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Effect of temperature on embryonic development of *Melanotaenia boesemani* (Allen and Cross, 1982)

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Summary

The present study aimed to provide data on the time required for *Melanotaenia boesemani* to complete embryonic development, and to investigate the influence that incubation at different temperatures caused in this species. The effects of temperature on the time and hatching rate are presented, as well as information related to embryonic development stages. After fertilization, the eggs were kept in incubators at 23, 26, 29 or 32°C and observed at predetermined times until the moment of hatching. Stages of development were identified and classified according to morphological and physiological characteristics. Oil droplets were visualized inside the eggs as well as filament adhesion present at the chorion. Embryonic development was similar to that observed in other species of the genus *Melanotaenia* with hatching and faster development in higher temperatures.

Keywords: Melanotaeniidae, Ontogeny, Ornamental fish

Introduction

Embryos and larvae are very vulnerable to variations in physico-chemical parameters that occur in the aquatic environment (Laurence & Howell, 1981). These changes have a remarkable influence on many metabolic processes, and may directly affect the survival of fish, especially during the initial stages of development (Laurence, 1975). Water temperature is considered to be one of the variables that most affects the development of aquatic organisms (Rogers & Westin, 1981), due to its importance in the control of physiological processes (Blaxter 1992; Fuiman 2002). Both organogenesis and somatic development are controlled by enzymes, and these are affected by variation in temperature *in situ*.

The length of the embryonic period and the rate of consumption of the yolk are strongly influenced by the temperature of the water surrounding spawning (Petereit *et al.*, 2008), abnormal development of structures may occur if the yolk is exhausted before the embryo is ready to hatch.

Knowledge of embryogenesis is a useful tool for locating spawning areas, as well as the study of growth of aquatic species in the natural environment (Anjos & Anjos, 2006), and provides relevant information on cultivation.

The melanotenideos are fish that are widely used in aquariums and their numbers have intensified in the past decades. *Melanotaenia boesemani* is one of the species in this family that is cultivated and is one of the most appreciated and valued on the market (Rainbowfish, 2014). Despite being popular, there are almost no records about its early development. Given its large production potential and high market value, studies on this species have become increasingly relevant, particularly those that concern embryonic and larval development of the species. These are the areas that can lead to improvements in survival squad rate, especially in the hatchery, thus increasing the

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efficiency of production, and also serve as a basis for conservation work in the species' natural habitat.

The present investigation describes the sequence of ontogenetic processes during the development of *Melanotaenia boesemani* embryos, as well as the time required for the occurrence of morphophysiological events at each test temperature.

Materials and methods

Eggs obtained from natural spawns of *Melanotaenia boesemani*, from the aquaculture sector of Universidade Estadual do Norte Fluminense Darcy Ribeiro, were used. Fish were maintained at a ratio of one male to every female, spread over three tanks of 40 l each, at a temperature close to 28°C. In total, 30 males and 30 females were used.

In each tank, two water hyacinths *(Eichhornia crassipes*), which served to stimulate reproduction and are a substrate for oviposition, were placed. The water hyacinths were observed every 30 min until eggs were found adhering to the roots; for each observation at which the presence of eggs was not noted the water hyacinths were discarded and replaced.

Spawning occurred spontaneously and eggs were removed from the water hyacinth roots with the aid of scissors and tweezers. Eggs were placed on glass slides and observed immediately under an optical microscope (Nikon® Eclipse e200 at ×25 magnification) for characterization and identification of the embryo stage. The egg diameter was determined with the aid of an ocular Nikon® equipped with a micrometric scale.

After the initial observation, the eggs were counted and transferred to four incubators with a capacity of 35 l, in which heaters with automatic thermostats (Boyu®) were used to maintain a stable temperature. Each incubator was equipped with aeration using, for this purpose, porous stone. After acclimatization, 105 eggs were divided among three floating sieves for each treatment, and were kept there during the incubation period to eclosion.

