Zygote **24** (October), pp. 733–741. © Cambridge University Press 2016
doi:10.1017/S0967199416000046 First Published Online 3 May 2016

Effect of temperature during embryonic development and first feeding of *Trichogaster leeri* larvae

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Date submitted: 03.12.2014. Date revised: 14.12.2015. Date accepted: 14.01.2016

Summary

Temperature is an environmental factor that influences the development of fish, and when changed abruptly can lead to high mortality. Some species of fish are influenced by this factor, exhibiting a longer time for embryonic development and time to first feeding. This study aims to evaluate the effect of water temperature on embryonic and larval development up to first feeding, to describe the time in hours post fertilization (hpf) of the emergence of different structures and to determine the best hatching rate and survival of animals under different treatments. Five different egg incubation temperatures were used (24, 26, 28, 30 or 32°C, respectively). The eggs were observed at regular intervals of 30 min up to 24 h, every 2 h until 48 h and every 4 h until the display of first feeding in all treatments. Embryonic development was longer for eggs incubated at 24°C and the best results for hatching rate and survival of spawning efficiency were at 28°C. We recommend that incubation of *Trichogaster leeri* eggs is carried out at 28°C up to the first feeding of larvae.

Keywords: Feeding, Larvae, Ornamental fish, Survival, Water temperature

Introduction

Trichogaster leeri is a good species choice to serve as a model for biological studies as it generates numerous offspring and reproduces easily. In addition, it is commonly sold in ornamental fish shops (Tonini *et al.* 2012). Despite the unique characteristics of this species, few studies have been conducted that describe aspects of its early ontogeny. Ontogenetic studies are important for understanding the physiological aspects of the embryo, generating information for conservation of fish populations and improving culture techniques (Godinho *et al.* 2003).

The description of morphological and physiological aspects of the early stages of embryonic development are extremely important to maximize its survivability rate during the early period of its life (Senhorine, 1993; Nakatani *et al.* 2001). Ontogenetic studies can be used as a tool to improve information on fish culture, especially when associated with water quality parameters that interfere directly on fish life stage.

Temperature is one of the main parameters measured in general during fish culture. Fish are classified as poikilothermic and changes in water temperature can significantly affect fish feeding, growth, survival and metabolism (Bustos *et al.* 2007). Increased temperature accelerates food intake, metabolism and ontogenetic development, and may disturb the balance between intake and energy expenditure, leading to mortality of the fish. In addition, yolk absorption is faster at higher temperatures, reducing the duration of endogenous supply (Fukuhara, 1990; Aritaki & Seikai, 2004; Dou *et al.* 2005; Fielder *et al.* 2005; Bustos *et al.* 2007).

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Some studies have investigated the effects on hatching of factors such as yolk sac absorption (slower yolk consumption at lower temperatures for tropical fish) and animal development (accelerated at higher temperatures within the thermally comfortable range), plus the ability to interfere with swimming capabilities of larvae, making them more susceptible to predation and limiting their ability to obtain food (Howell & Caldwell, 1984; Berlinsky *et al.* 2004). Thus the aim of this study was to determine the effect of temperature throughout embryonic and larval development up to the period of first larval feeding.

Material and methods

The eggs used to describe embryonic development were obtained from the natural spawning of a pair of *T. leeri.* The pair was kept in the aquaculture sector of the Laboratory of Animal Science and Animal Nutrition (LZNA) of Science Center and Agricultural Technologies (CCTA) from State University of North Fluminense Darcy Ribeiro (UENF), Campos dos Goytacazes, Rio de Janeiro, Brazil.

The pair was kept in a tank with a 20 l capacity, consisting of a re-circulating water system, and equipped with a mechanical and biological filter. After the separation of the pair, the female was kept locked in a transparent container with an opening above the water surface to avoid fighting with other animals. The male built a bubble nest around the female in a floating substrate of expanded polystyrene (EPS), and then the female and the male were released to perform courting, then breeding and until spawning.

