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The effect of rearing temperature in larval development of pejerrey, *Odontesthes bonariensis* - Morphological indicators of development

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It is well known that in pejerrey water temperature not only affects growth rates but also directs the sexual differentiation process. This fact rise the question of how different the development of pejerrey larvae of the same age is when reared at different temperatures. A description of developmental stages for the embryonic and larval periods of the pejerrey, *Odontesthes bonariensis*, and the influence of rearing temperature on larval development are presented. Then, larval development was studied at three rearing temperatures, and changes in general morphology, fin morphology, and caudal fin structure have been taken into consideration within the thermal range involved in the temperature sex determination of this species. Fin fold reabsorption, caudal fin formation, and body shape were selected to follow the events leading to the acquisition of the juvenile morphology. The juvenile phenotype was defined when the fin fold was reabsorpted and the caudal fin acquired its definitive homocercal structure. The moment at which the juvenile phenotype was achieved, was evaluated in relation to larval age, size and, shape. The size resulted as the best indicator of development in pejerrey.

A temperatura da água não afeta apenas as taxas de crescimento no peixe-rei, mas também direciona o processo de diferenciação sexual. Este fato levanta o questionamento de quão diferente é o desenvolvimento de larvas do peixe-rei da mesma idade quando criadas em temperaturas diferentes. Este trabalho teve como objetivo apresentar uma descrição do de desenvolvimento de embriões e larvas do peixe-rei, *Odontesthes bonariensis*, e a influência da temperatura de criação no desenvolvimento das larvas. Neste trabalho, o desenvolvimento das larvas foi estudado em três temperaturas diferentes de cultivo. Foram consideradas as alterações ocorridas na morfologia geral, assim como na morfologia e na estrutura da nadadeira caudal dentro da variação termal da temperatura de determinação sexual desta espécie. A taxa de reabsorção da membrana embrionária, a formação da cauda e o formato do corpo foram selecionados para acompanhar os eventos que levam à aquisição da morfologia juvenil. O fenótipo juvenil foi definido quando a nadadeira caudal foi reabsorvida e a cauda adquiriu a estrutura homocerca. O momento no qual o fenótipo juvenil foi atingido, foi avaliado quanto à idade, tamanho e formato da larva, sendo que o tamanho resultou no melhor indicador do desenvolvimento do peixe-rei.

Key words: Atherinopsidae, Fin fold, Larvae, Sexual determination.

Introduction

The early-life history of teleost fish includes a series of morphological, physiological, and ecological changes (Berlinsky *et al.*, 2004; Al Hazzaa & Hussein, 2007; Fujimura & Okada, 2007; Murphy *et al.*, 2007; Kawakami *et al.*, 2008). In bony fish species characterized by an indirect development, three main consecutive periods can be recognized: embryo, larva, and juvenile (Youson, 1988, 2003). After hatching, the absorption of the yolk sac resources continues without exogenous feeding for a variable lapse depending on the species (Webb, 1999). Some authors propose the change from endogenous to exogenous feeding as the event indicating the transition from embryos to larvae (Balon, 1990). Nevertheless, other authors propose different events to define this moment: hatching, in the view of Blaxter (1988). In the same way, complete fin ray development (Kawakami *et al.*, 2008), the mouth shift from an upper to lower position (Gozlan *et al.*, 1999), or the change in the use of the habitat (Balon, 1999), have been taken as indicators of larval-juvenile transition (metamorphosis) in different fish species. The lack of agreement in the use of the word larvae (Balon, 2001; Blaxter, 1988; Kováè & Copp, 1999; Hensel, 1999; McElman & Balon, 1979) has impaired but only in part, the comprehension of the ecological and physiological

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significance of this period (Copp *et al.*, 1999). Despite development has a stronger correlation with size than with age (Fuiman *et al.*, 1998; Adriaens & Verraes, 2002; Sæle & Pittman, 2010), the last is still used as an indicator of development, *i.e.* the larval-juvenile transition.

Water temperature is known to be the most important environmental factor influencing fish development (Blaxter, 1992; Chambers & Leggett, 1987). A close relationship between temperature and developmental rates has been reported in many fish species (Pepin, 1991; Martell *et al.*, 2005; López-Olmeda & Sánchez-Vázquez, 2010).

In the pejerrey *Odontesthes bonariensis*, water temperature not only affects growth rates but also directs the sexual differentiation process (Strüssmann & Patiño, 1995; Ito *et al.*, 2005). This species presents a strong temperature sex determination (TSD), a form of environmental sex determination (ESD) where 100% female or male populations can be obtained simply by controlling water temperature (Strüssmann *et al.*, 1996a). In pejerrey, the critical or temperature-sensitive period occurs from the first to the fifth week after hatching, depending on temperature. Pejerrey larvae reared from hatching at 13-19ºC produced 100% females, at 29ºC 100% males, and mixed sex proportions at intermediate temperatures, 24-25ºC (Strüssmann *et al.*, 1996b, 1997; Karube *et al.*, 2007; Fernandino *et al.*, 2008a). Sex differentiation is also accelerated by rearing temperature; the ovarian differentiation occurs before at 24ºC than at 17ºC, whereas testis development takes place before at 29ºC than at 24ºC (Ito *et al.*, 2005; Fernandino *et al.*, 2008b). Also, germ cell degeneration usually became histologically recognizable after one to two weeks of exposure to temperatures higher than 29ºC and became more severe with increasing duration of exposure (see Strüssmann *et al.*, 2010).