Observation of the development of embryos was conducted as follows: (i) every 30 min from the time of fertilization until the end of the gastrula; (ii) every 1 h from the start of organogenesis until the emergence of the heart; and (iii) every 2 h from emergence of the heart until the time of hatching. At each observation, some eggs were removed from the sieves, placed on glass slides and examined under an optical microscope (×25 magnification). At the end of the observation, the eggs were returned to their respective sieves. The unfertilized eggs and/or those with fungus were discarded and noted for quantity control. In order to avoid the frequent handling of the same egg, an order for collection and return to the other sieve was kept, so that one group of eggs was not handled in a <12 h interval, based on the assumption that excessive handling could affect its development.

Embryonic development stages were identified and characterized from the time at which >50% of the eggs observed reached a certain stage of embryonic development. The classification of embryonic stages was made according to that proposed by Fujimoto *et al.* (2006) and adapted by Radael *et al.* (2013) for *Melanotaenia praecox*.

For assessment of time and correlation with morphophysiological events, measurement of hours post-fertilization (PFH) was used, fertilization being considered as time 0; only one cell in the egg was viewed.

For the determination of PFH for hatching and hatching rate after the period of description of the embryos, new spawning was conducted. This was maintained in the same conditions as the other incubators, however, were kept without manipulation, thereby representing more accurately the time required to hatch.

At every egg observation, water temperature was measured using a digital thermometer; oxygen was measured once a day in the morning using an oximeter. Water pH was measured using a digital pH meter four times per day, at 06.00 h, 12.00 h, 18.00 h and 00.00 h.

Descriptive statistics of variables was performed using the statistic program SAEG and Pearson correlation between the variables was calculated.

Results

The values observed for physico-chemical parameters of water from the incubator during the experimental period were 31.9 ± 0.052°C for temperature; 6.9 ± 0.022 for pH and 9.3 ± 0.193 mg/l for dissolved oxygen in the incubator at 32°C. For the incubator at 29ºC, the parameters were 29.0 ± 0.0307ºC for temperature; 6.9 ± 0.0081 to ± pH 9.3 and 0.2156 mg/l for dissolved oxygen. For the incubator at 26°C the parameters were 26.0 ± 0.0327ºC for temperature; 6.9 ± 0.0099 to ± pH 8.8 and 0.1745 mg/l for dissolved oxygen. In the incubator at 23ºC, the parameters observed were 23.0 ± 0.0314ºC for temperature; 7.0 ± 0.0100 to pH 8.0 and 0.1361 mg/L for dissolved oxygen. The values of the physico-chemical water parameters observed in incubators are considered indicated for this species.

The newly fertilized *M. boesemani* oocytes had a spherical shape with a chorion, a translucent yolk sac and were slightly yellowish. They were 1.09 ± 0.0158 mm in size. The eggs contained drops of oil of varying

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Table 1 Events and embryonic stages of *Melanotaenia boesemani* hours post-fertilization. incubated at 23, 26, 29 or 32°C

	Number of hours post-fertilization			
Event/stage	23°C	26°C	29°C	32°C
4 cell	0.83	1.33	1.50	0.83
64 cell	5.63	4.50	3.22	2.83
128-cell	6.13	5.50	4.22	3.83
256-cell	7.83	6.43	5.53	4.63
Gastrula 40	19.83	13.81	11.62	9.63
Gastrula 50	21.33	14.81	12.40	10.23
Gastrula 60	23.83	15.98	13.45	11.63
Differentiation of the embryonic axis	24.83	15.31	13.45	12.63
Blastopore closure	28.13	19.31	15.45	14.13
Differentiation of head and tail	29.17	20.81	15.95	14.63
Optic primordium	32.83	21.81	17.12	14.63
Optic vesicle	33.83	22.81	18.12	16.63
Kupffer vesicle	34.83	21.81	17.45	15.13
Emergence of somites	36.83	29.51	21.45	18.63
Chondrocranium	38.83	31.31	25.67	21.63
Melanophores	40.83	34.31	27.88	23.92
Optic vesicle	50.83	30.58	24.45	23.92
Otoliths	56.83	33.31	30.68	25.05
Heartbeat	62.83	36.31	29.12	25.05
Circulation	66.83	42.31	34.67	28.88
Fins	128.83	70.31	54.47	55.13
Release of tail	144.83	72.41	56.47	48.63
Movement of fins	206.83	114.31	88.47	77.93
Movement of eyes	240.83	130.31	84.40	77.93
Opening of the mouth	250.83	136.31	114.47	103.65
First outbreak	253.58	157.88	123.97	107.15
Last outbreak	342.30	251.56	167.32	117.65

size initially spread by the yolk sac. During embryonic development, these drops assembled into the vesicle region opposite the blastodisc.