After the start of spawning, about 20 eggs were examined under an optical microscope (×25 magnification) to evaluate the embryonic spawning stage. After the first observation, 30 eggs were inserted using a floating sieve into five tanks with a capacity of 18 l, with six sieves per incubator. Each tank possessed a heater coupled to a thermostat, keeping the temperature constant during the whole experimental period. Temperatures in the incubators were set at 24, 26, 28, 30 or 32°C. Each tank had an oxygenation system for water, connected to an air blower, hose and porous stone.

Observations were performed frequently from fertilization until the time that the first feeding of brine shrimp (*Artemia* spp.) occurred. Observation intervals varied according to the ontogenetic phase, e.g. 30 min intervals during the incubation period (0–24 h), every 2 h (24–48 h), and every 4 h until the first feeding of the animals was observed.

At each observation, a sample of 10 eggs was taken from the total number of eggs from three sieves at random and placed on a glass slide for evaluation under an optical microscope (×25 or ×100 magnification). The images were obtained using an optical microscope with an attached camera.

The embryonic development stages were described only when 50% of the eggs observed in the sample reached the same stage. The values of temperature, pH and dissolved oxygen were measured every 2 h with the aid of a digital thermometer, pH meter and a digital oximeter, respectively. Event classification is based on morphologic characteristics according to the methodology used by Fujimoto *et al.* (2006) for the loach (*Misgurnus anguillicaudatus*). Hours post fertilization (hpf) were used to correlate morphophysiological events. The hpf corresponded to the time spent after fertilization from time 0 (H0 – initial). Upon completion of the embryonic and larval development stages, another spawning was performed from the same family to obtain the hatching rate and larval survival rate as described by Omitogun *et al.* (2012) and the efficiency index calculated using the following equations:

$$\%\ \text{Hatchability} = \frac{\text{Total no. of fertilized eggs} - \text{No. of unhatcched eggs}}{\text{Total no. of fertilized eggs}} \times 100\%$$

$$\%\ \text{Survivability} = \frac{\text{Total no. of larvae} - \text{No. of dead larvae}}{\text{Total no. of larvae}} \times 100\%$$

$$\%\ \text{Spawning efficiency index} = \text{Hatchability} \times \text{Survivability} \div 100$$

The results obtained during the experimental period were subjected to statistical analysis, used Pearson's correlation to determine the correlation between variables and polynomial regression to specify a better time of occurrence for the variables studied.

Results

The physicochemical parameters of the aquarium water are shown in Table 1. There were no major changes in temperature during all treatments, the temperature remained near the set value, showing a thermal equilibrium during the experimental period. Oxygen levels and pH of the water were recorded. pH and dissolved oxygen showed acceptable values for culture of this fish species. The average diameter of *Trichogaster* eggs at different temperatures was 0.863 ± 0.2603 mm.

The fertilized *T. leeri* eggs had a spherical shape, with the chorion and the yolk sac translucent (Fig. 1*A*). The chorion was described as somewhat hard and its texture was similar to that of small 'gelatinous spheres'.

https://doi.org/10.1017/S0967199416000046 Published online by Cambridge University Press

Table 1 Average values of temperature (°C). Dissolved oxygen (DO) and pH of water used in the incubators during the experimental period

	Incubator temperature				
Treatment	24°C	26°C	28°C	30°C	32°C
Temperature range (°C)	24.01 ± 0.20	26 ± 0.17	28.29 ± 0.17	30.09 ± 0.14	32.01 ± 0.18
pH	7.33 ± 0.17	7.23 ± 0.19	7.24 ± 0.14	7.19 ± 0.28	7.28 ± 0.21
Dissolved oxygen (mg/L)	7.73 ± 0.62	7.28 ± 0.39	6.71 ± 0.58	6.60 ± 0.49	6.23 ± 0.53

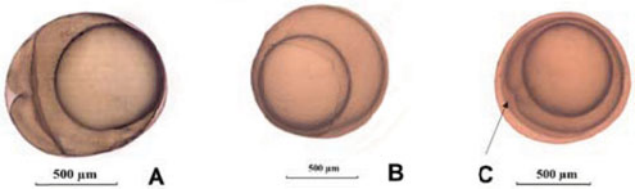

Figure 1 Stages from early cleavage to blastopore closure. (*A*) Visualization of the beginning of cleavage (two blastomeres). (*B*) Visualization of the egg in gastrulation. (*C*) Blastopore closure.