The above-mentioned concepts arise the question of how different is the development of pejerrey larvae of the same age reared at different temperatures. Therefore, the aims of the present study were: a) to provide a brief description of the embryo period and a morphological and quantitative description of the larval development of *O. bonariensis* to complete previous information on this species (Minoprio, 1944; Muñiz Saavedra & Piacentino, 1991); b) to describe the effects of the thermal range involved in TSD on larval development, and c) to generate an operative definition of pejerrey larva and juvenile.

Material and Methods

Fertilization and incubation

For the characterization of embryo development, the eggs were obtained from six years old pejerrey brood-stock at the "Instituto Tecnológico de Chascomús" aquatic facilities in spring, by controlled fertilization. Before manipulation, the fish were anesthetized by immersion in a 100 ppm benzocaine solution for five minutes approximately. Fertilization time was taken as the moment when the oocytes took contact with the milt. The fertilized oocytes were then incubated at 19 ± 0.5°C in a flow-through water (4 - 5 NaCl g/L) incubator.

Larval rearing

For the characterization of larval development, fertilized eggs were obtained from spontaneous spawning events of the same captive-reared brood-stock. Newly spawned eggs were collected early in the morning and incubated in the previously described conditions. After hatching, three groups of 1,500 larvae each one were kept in 130 l fiberglass tanks with flow-through water under 12L:12D light cycle, and salinity 15 g/L. The tanks were kept at three different temperatures: female producing temperature (FPT = 17ºC), mixed-sex producing temperature (MixPT = 24ºC) or male producing temperature (MPT = 29ºC) according to Strüssmann *et al*. (1997). Water temperature in the tanks was recorded with data-loggers and maintained with heaters with electronic control.

The larvae were feed daily at satiation with *Artemia* nauplii until day 42, and then supplied with artificial fish food (Shullet®, Argentina).

Embryonic development

The embryo development was registered using a Nikon SMZ800 stereomicroscope connected to a digital camera. In order to facilitate the correct orientation of the embryos, they were set into a Petri dish full of a 0.01% agar solution. The time at which each developmental stage took place was considered as the moment where at least five out of ten embryos sampled from the same pool presented the same characters.

Larval development

The morphological changes occurring during larval development stages were only described from larvae reared at MixPT. After hatching, ten larvae from each tank were photographed once a week with a digital camera using the same stereomicroscope. To study fin development, ossification of caudal fin rays, and the formation of the first and second dorsal and anal fin rays, Alizarin Red and Alcian Blue staining were carried out using a protocol adapted from Potthoff (1984). The number of caudal fin segments was counted on digital images on a dark field.

To describe the effects of temperatures on larval development the following morphometrics measurements (orbital diameter, inter-orbital distance, head length, pectoral fin length, head width, pre-orbital distance, post-orbital distance, head depth, caudal peduncle depth, body depth, anal distance, total length, and standard length), were taken from larvae reared at MPT, MixPT and FPT and measured to the nearest 0.001 mm using digital images and the Image-pro plus 4.0 software.

Body weight (BW), total length (TL), and the mentioned morphometric measurements were transformed to decimal logarithms in order to obtain linear relationships. Un-standardized residuals were obtained from the regression of morphometric measurements with logTL in order to eliminate the effect of size on the body shape analysis.

The Condition Factor ($CF = 1000 \times BW(mg) \times SL(mm)^{-3}$) was used as an indicator of body shape. The effects of temperature (as a fixed factor) on TL, BW, and CF were analyzed using ANCOVA, considering time or TL as co-variables. Multivariate

analysis of body shape (see morphometric measurements) was conducted using Discriminant Analysis (DA) in order to show the effects of developmental temperatures.

Results

Embryonic development

Just spawned oocytes were yellow-green, surrounded by a transparent *zona radiata* (*sensu* Kunz, 2004). The oocyte diameter before fertilization was around 1.68 ± 0.01 mm (mean ± standard error, N = 84) and most of the volume was occupied by the yolk. At that moment the oocytes presented a series of oil droplets (40.7 ± 1.2; N = 41) with sizes ranging from 30 to 250 µm. They also presented some, among 3-10, adherent filaments per egg.

Just after fertilization the perivitelline space (68 ± 12 µm; N = 81) was clearly defined, and 1.5 hours post fertilization (hpf) the cytoplasm streamed towards the animal pole forming a cap-shaped bulge, the blastodisc (Fig. 1A).