The eggs had long, cylindrical and numerous filaments that originated from a certain area of the corium, near the animal pole. These filaments showed adhesive characteristics, as they adhered to the substrate with which they were in contact (roots of water hyacinth, tweezers etc.) and therefore had the task of joining the egg to the support during spawning and the incubation period. Unfertilized eggs were easily observed due to their opaque white appearance.

M. boesemani eggs could be classified as telolecitos as the yolk was focused at the vegetal pole and the cytoplasm with its organelles was at the animal pole.

The cleavage pattern was meroblastic. The observation of the succession of events that happened during embryonic development allowed division into the following stages: cleavage, blastula, gastrula, organogenesis and pre-hatching.

The initial cleavage pattern presented blastomeres by division every 30 min from the time when temperature began to effect incubation, spacing changed according to the test temperature.

The times of appearance of each stage or structure in embryonic development are described in Table 1.

In the cleavage period (Fig. 1*A—F*), it was possible to clearly observe exponential division of blastomeres, until these reached the blastula, after which it was no longer possible to determine the number of cells, although it was possible to observe their division. The cells decreased in size as the divisions took place. The blastoderm was initially organized, semi-spherical and high up on the yolk sac. Blastoderm development occurred with increase in the number of their cells without causing further increase in size until it was possible to observe the advancement of the blastoderm on the yolk sac.

The epibolia process, in which the motion of blastodermal cells shrouded the vitelline mass and for migration, when the inner blastoderm cells moved back and converge, forming the embryonic axis, is characterized at the gastrula stage. At this stage, the blastoderm formed a small dome on the yolk sac, this dome increased gradually with each observation, thus characterizing the period of gastrula formation (Fig. 1G), which was measured as a percentage.

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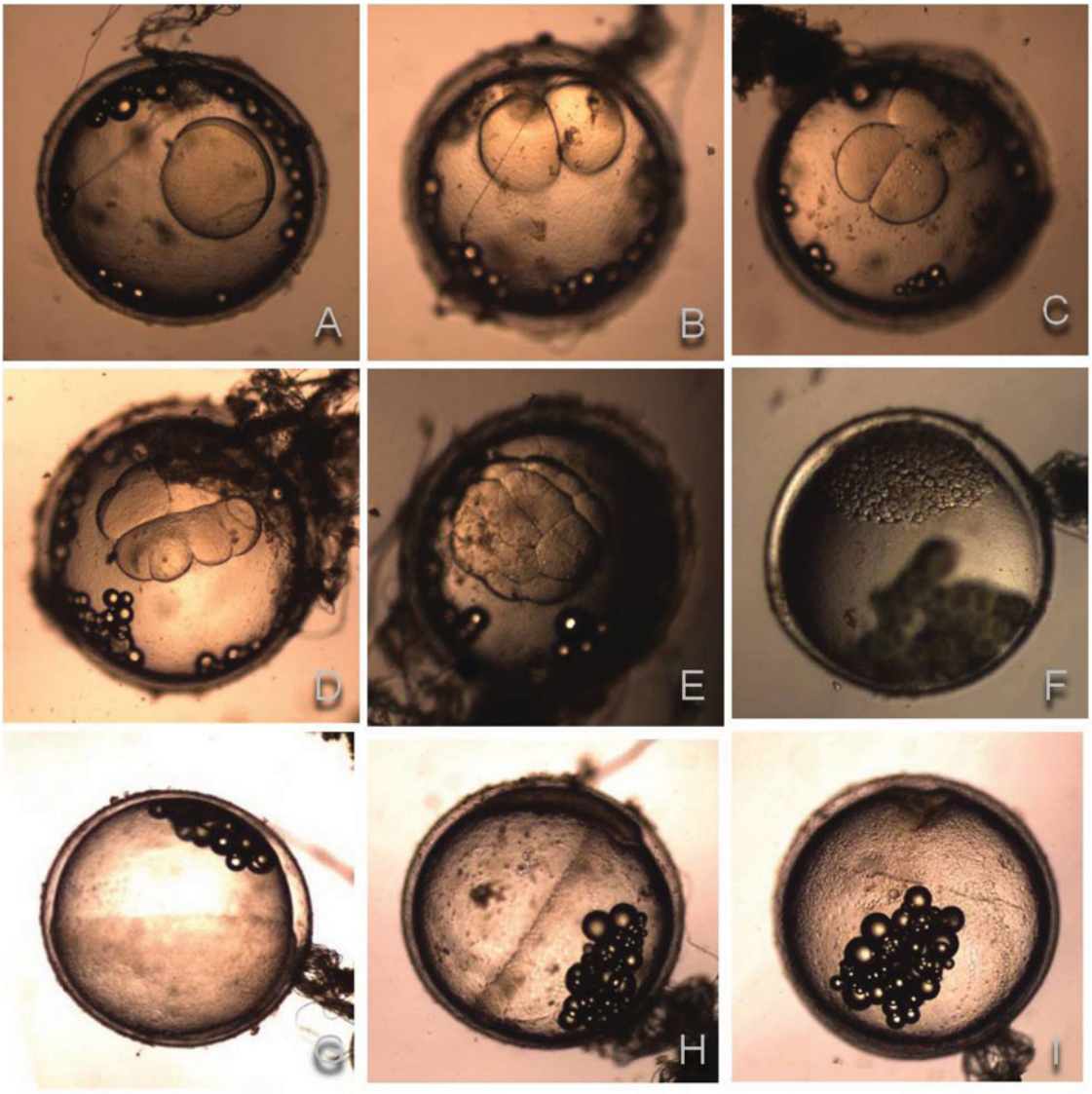