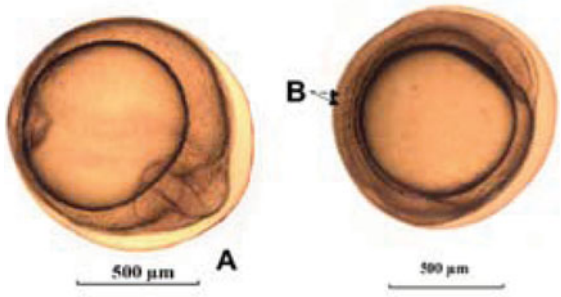

Figure 2 Beginning of differentiation of structures during organogenesis. (*A*) Optical primordium. (*B*) Visualization of somites in the embryonic axis.

The cleavage pattern observed in the embryos studied, as well as in other teleosts, was meroblastic. The initial division of blastomeres was very rapid and intense, making it difficult to see and preventing any count of blastomere numbers, marking the start time of cleavage from the moment when the eggs had two blastomeres until the moment when the eggs had a spherical-shaped blastodisc, marking the beginning of gastrulation.

Hpf values for each temperature for all the events are listed in Table 2. Steps such as cleavage, gastrulation and blastopore closure occurred rapidly at all the evaluated temperatures (Fig. 1*A*, *B*). The end of gastrulation was taken to be at blastopore closure in *T. leeri* eggs (Fig. 1*C*). At the end of the gastrula period, organogenesis began, at which time the tissues and organs differentiated. The beginning of differentiation of the head and tail was observed at 6.63, 7.37, 8.97, 9.38, or 11.05 hpf at temperatures of 32, 30, 28, 26 or 24°C, respectively. It can be seen that shortly after visualization of the head and tail of the embryo, the emergence of the optical primordium occurred, which showed a positive correlation of 97.96% with the beginning of visualization of the somites (Fig. 2*A*, *B*).

During organogenesis, the start of visualization of the somites was positively correlated (97.53%) with development of the chondrocranium. Another correlation is listed in Table 3. At this stage it was possible to see the emergence of the chondrocranium, a cartilaginous cranial structure of the embryo in the cephalic region, which gives rise to the skull base that sustains the brain. An even higher positive correlation than that of the chondrocranium was observed with the emergence of the Kuppfer vesicle (98.93%).

In the present study the emergence of melanophores occurred before the rise of the chondrocranium, except at 32°C. It was possible to observe the beginning of the emergence of the otic vesicle, in the posterior region of the optic vesicle, and then the emergence of the otolith, seen just a couple until moments before hatching. The correlation between the otic vesicle and the otolith was 97.51%. (Fig. 3*A*, *B*).

When the animals began contractions, usually near the hatching period, large amounts of dendritic melanophores were observed throughout the embryonic axis (Fig. 3), muscle contraction began to occur during the embryo's preparation to hatch, in order to assist chorion breakage.

The beginning of the heartbeat showed a positive correlation (98.14%) with the beginning of embryo contractions, followed by the beginning of hatching at hpf of 14.55; 19.60; 21.30; 27.22 or 32.63 at temperatures of 32, 30, 28, 26 or 24°C, respectively. The end of hatching occurred over a large time interval between 32 and 24°C, recorded at 19.22 and 39.13 hpf, respectively.

The caudal fin was viewed in the larvae at 32.72, 33.48. 39.22, 42.22 or 48.13 hpf at temperatures of 32, 30, 28, 26 or 24°C, respectively. Eye movement was viewed only after the development of pigmentation of the lens and of retinal pigmentation.