The first cleavage occurred 2 hpf originating two rounded cells (Fig. 1B). The cells kept their rounded shape until the fourth cleavage that produced 16 blastomeres arranged in 12 peripheral and 4 central cells. At 64 blastomeres, the symmetry

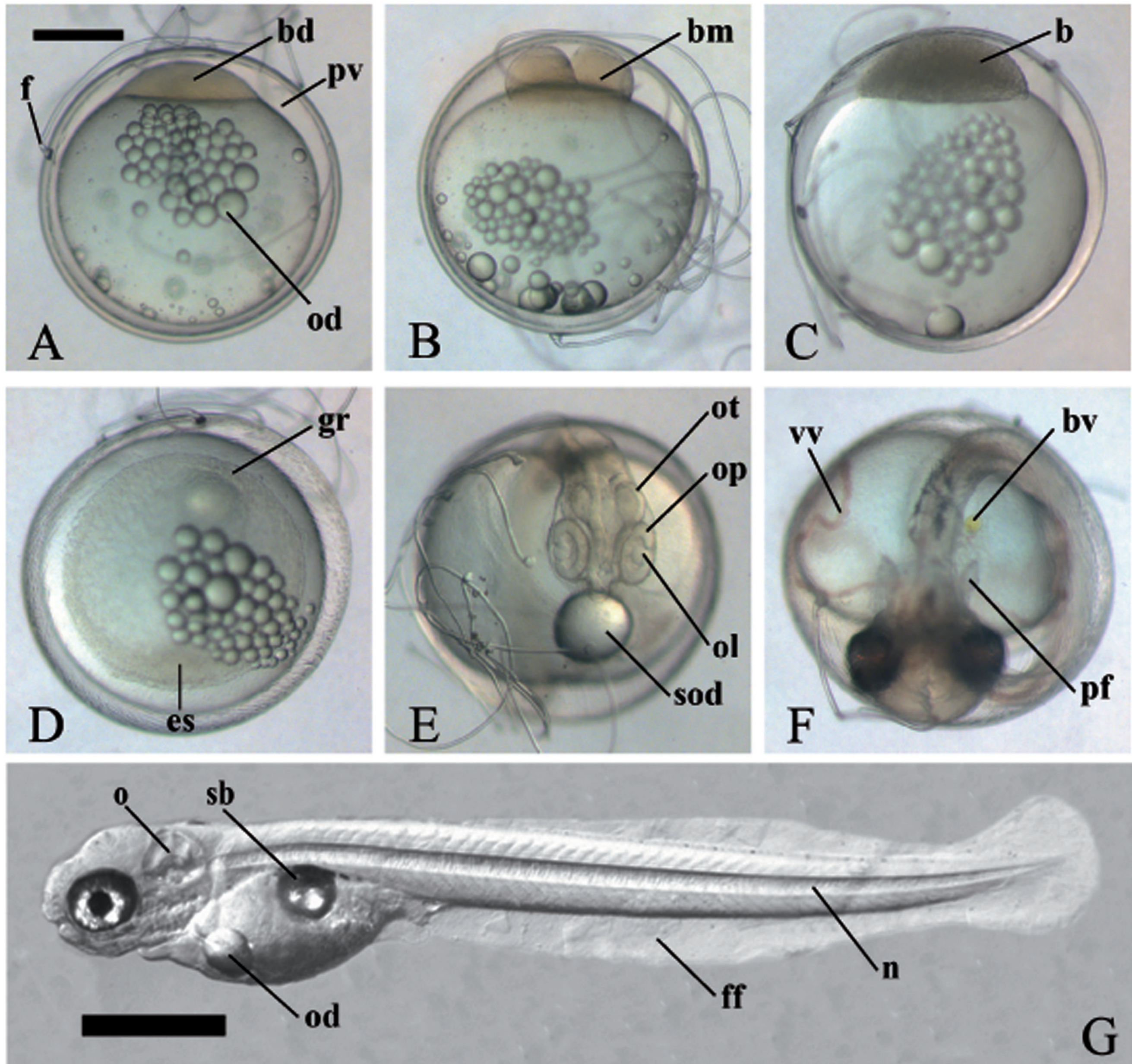

Fig. 1. Embryonic stages of pejerrey. **A**) One cell stage: bd, blastodisc; f, adherent filaments; od, oil droplets; pv, perivitelline space; **B**) Two cells stage: bm, blastomeres; **C**) Blastula stage: b, blastula; **D**) Animal pole view at 25% epiboly stage, es, embryonic shield; gr, germinal ring; **E**) Vitelline veins stage: ol, ocular lenses; op, optic capsule, ot; otic capsules; sod, single oil drop; **F**) Pectorals fins stage: bv, bile vesicle; pf, pectoral fins; vv, vitelline veins. **G**) Hatching: n, notochord; o, otoliths; sb, swim bladder; sod, single oil droplet. **A-F**) bar = 0.5 mm; **G**) bar = 1 mm.

Table 1. Embryonic development. Stages, hours post fertilization (HPF) and characteristics.