Figure 1 (*A–F*) Period of cleavage. (*A*) 1-cell. (*B*) 2-cell. (*C*) 4-cell. (*D*) 8-cell. (*E*) 16-cell. (*F*) 246-cell. (*G*) Gastrula 50%. (*H*) Differentiation of the embryonic axis. (*I*) Blastopore closure.

Still in the gastrula, there was thickening of the marginal edge of the blastoderm forming the germinal ring, which began a buildup of cells perpendicular to the ring, giving rise to the embryonic shield on which later formed the embryonic axis (Fig. 1*H*). The time of this event varied with the incubation temperature, but for all embryos it occurred in the gastrula stage (Table 1).

With the gradual evolution of the gastrula, the blastoderm edges approached together. When the blastoderm edges merged, and fully covered the yolk sac, this event characterized the end of the blastopore (Fig. 2).

After this time organogenesis occurred, with visible differentiation of embryonic tissues and organs. Differentiation at all temperatures followed a similar order of events, however, with change in development time of each structure, subject to the temperature tested.

Following the development of the embryonic axis, one end began to differentiate in the anterior portion of the embryonic body, showing up more prominently, giving rise to the cephalic region of the embryo (Fig. 2*A*). In this region, after evagination of the sidewall, the optical primordium emerged (Fig. 2*B, C*), initially viewed as a pair of optical rudimentary vesicles without coloration and delineation of structures. Throughout development, we observed the differentiation of the retina in the optic primordia that appeared as a more defined shape and slightly darker in the central region, and from that time called the optic vesicle (Fig. 2*F*).