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Table 2 Events reported during embryonic development of *Trichogaster leeri* and characteristics of larvae up to first feeding

Events (hours post infection)	Incubator temperature				
	24ºC	26ºC	28ºC	30ºC	32ºC
	0	0	0	0	0
End of cleavage	4.83	4.17	3.3	3.22	2.63
Beginning of gastrulation	4.83	4.17	3.3	3.22	2.63
End of gastrulation	8.22	8.05	7.38	6.72	5.85
Blastopore closure	8.22	8.05	7.38	6.72	5.85
Beginning of differentiation of head and tail	11.05	9.38	8.97	7.37	6.63
Emergence of optical primordium	13.9	10.05	9.38	8.02	7.13
Initial view of somites	17.77	13.77	12.25	8.93	8.22
Emergence of chondrocranium	18.13	15.02	14.75	10.35	8.72
Kuppfer vesicle	17.77	14.78	13.35	9.52	8.72
Melanophores	18.13	13.77	13.35	10.02	9.22
Otic vesicle	21.4	17.28	15.22	10.77	9.22
Otoliths	24.3	18.02	18.72	14.02	11.88
Beginning of heartbeat and blood circulation	24.75	19.92	19.82	14.48	12.65
Beginning of the embryo contraction	28.05	19.92	19.82	15.08	13.18
Beginning of outbreak	32.63	27.22	21.3	19.6	14.55
End of eggs hatching	39.13	27.72	24.47	21.98	19.22
Newly hatched larvae	39.63	28.22	24.97	22.48	19.72
Caudal fin	48.13	42.22	39.22	33.48	32.72
Pectoral fin	64.13	46.22	43.22	37.48	32.72
Eye movement	72.13	66.22	51.22	45.48	40.72
Mouth opening	84.13	66.22	67.22	45.48	40.72
Horizontal swimming	84.13	66.22	67.22	49.48	44.72
Opening of anus	100.13	71.22	70.22	53.48	52.72
First feeding	120.13	95.22	98.22	91.48	76.72

The time of first feeding was described as the time at which the *T. leeri* larvae began to capture the nauplii. Larvae that were capturing nauplii generally had an orange pigmentation throughout the region of the digestive tract, even in formation. To prove that the animals were feeding, they were removed from the floating sieves and observed under an optical microscope.

Hatching rate was best from 26 to 30°C, with a maximum at 28.79ºC (Fig. 4). The mean survival values for the larvae were observed at 26 and 28ºC, with maximum at 27.40°C (Fig. 5).

In relation to the efficiency of spawning, obtained through the correlation between the hatching rate and larvae survival, it was possible to observe the best results at temperatures of 26 and 28°C, with the maximum at 28.15°C (Fig. 6).

Discussion

At the time of spawning, the male *T. leeri* puts the eggs into a bubble nest, showing parental care, as seen in *Betta splendens* and *Hoplosternum littorale* species (Nakatani *et al.* 2001; Duarte *et al.* 2012).

Embryonic development time in this study showed a difference in function at the experimental temperatures used in all treatments. In general, the time to occurrence to events was faster than that observed in other tropical fish (Ferreira, 2007; Radael *et al.* 2013; Mattos *et al.* 2015).

Cleavage period was characterized by blastodisc successive cell divisions and began soon after the oocyte fertilization. Cell divisions occurring during cleavage continued up to the beginning of gastrulation, a step in which by epibolic movements the blastodermal cells overly the vitelline mass and migration occurs over most internal cells of the blastoderm, moving back and convergently to form the embryonic axis. The embryo axis of *T. leeri* could be observed when the gastrula was 60%, this result differed from that of Humphrey *et al.* (2003), who reported that *Melanotaenia splendida* differentiation of the embryonic axis occurred during the period when the gastrula was 70%. The end of gastrulation was deemed to be at blastopore closure (Olaniyi & Omitogun, 2014)

This phase has been described by other authors such as Puvaneswari *et al.* (2009) in *Heteropneustes fossilis* at 7 hpf and by Reynalte-Tataje *et al.* (2004) in *Brycon*

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Table 3 Correlation between events observed during embryonic and larval development of *Trichogaster leeri*