	Stage	HPF	Characteristics
Zygote	Just fertilized egg	0	Cortical cytoplasm equally distributed.
	Blastodisc	1.5	Cortical cytoplasm concentrated on the animal pole.
Cleavage	2 Cells	2	First cleavage.
	4 Cells	3	Second cleavage. 2x2 blastomeric array.
	8 Cells	4	Third cleavage. 2x4 blastomeric array.
	16 Cells	5	Fourth cleavage. 4 central and 12 peripheral blastomeres.
	32 Cells	6.5	Fifth cleavage. 4x8 blastomeric array.
	64 Cells	7.5	First equatorial cleavage.
	Blastula	8.5	Blastoderm dome-shaped with several cellular layers.
Gastrulation	25% Epiboly	26	The blastoderm margin expands ¼ over the yolk. The germ ring and embryonic shield are formed.
	50% Epiboly	29	Beginning of the embryonic axis formation on the embryonic shield.
	90% Epiboly	38	The notochord rudiment starts to be visible.
Neurulation	Optic primordial	47	The somitogenesis begins. The eye primordium and Kupffer's vesicle can be distinguished.
	Otic primordial	52	The otic capsule is visible.
	Ocular lenses	63	The number of somites increases and the ocular lenses is visible.
	Oil drop	95	The oil droplets coalesce into a single drop.
	Vitelline veins	97	The vitelline veins, optic and otic capsules are clearly distinguished.
	Heart	131	The heart is visible and blood flow can be clearly seen. First movements of the tail.
	Ocular pigments	147	The olfactory bulbs, telencephalon and the optic cups with pigmented retina are distinguished.
	Pectoral fins	170	The pectoral fins and bile vesicle can be observed. The body pigmentation begins.
	Swim bladder	220	Cephalic melanophores and an inflated swim bladder can be observed.
	Hatching	270	

of the cleavage planes was difficult to observe. Until the mid-blastula transition, the cleavage rate was approximately of 1 division per 1 to 1.5 h. Finally, 8.5 hpf a "ball-like" blastoderm, the blastula, was formed (Fig. 1C).

At 26 hpf, the blastoderm cells produced 25% epiboly and the germinal ring and the embryonic shield were clearly visible (Fig. 1D).

At the end of gastrulation and just before the beginning of the formation of the first somite, the optic primordia, the head, and the Kupffer's vesicle were observed. With the formation of the ninth somite, the otic capsules appeared behind the optic cups (52 hpf). Then, at 63 hpf 17 somites were clearly recognized and the ocular lens development began.

At 95 hpf the original multiple oil droplets completely coalesced in a single large drop. In a dorsal view, this oil drop can be seen located just anterior to the head. Two hours later, well developed optic and otic capsules can be seen (97 hpf; Fig. 1E). At 131 hpf, the heart was actively bombing and the tail coiled. One day after (147 hpf) some chromatophores could be seen on the dorsal part of the body, as well as the ocular pigmentation.

Table 2. Total length (TL) and days post hatching (DPH) corresponding to developmental events of free embryos and larvae reared at MixPT.

Event	TL (range, mm)	DPH
Hatching	6.5-7.6	0
Exogenous feeding	7.4-8.2	2
First rays fin caudal	8.3-8.9	7
Notochord flexion and the rays became segmented	9.1-10.4	7-21
Caudal fin rays complete	11.1-11.7	14-28
First dorsal fin rays	12.6	14-28
Forked homocercal caudal fin	13.9-15.0	21-35
Caudal fin rays bifurcated	18.4-19.4	28-49
Fin-fold absorption	20.1-23.0	42-56

Between 170 and 220 hpf the pectorals fins, bile vesicle, and the swim bladder could be clearly observed (Fig. 1F).

Finally, hatching ocurred at 270 hpf. This point represents the time at which 50% of the larvae have already hatched. The free embryos (6.5-7.6 mm) presented a characteristic median finfold, big otoliths, a conspicuous notochord, and a single ovoid oil droplet (Fig. 1G).

A summary of the developmental stages of pejerrey embryos is shown in the Table 1.

Larval development

Total length and days post-hatching (dph) of free embryos and larvae corresponding to ontogenetic events are shown in Table 2.

Beginning of exogenous feeding: At 2 dph (7.4-8.2 mm), the larvae showed active movements and swan in the superficial layer of the water. The exogenous feeding on *Artemia* nauplii started.

Finfold development: The caudal fin development was characterized by a restructuration from a rounded primary structure to a definitive forked homocercal shape (Fig. 2). During this transition, the first fin rays appeared on the ventral portion of the caudal fin (8.3-8.9 mm, 7dph). At this moment, the larvae still presented a straight notochord (Fig. 2A). With the beginning of the formation of the first caudal rays, the notochord flexion started (9.1-10.4 mm, 7-21 dph). This movement resulted in a ray alignment with the antero-posterior axis (Fig. 2B; 2C). At this moment the medial-fin-fold reabsorption started. Finally, the caudal fin acquired the definitive forked homocercal structure (13.9-15.0 mm, 21-35 dph, Fig. 2D). Then, the bifurcation of the central fin rays in the sixth ray's segment began (Fig. 2E). At this moment (20.1-23.0 mm, 42-56 dph), the original fin-fold was almost completely reabsorpted. A remnant fin-fold could be observed between the anus and the anal fin (Fig. 2F), and the body shape acquires the juvenile conformation (Fig. 2G).