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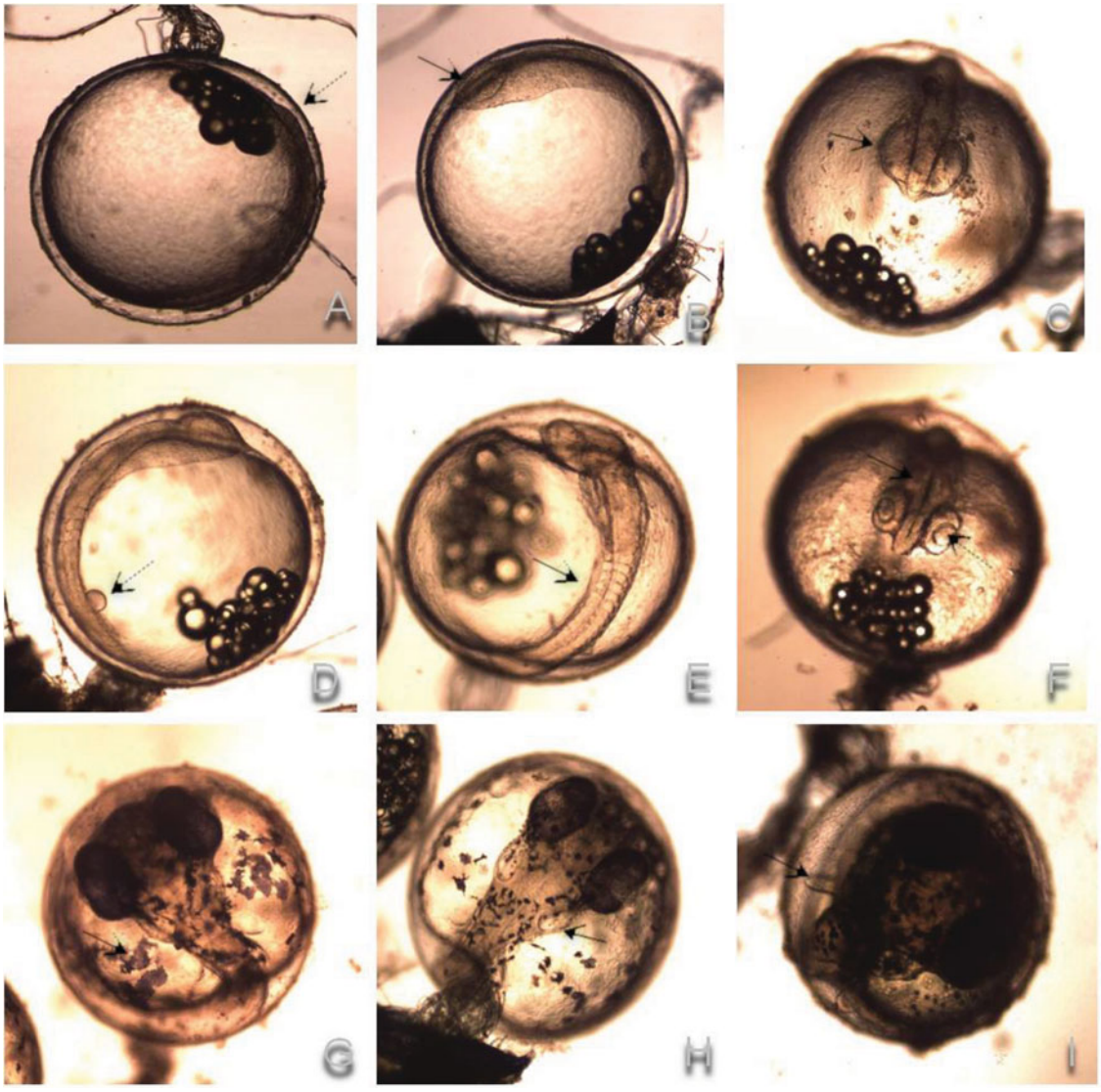

Figure 2 The arrows indicate the structures described. (*A*) Differentiation of head and tail with an arrow indicating the head. (*B*, *C*) Optic primordium side and top view, respectively. (*D*) Kupffer vesicle. (*E*) Somites. (*F*) Chondrocranium and early formation of the retina (optic vesicle). (*G*) Melanophores. (*H*) Otoliths in the otic vesicle. (*I*) Fins.

Differentiation of rudimentary somites occurred in the middle region of the embryo (Fig. 2*E*), which give rise to the vertebrae, ribs and axial muscles. These at first showed up in a few pairs and had elliptical shape. During development other pairs emerged and, after some time, acquired the form of a 'v'. The Kupffer vesicle (Fig. 2*D*) appeared on the opposite side and presented itself as a bubble shape.

In the cephalic region of the embryo, it was possible to visualize the cartilaginous cranial structure that give rise to the cranial base that would sustain the brain. This structure was located in the sagittal region, with the darker middle portion called the chondrocranium (Fig. 2*F*). The otic vesicle was visualized in the cephalic region, where a pair of otoliths initially presented (Fig. 2*H*). At the end of development two pairs were visible in each vesicle.

The embryonic heart was initially observed as a single region of the axial vein in the mesoderm that started slow peristalsis movement, promoting blood circulation. Later, the heart had two defined chambers, working in antagonistic movements of systole and diastole, representing the atrium and ventricle.