Correlated events		Correlation (%)
End of cleavage	Beginning of gastrulation	100.00
End of cleavage	End of gastrulation	93.29
Beginning of gastrulation	Closing of blastopore	93.29
Beginning of gastrulation	Differentiation of head and tail	94.66
End of gastrulation	Closing of blastopore	100.00
End of gastrulation	Differentiation of head and tail	94.76
Closing of blastopore	Differentiation of head and tail	94.76
Closing of blastopore	Chondrocranium	95.34
Differentiation of head and tail	Optical primordium	96.68
Differentiation of head and tail	Beginning of visualization of somites	99.24
Differentiation of head and tail	Chondrocranium	99.31
Differentiation of head and tail	Kuppfer vesicle	99.50
Differentiation of head and tail	Melanophores	99.22
Differentiation of head and tail	Optical vesicle	99.71
Differentiation of head and tail	Beginning of hatching	96.50
Optical primordium	Beginning of visualization of somites	97.96
Optical primordium	Melanophores	98.45
Optical primordium	Optical vesicle	96.20
Beginning of visualization of somites	Kuppfer vesicle	99.38
Beginning of visualization of somites	Melanophores	99.49
Beginning of visualization of somites	Optical vesicle	99.55
Chondrocranium	Kuppfer vesicle	98.93
Chondrocranium	Optical vesicle	98.78
Chondrocranium	Otoliths	98.15
Kuppfer vesicle	Optical vesicle	99.89
Kuppfer vesicle	Heartbeat	99.01
Kuppfer vesicle	Beginning of hatching	95.94
Melanophores	Otoliths	99.23
Melanophores	Beginning of hatching	94.88
Optical vesicle	Otoliths	97.51
Optical vesicle	Beginning of hatching	97.08
Otoliths	Heartbeat	99.22
Otoliths	Beginning of contractions	99.39
Otoliths	Beginning of hatching	93.15
Otoliths	End of hatching	95.16
Heartbeat	Beginning of contractions	98.14
Heartbeat	Beginning of hatching	94.02
Heartbeat	End of hatching	93.13
Beginning of hatching	End of hatching	95.69
Beginning of hatching	Survival of newly hatched larvae	95.69
End of hatching	Survival of newly hatched larvae	100.00
End of hatching	Caudal fin	95.91
End of hatching	Pectoral fin	99.70
Survival of newly hatched larvae	Caudal fin	95.91
Survival of newly hatched larvae	Pectoral fin	99.70
Survival of newly hatched larvae	First feeding	95.19
Caudal fin	Pectoral fin	96.63
Caudal fin	Mouth opening	97.35
Caudal fin	Horizontal swimming	97.97
Eye movement	Mouth opening	85.36
Eye movement	Horizontal swimming	83.86
Mouth opening	Horizontal swimming	99.79
Mouth opening	Anus opening	96.03
Mouth opening	First feeding	94.87
Horizontal swimming	Anus opening	97.45
Horizontal swimming	First feeding	94.62
Anus opening	First feeding	94.30

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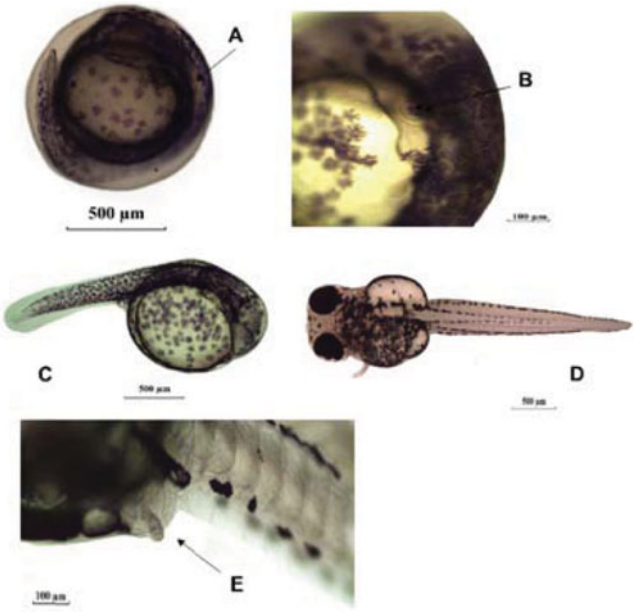

Figure 3 Photomicrographs of the embryo during pre/post-hatching. (*A*) Optic vesicle of pre-hatching embryo. (*B*) Optic vesicle with higher magnification of the image. (*C*) *Trichogaster leeri* larva newly hatched. (*D*) Larvae with mouth opening, developed fins and exhausted calf. (*E*) Anus opening.