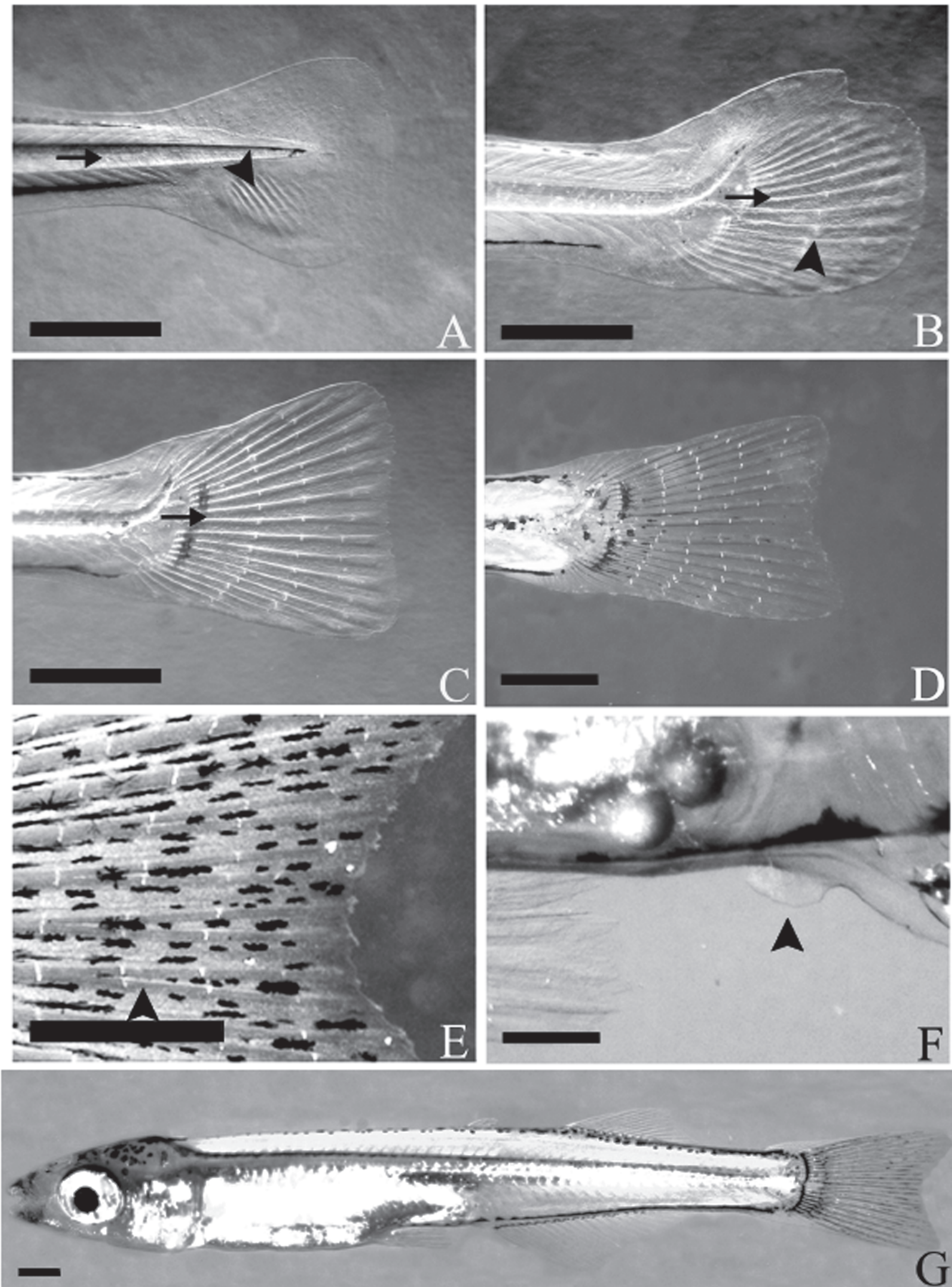

Fig. 2. Fin fold reabsorption during larvae-juvenile transition. **A**) The characteristic lobulated caudal fin showing the first fin rays (arrowhead) and the straight notochord (arrow); **B**) The second segment appeared (arrowhead) and the ray started to be aligned with the rostro-caudal axis (arrow); **C**) The ray aligned with the rostro-caudal axis (arrow); **D**) The forked homocercal caudal fin; **E**) Bifurcation of the central fin rays (arrowhead); **F**) The remnant fin-fold between the anus and the anal fin; **G**) The body shape acquires the adult conformation. **A-F**, bar = 0.5 mm; **G** = 1 mm.