Melanophores (Fig. 2*G*) appeared randomly along the embryonic axis, passing just near to the yolk sac. Initially they were dendritic, assuming the pitting format throughout development. Circulation started on the embryonic axis, at first slowly and with elliptical red blood cells, becoming rounded and with increasing flow throughout development. Initially movement

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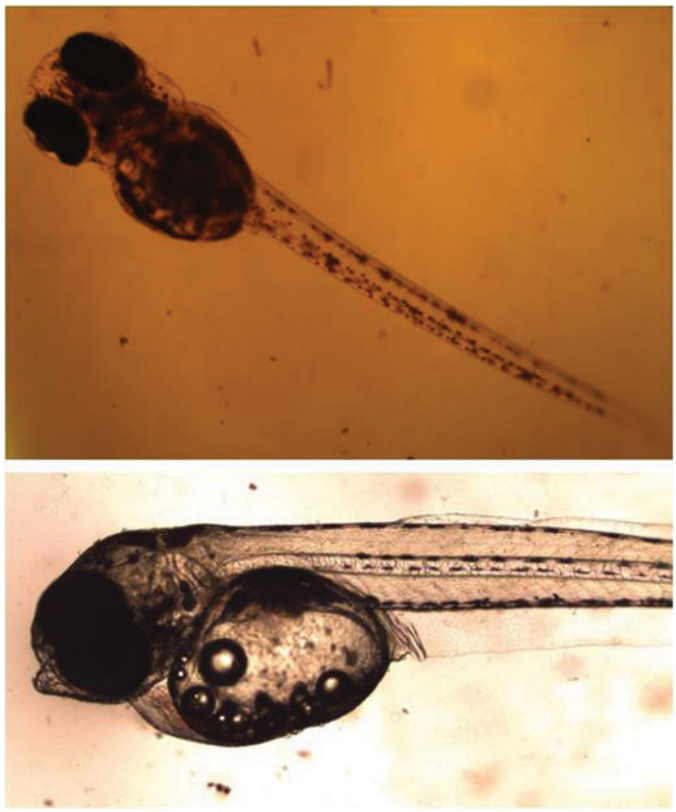

Figure 3 Newly hatched *M. boesemani* larvae.

was colorless, but developed a reddish tinge as the emerged branches of blood vessels promoted blood flow in the periphery region of the yolk sac.

Muscle contractions showed a reduced frequency of spasm repetition, but these intensified, particularly near thatching time. In this phase, the animals moved intensively, including rotational movements within the egg. The pectoral fins (Fig. 2*I*), which started development at the embryonic stage, moved with quick and well spaced beats near to breakout.

After the development of len pigmentation and the retinal pigment the eyes began to move. It was possible to identify the outline of the mouth, which opened even before breakout and showed slow and spaced movements.

In recent observations of larvae before hatching, many embryos were pigmented and the egg perivitelline space was greatly reduced. The output of the larvae happened very quickly after chorion rupture. The swim bladder inflated quickly, allowing the larvae to swim with great skill and agility. The larvae had a rapid capping movement, movement of the mouth and digestive tract and also had evident otoliths. Newly hatched larvae contained a residual volume of yolk and it was also possible to visualize lipid droplets in this residue (Fig. 3). Larvae were very pigmented, had dark eyes and many melanophores spread throughout the body.

The embryos that hatched late (incubation temperature of 23°C) had lesser amounts of apparent yolk, more pigment, and larger and higher concentration of melanophores. These embryos were less agile than those that born after a shorter incubation time.

Among the events described, in some there was a large difference in the time of occurrence between the highest and lowest temperature. For example, blastopore closure occurred in almost half the time at 32°C compared with at 23°C and also time to the first outbreak at a temperature of 32°C was 107.15 pfh compared with at 23°C when it was 253.58 pfh.

Time of appearance of morphophysiological events observed during *M. boesemani* embryo development was correlated and the most significant results can be seen in Table 2.

Eggs in the 32°C incubator had a hatching rate of 20.9%, while those in the 29°C incubator had 57.3% hatching rate, at 26°C the hatching rate was 51.8% and at 23°C it was 49.1%.

