastopore closure in *M. nigrans* and *M. s. inornata* at 18.00 hpf, whereas Humphrey *et al.* (2003) reported it at approximately 12.50 hpf in *M. s. splendida*. Faster embryonic development has been observed in Brazilian rheophilic fishes, such as the embryos of *Brycon orbignyanus*, with blastopore closure at 6.50 hpf (Reynalte-Tataje *et al.*, 2004), or *Brycon insignis*, at 5.50 hpf/161.00 hgpf

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(Souza, 2004). Ferreira *et al.* (2006) also observed blastopore closure for *Astyanax cf bimaculatus* at 5.50 hpf, but these animals have a relatively shorter hatching period than melanotaenids and usually hatch earlier at a less advanced stage of body development.

During the organogenesis period, head and tail specification was observed at 16.00 hpf/464.00 hgpf. Head and tail differentiation of *Pterophyllum scalare* was also observed at 16.0 hpf (Radael, 2009), whereas in *G. incisus*, this specification occurs at 12.83 hpf (Ferreira, 2007).

Reid & Holdway (1995) observed the initial heart rate in *M. fluviatilis* at 72 beats per minute. According to the same authors, heart of *M. fluviatilis* began beating at 46.00 hpf, whereas Crowley & Ivantsoff (1982) observed the first heartbeats of *M. nigrans* and *M. s. inornata* at 47.00 hpf at 25°C. In this work, *M. praecox* initially possessed a heartbeat of 92 beats per minute on average at 32.82 hpf/963.78 hgpf, slightly earlier than that observed by other authors for melanotaenid species. This discrepancy might be explained by the 28°C incubation temperature of *M. praecox*, which may have accelerated the heartbeat process.

At the end of the differentiation of pectoral fins (105.00 hpf and 2887.50 hgpf), embryos began moving the fins with fast and well spaced beats. Ferreira (2007) described this embryonic event for *G. incisus* at 83.00 hpf. The timing of mouth opening and intense movement of the fins appears to be correlated in animals from the Melanotaeniidae Family, because hatching usually occurs very close to this event.

Embryo hatching began at 119.50 hpf and 3286.25 hgpf and continued until 126.53 hpf and 3542.93 hgpf. This period of embryonic development is considered relatively long when compared to national fishes like Cichlidae, Characidae, Anostomidae and others (Nakatani, 2001; Reynalte-Tataje *et al.*, 2004), whereas for animals of the genus *Melanotaenia*, the literature considers the period between 6 and 7 days of embryonic development as the normal time for hatching (Reid & Holdway, 1995; Humphrey *et al.*, 2003). The period of hatching for *M. fluviatilis* was observed to be between 7 and 9 days, and Humphrey *et al.* (2003) observed hatching between 4 and 8 days for *M. s. splendida*. Ivantsoff *et al.* (1988) observed that in *M. s. australis*, *M. s. inornata* and *M. nigrans*, all species of the genus *Melanotaenia*, the embryonic period lasted between 4 and 5 days at 26°C, whereas for *G. incisus*, a hatching period between 5 and 6 days at 28 °C was observed (Ferreira, 2007).

Disruption of the corium by the larvae occurred very quickly, similar to observations of *M. s. splendida* by Humphrey *et al.* (2003). Newly hatched larvae were very active and possessed excellent swimming ability, sometimes making collection difficult. This feature is similar in *M. s. splendida* (Humphrey *et al.*, 2003) and *M. fluviatilis* (Reid & Holdway, 1995).

The recently hatched larvae showed rapid movement of the opercula, mouth and digestive tract and had prominent otoliths, as observed in *M. s. splendida* (Humphrey *et al.* 2003) and *M. fluviatilis* (Reid & Holdway, 1995). Newly hatched larvae still contained yolk residue with droplets of oil. Filling of the swim bladder could not be observed in the newly hatched larvae of *M. praecox*, but sinking did not occur when they stopped swimming. Larvae of *M. s. splendida* (Humphrey *et al.*, 2003) fill their swim bladder at the moment of hatching.

The embryonic development of *M. praecox* was similar to the embryonic development observed for members of the genus *Melanotaenia*. In this work, *Melanotaenia praecox* hatched at 119.50 hpf and 3405.75 hgpf. Hatching occurs in fully formed animals. The opening of the mouth and anus were observed, as were peristaltic movements in the intestine, demonstrating that these animals are approaching the onset of feeding. Newly hatched larvae have few yolk reserves, excellent swimming ability and can float.

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