Effects of temperature on growth, condition, and meristic characters

The quantitative characterization of the larval development was achieved during 77 days at FPT, 63 days at MixPT and 56 days at MPT in order to reach approximately the same size. All, logTL (ANCOVA, N = 291, P < 0.001), logBW (ANCOVA, N = 291, P < 0.001), and CF (ANCOVA, N = 291, P < 0.001) differed significantly between temperatures, considering time as a significant co-variable (ANCOVA, N = 287, P < 0.001) and being the larvae longer, heavier, and more robust at higher temperatures (Fig. 3A-C, Table 3). It is interesting to note that considering size instead time as the independent variable, body size explains the Condition Factor (a measure of body shape) with a F value of co-variable changing from 383 (co-variable = Time) to 443 (co-variable = Body size), *i.e.* the degree of development can be directly related to size irrespective of temperature better than time (Fig. 3D, Table 3).

Although less conspicuous, the relationship of logBW versus logTL also differed significantly between developing temperatures (ANCOVA, N = 287, P < 0.001), with a common slope slightly but significantly higher than 3 (95%

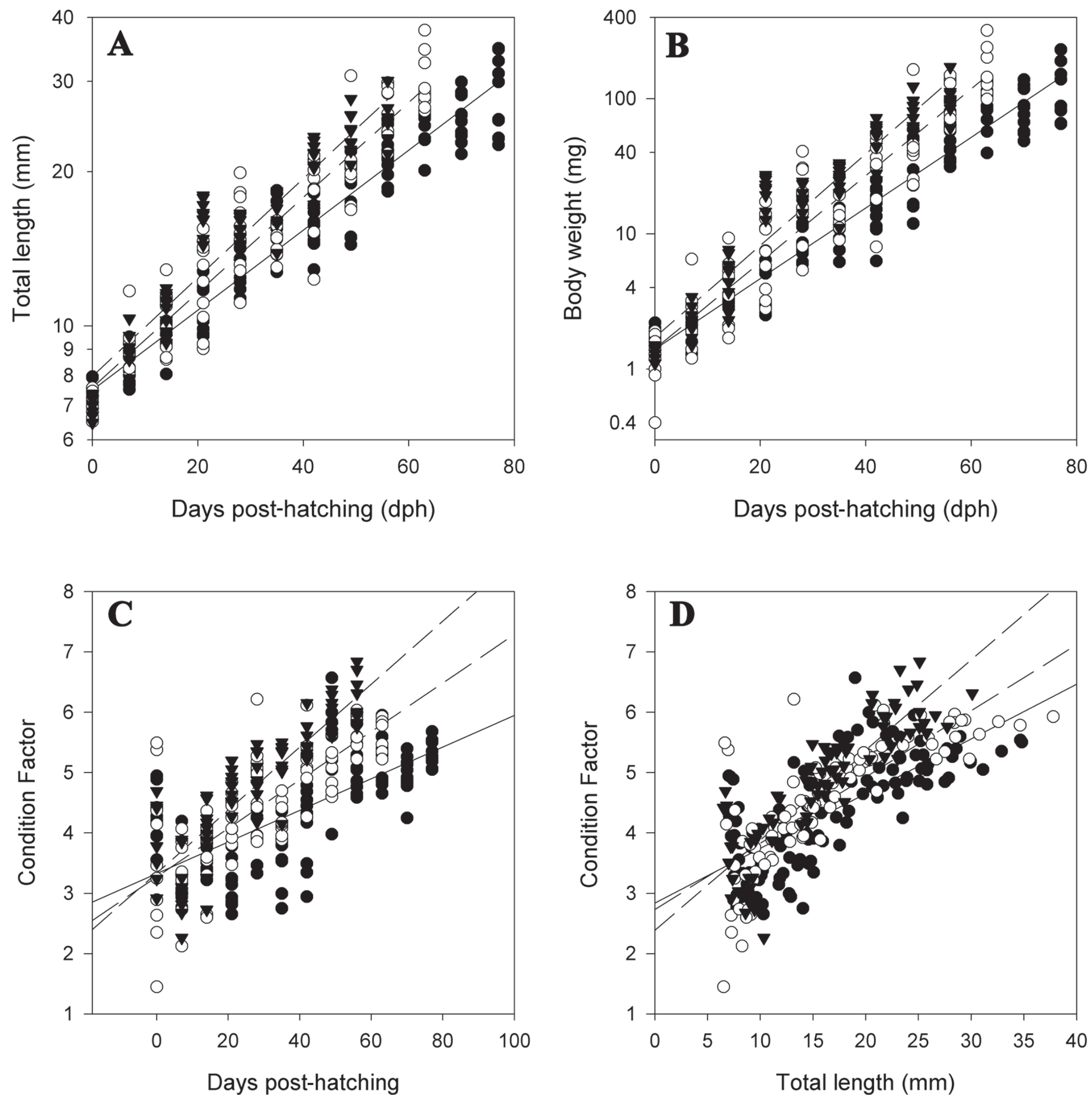

Fig. 3. Total length (**A**), body weight (**B**), both in logarithmic scale, and Condition Factor (**C**) in relation to days post hatching (dph) and water temperature. Female producing temperature (FPT, filled circles and solid line), mixed-sex producing temperature (MixPT, empty circles and long dashed line), and male producing temperature (MPT, triangles and medium size dashed line). Note that when Condition Factor is considered as a function of Total length (**D**) dispersion diminishes. Lineal regression lines are indicated in order to show the tendency. Regression coefficients and r^2 are indicated in Table 3.