Discussion

The embryonic development of *Melanotaenia boesemani* is similar to that of most freshwater teleosts, being evident for the segmentation stages, formation of

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Table 2 Correlation between ontogenic events in the embryonic development of *Melanotaenia boesemani*

Correlated events		Correlation (%)	Significance
64-cell	Differentiation of the embryonic axis	92.37	3.81
	Blastopore closure	97.20	1.40
	Differentiation of head and tail	98.44	0.78
	Emergence of somites	99.99	0.00
	Chondrocranium	99.32	0.00
	Melanophores	99.53	0.00
	Circulation	96.26	1.87
	First outbreak	96.41	1.79
	Circulation	96.26	1.87
128-cell	Emergence of somites	99.17	0.00
	Melanophores	98.90	0.55
256-cell	Gastrula 40	98.52	0.74
	Gastrula 50	98.57	0.71
	Optic primordium	97.89	1.06
	Optic vesicle	96.75	1.63
	Chondrocranium	99.87	0.00
	Emergence of somites	98.86	0.57
	Melanophores	99.61	0.00
	Circulation	97.24	1.38
	First outbreak	96.81	1.60
Gastrula 50	Blastopores closure	99.50	0.00
	Optic primordium	99.83	0.00
	Optic vesicle	99.41	0.00
	Kupffer vesicle	99.56	0.00
	Otolith	98.59	0.71
	Heartbeat	99.17	0.00
	Circulation	99.78	0.00
	Release of tail	98.39	0.80
	First outbreak	99.58	0.00
Differentiation of the embryonic axis	Kupffer vesicle	99.28	0.00
	Otoliths	99.44	0.00
	Mouth opening	100	0.00
	First outbreak	99.18	0.00
	Movement of fins	99.80	0.00
Blastopore closure	Differentiation of head and tail	99.81	0.00
	Optic vesicle	99.99	0.00
	Kupffer vesicle	99.88	0.00
	Optic vesicle	99.22	0.00
	Circulation	99.76	0.00
	Release of tail	99.15	0.00
	Movement of fins	99.57	0.00
	Movement of eyes	99.82	0.00
	First outbreak	99.92	0.00
Kupffer vesicle	First outbreak	100	0.00
Movement of fins	First outbreak	99.78	0.00
Movement of eyes	First outbreak	99.75	0.00
Opening of mouth	First outbreak	99.20	0.00

blastula, gastrula, blastopore closure, organogenesis, release of tail and hatching (Nakatani *et al.*, 2001).

The types of egg (telolecito) and segmentation (meroblastic) found in the fish studied, are also typical in teleost fish, with large amount of yolk and blastomeres at the animal pole (Leme dos Santos & Azoubel, 1996; Garcia & Fernandez, 2001).

M. boesemani eggs showed an average diameter close to that of other melanotenideos, such as *Melanotaenia praecox*, which was 1.02 mm ± 0.01 (Radael *et al.*, 2013) *Glossolepis incisus* which was 0.90 mm 1.10 mm (Ferreira, 2007), *Melanotaenia splendida australis* with 1.07 mm (*Ivantsoff et al.*, 1988), *Melanotaenia nigrans* averaging 1.05 mm and *Melanotaenia splendida*

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inornata with an mean of 0.88 mm (Reid & Holdway, 1995).

The adhesive filaments observed on the eggs in this study have been described in papers regarding other melanotenideos, showing that egg adhesiveness is a reproductive strategy characteristic of the family (Ferreira, 2007; Radael *et al.*, 2013).

The arrangement and aggregation of oil droplets observed in newly fertilized eggs was the same as that observed and described for *M. praecox*, *Betta splendens* and *Glossolepis incisus* (Ferreira, 2007; Duarte *et al.*, 2012; Radael *et al.*, 2013).

The initial stages of cleavage showed no major difference in the times of occurrence at the temperatures used. This fact may be because eggs were not influenced by temperature, as the distribution into different incubators only occurred after fertilization.

In the gastrula stage, differences in event time started to be evident based on incubation temperatures. At this stage, differentiation of the embryonic axis primarily occurred at 32°C and followed in descending order until embryos finally emerged from the incubator set at 23°C. Humphrey *et al.* (2003) reported that in *Melanotaenia splendida* differentiation of the embryonic axis was also observed in the gastrula period, occurring in that species near the time of gastrulation 70%.