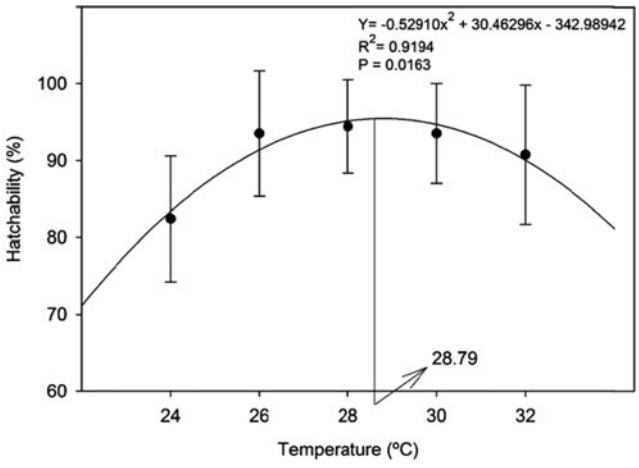

Figure 4 Effect of temperature on hatchability of *Trichogaster leeri* eggs. Arrow indicates the maximum point obtained by the equation.

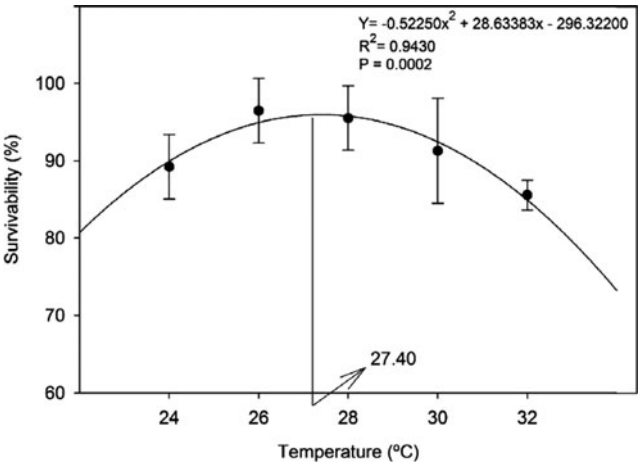

Figure 5 Effect of temperature on survivability of *Trichogaster leeri* larvae. Arrow indicates the maximum point obtained by the equation.

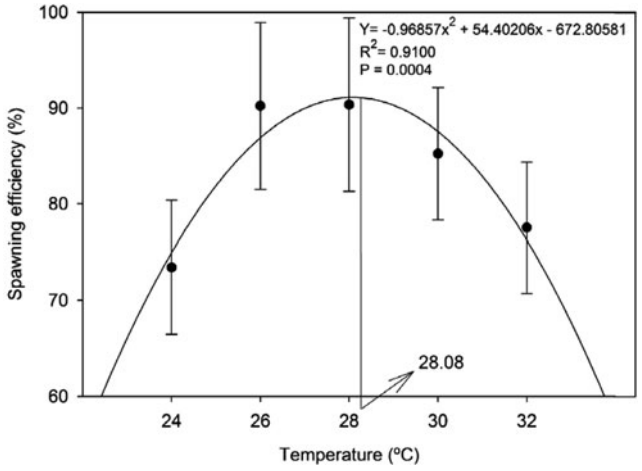

Figure 6 Efficiency of spawning at different temperatures. Arrow indicates the maximum point obtained by the equation.

orbignyanus at 6.5 hpf to blastopore closure. This step in embryonic development is an important event and it is considered the period at which oocyte fertilization can be confirmed (Woynarovich & Horváth, 1983).

These events occurred faster at all temperatures when compared with the study of Duarte *et al.* (2012), who observed time until the end of cleavage and end of gastrulation to be 7.5 and 14.5 hpf respectively for *B. splendens.* In eggs of surubin hybrids (*Pseudoplatystoma corruscans* × *Pseudoplatystoma fasciatum*) observed by Faustino *et al.* (2007), it was possible to observe faster occurrence in relation to that observed at all temperatures in this experiment. However, this same author pointed out that the time of occurrence of the events in his experiment varied depending on the water temperature.