Table 3. Regression coefficients (Y= a + b. X), temperatures (TºC) and r^2 of the equations fitted in Fig. 3 and 4.

Y =	a	+ b	. X	T ºC	r^2
log Total length (mm)	0.8750125826	7.806587936e-3	Days post hatching (dph)	17	0.9362491701
	0.8800653739	9.270223130e-3		24	0.8932340519
	0.9019366886	9.644828454e-3		29	0.9081459834
log Body weight (mg)	0.1487252087	0.0261069603	Days post hatching (dph)	17	0.9283523265
	0.150164111	0.0320462293		24	0.8852029371
	0.2352059927	0.0339170351		29	0.9137481102
Condition Factor	3.328951688e-3	2.622077894e-5	Days post hatching (dph)	17	0.4808749279
	3.274492228e-3	4.012380058e-5		24	0.611313859
	3.338880945e-3	5.212077784e-5		29	0.7722985899
Condition Factor	2.837346867e-3	9.075799020e-5	Total length (mm)	17	0.5187828299
	2.730902885e-3	1.097818274e-4		24	0.6248020549
	2.387184154e-3	1.495034898e-4		29	0.7552203085
Number of segments	-0.7320136925	0.1278166394	Days post hatching (dph)	17	0.9073954999
	-0.0554012424	0.1374750193		24	0.8200485421
	0.2411666159	0.1454244811		29	0.9294294466

Confidence Interval = 3.375, 3.458). In the same way, the number of caudal fin segments showed a significant dependence with temperature, in addition to the significant co-variation with time and total length (ANCOVA, N = 234, P < 0.001, Fig. 4, Table 3).

Fish reared under different temperatures were also discriminated based on their body shape (DA applied on un-standardized residuals, N = 287, P < 0.001; Fig. 5). During the rearing period, clear differences among larvae were observed in the rate of fin fold restructuration (Fig. 6).

Discussion

Just like all teleosts (Kunz, 2004), pejerrey showed a discoidal meroblastic cleavage pattern, where the large yolk volume restricts cell divisions to a small area at the animal pole. Twenty two developmental stages could be recognized during the embryogenesis which can be used to compare to closely related species. In this way, some similarities were observed when compared to medaka's, *Oryzias latipes*, embryogenesis (Iwamatsu, 2004). For example the array of 4 central and 12 peripheral cells at the fourth cleavage event, the formation of embryonic shield at 25% of epiboly, and the development of optic primordial before the somitogenesis started. In addition, some similarities were observed when compared to other Atheriniformes, which embryos shared the presence of a single row of melanophores over the dorsal portion of the body and a short preanal length, less than 40% of standard length until the notochord flexion (White *et al.*, 1984). There were also some ontogenetic characters: presence of adherent filaments, oil droplets coalescing and position of developing heart; shared with other Atheriniformes, Beloniformes, and Cyprinodontiformes. These morphological characteristics may support the concept of Atherinomorpha as a monophyletic group (Rosen, 1964; Dyer, 2006; Setiamarga *et al.*, 2008).

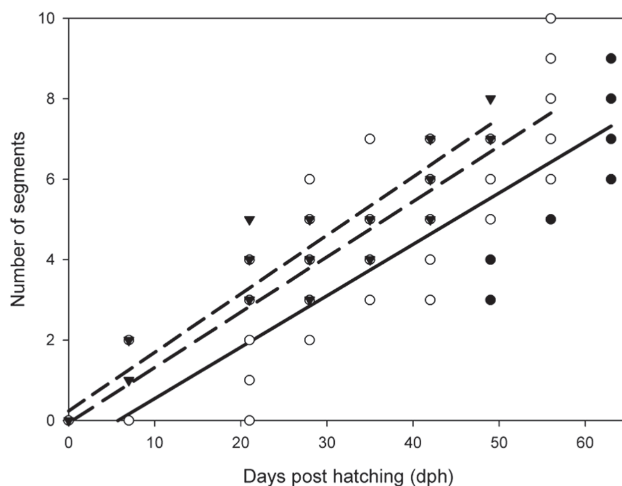

Fig. 4. Number of caudal fin segments in relation to days post hatching (dph) and water temperature; 17°C (filled circles and solid line), 24ºC (empty circles and long dash line), and 29ºC (triangles and medium dash line). Regression coefficients and r^2 are indicated in Table 3.