Blastopore closure is an important event to be considered as the period at which fertilization of the oocyte can be confirmed (Woynarovich & Horváth, 1983) and characterizes the end of the epibolia process. In *M. boesemani* embryos incubated at 32°C the blastopore closure occurred at 14.13 pfh, before closure at other test temperatures (Table 1), this phase has been described for *M. praecox* (30°C) at 13.99 pfh; for *M. nigrans* and *M. s. inornata* this event was observed at near 18.0 pfh (Reid & Holdway, 1995), in *M. s. splendida* close to 12.5 pfh (Humphrey *et al.*, 2003) and *G. incisus* at 11.8 pfh (Ferreira, 2007). In endemic fishes in Brazil, there is noticeable quicker embryonic development. For example, *Brycon orbignyanus* embryos were observed at 6.5 pfh (Reynalte-Tataje *et al.*, 2004) and *Brycon insignis* at 5.5 pfh (Souza, 2004).

The events of organogenesis showed the same trend of development, with differentiation occurring usually first in a higher temperatures and then in the other groups. In a study of *Sprattus sprattus* (Petereit *et al.*, 2008), egg development was also dependent on temperature, indicating that, with increasing of temperature, incubation period from fertilization to hatching is reduced.

M. boesemani hatched embryos occurred primarily in the incubator at 32°C (higher temperature), at approximately 4 days and with a difference of 146.43 pfh when compared with first hatch from the incubator at 23°C (lower temperature). The greatest time span for hatching was at 26°C with a 93.68 pfh interval between the first and the last breakout.

Time for embryo development was relatively high compared with Brazilian native fish (Nakatani *et al.*, 2001; Reynalte-Tataje *et al.*, 2004) such as *Symphysodon aequifasciatus* that hatched in water at approximately 28°C, beginning after 55.5 pfh (Mattos *et al.*, 2014). However, a time for embryonic development of between 6 and 7 days was commonly reported for genus *Melanotaenia.* Ivantsoff *et al.* (1988) was observed that *M. s. inornata* and *M. nigrans* hatched within 4 to 5 days at a temperature of 26°C. For *M. fluviatilis* the hatching period was between 7 and 9 days (Reid & Holdway, 1995) and in *M. s. splendida* the hatching time was within 4 to 8 days (Humphrey *et al.*, 2003).

In this study the larvae emerged following rupture of the chorion, this event was very swift and similar to that observed by Humphrey *et al.* (2003) for *M. s. splendida.* Newly hatched larvae behaved in the same manner as did *M. s. splendida* (Humphrey *et al.*, 2003) and *M. fluviatilis* (Reid & Holdway, 1995), were very active and had good swimming ability. The newly hatched larvae had rapidly moving cappings, movement of the mouth and of the digestive tract, and the otoliths were prominent, as observed in *M. fluviatilis* (Reid & Holdway, 1995), *M. s. splendida* (Humphrey *et al.*, 2003) and *M. praecox* (Radael *et al.*, 2013).

The most listed embryonic stages (expressed in hours after fertilization) had more than one significant correlation (Table 2). Correlations were only expected between events that were temporally adjacent, however correlation was observed between the 64-cell stage (recent development) and the differentiation of head and tail, near blastopore closure. Also, correlation was observed between the 128-cell stage (recent development) and the appearance of somites, which occurred only after blastopore closure during organogenesis. A high correlation was observed between events close timewise, and earlier events, between blastopore closure and differentiation of the head and tail. However between differentiation of the embryonic axis and the first hatching, high correlation was also observed between distant events, therefore correlation exists both between adjacent events and events distant in time of development.

Therefore, in general, for eggs incubated in the ecological boundaries for the species, higher temperatures accelerated the biological processes, resulting in a shorter time for incubation than that observed at lower temperatures (Lasker, 1964). However, it is important that the temperature adopted, besides accelerating the

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incubation process, allows the birth of viable larvae and a good rate of hatching.

For *M. boesemani*, incubation at 29°C, although not the shortest incubation time, was the temperature that gave the best percent hatching with higher visually viable larvae. Therefore 29°C is the suggested temperature for embryo incubation for this species.

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