The differentiation of head and tail in embryonic development, and the appearance of the optic primordium and the somites had lower hpf values at all temperatures when compared with results obtained by Marimuthu & Haniffa (2007), Radael (2009) and Mattos *et al.* (2015). This difference is probably related to the characteristics of the studied species and larval development level at the time of hatching. *T. leeri* larvae are altricial and therefore not fully differentiated

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at the time of hatching, therefore hatching time was shorter than that of the species used by these authors.

It is noteworthy that structures such as somites, resulting in the formation of vertebrae, ribs and axial muscles, develop before emergence of structures such as Kuppfer vesicles that have an excretory function (Alves & Moura, 1992), the optic vesicle and the otolith. This behaviour can be observed in this present study, as well as observed in kinguios 'var. *comet*', by Mahmud *et al.* (2011), in *Carassius auratus* by Tsai *et al.* (2013) and in discus fish, described by Mattos *et al.* (2015).

The pigmentation that is clearly visible before hatching is a form of protection after hatching, as it camouflages the animal from predators (Bemvenuti & Fischer, 2010; Olaniyi & Omitogun, 2013). Furthermore, Olaniyi & Omitogun (2014) described that this pigmentation is essential for taxonomic identification of species.

Events at the beginning and end of hatching happened quickly at 26°C. It is possible that this result is associated with proximity to the optimum temperature for larval survival and spawning efficiency. Despite the lack of higher absolute values for hatching rate in 26°C, larvae survival and spawning efficiency was better at this temperature.

In this study, the *T. leeri* larvae after hatching developed a large area of pigmentation in the yolk and in the embryonic axis, had a large reserve of yolk and exhibited little movement. The *T. leeri* larvae showed little morphological development at hatching, low swimming motility and little yolk reserve, besides having incomplete digestive tract morphology at the first feeding that may impair the development of animals during and after hatching (Portella, 2004; Mattos *et al.* 2015). Until the digestive tract is fully developed, the animal is still dependent on endogenous feed. Digestive tract development corresponds with the necessity for exogenous food, and other changes such as development of the mouth opening, vision and improved swimming, which are all necessary for obtaining a food source (Santin *et al.* 2004).

At all temperatures, *T. leeri* larvae exhibited mouth opening shortly after eye movement, indicating that the larvae would be able to search for food. However, although the larvae exhibited mouth opening, first feeding was observed only after a long period, and this behaviour indicated that the larvae required food smaller than the brine shrimp nauplii used as the initial food in this study (Guevara & Guevara, 2008).

Temperature influenced embryonic development in *T. leeri*, best results were at the thermally comfortable range of 26 to 29°C. In studies performed with species from other fish families the effect of temperature on incubation has also been measured. As noted by Dionısio *et al.* (2012), for the effect of incubation temperature on *Solea senegalensis* eggs, generally grown at 18–22°C and in accordance with Engrola *et al.* (2005, 2009), Fernandez *et al.* (2009), Blanco-Vives *et al.* (2010), egg incubation temperature had a significant effect on the occurrence of abnormalities in *S. senegalensis* larvae.

Although these are different species, water temperature influenced the time of occurrence of events, and may even have affected hatching rate and larval survival, as observed in this study. In addition, for effects on larval performance, temperature may play an important role in the incidence of defects, resulting in lower quality larvae, as observed by Dionısio *et al.* (2012).

Water temperature is one of the most important factors that should be monitored in fish hatcheries because it can change the physiological characteristics of animals, decrease activities such as swimming in some species, provide high mortality rates with stringent changes in temperatures, or be out of the thermally comfortable range of the species of interest (Sfakianakis *et al.* 2011).

In conclusion, water temperature influenced embryonic and larval development of *T. leeri*, events occurred sooner at higher temperatures and were delayed at lower temperatures. Despite the effect that temperature had on the timing of occurrence of events, the best results for hatching rate, larval survival and efficiency of spawning were observed at 28°C, which is the temperature recommended for incubation of *T. leeri* larvae eggs.

Acknowledgements

The authors acknowledge CNPq for encouraging, and UENF for providing, the conditions for these experiments.

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https://doi.org/10.1017/S0967199416000046 Published online by Cambridge University Press