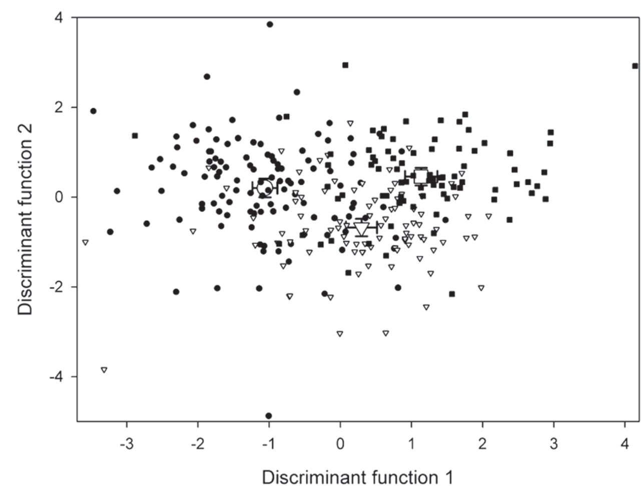

Fig. 5. Body shape (DA applied on un-standardized residuals, N = 287, P < 0.001). Discriminant function 2 *versus* discriminant function 1. Rearing temperature is indicated as black circles (FPT), triangles (MixPT) and black squares (MTP). Means and 95% confidence intervals correspond to FPT (circle), MixPT (triangle), and MTP (square).

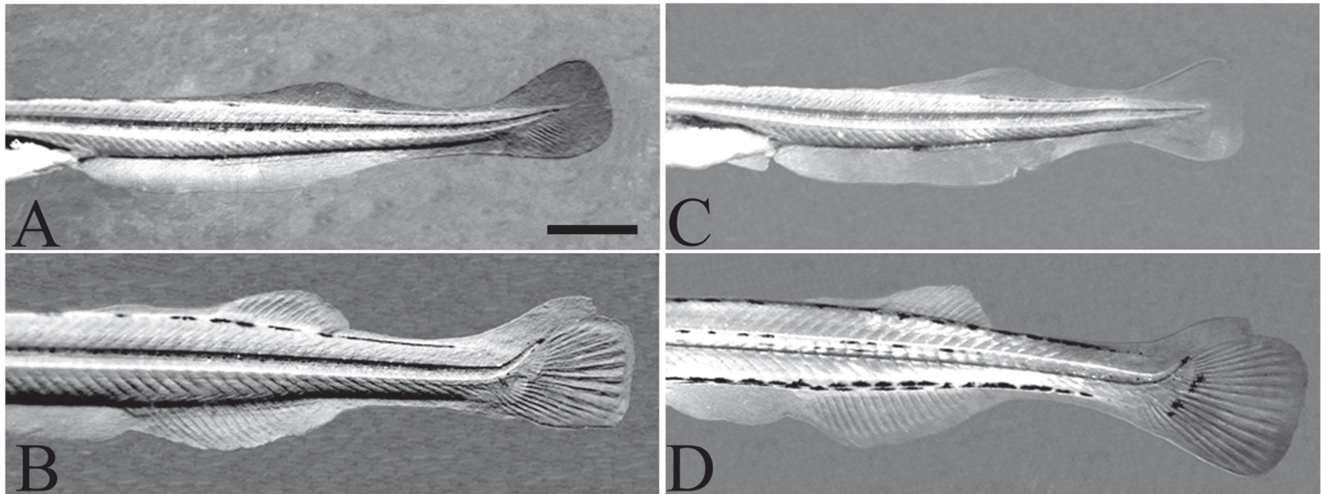

Fig. 6. Differences in the rate of fin fold restructuration among larvae. **A-B**) same age, same temperature and different finfold stages; **C-D**) same age, different temperature and different finfold stage; **B-D**) different temperature, same age and same finfold stage. **A**) 24ºC, 14 dph, TL=9.1 mm; **B**) 24ºC 14 dph, TL=11.7 mm; **C**) 17ºC 14 dph, TL=8.1 mm; **D**) 29ºC 14 dph, TL=11.1 mm. Bar = 1 cm.

Metamorphosis, the transition from larvae to juveniles, has great importance as it displays development programs under selective pressures operating during free life. In pejerrey, one informative metamorphic change was fin development and, especially, the caudal fin. Like most teleost fish (Kendall *et al.*, 1984), pejerrey larvae possess at hatching a median-fin-fold from which the odd fins (anal, caudal, first and second dorsal) develop. Fin growth takes place by sequential addition of new ray segments to the distal end of each ray (Santamaria & Becerra, 1991). During the caudal fin transition from a lobulated to a homocercal structure, these rays should change their growth in order to develop longer dorsal and ventral and shorter central rays. This could be accomplished in two ways: a) all rays increase segment number synchronously but there is a differentially higher rate of rays growing of dorsal and ventral fin rays over central fin rays or b) through an asynchronycal addition of segments, skipping cycles of segment addition in the central rays (Goldsmith *et al.*, 2003; 2006). In pejerrey larvae, all caudal rays had the same number of segments implying that the caudal fin shape is achieved by differential growth rate.

During fin fold reabsorption not only the caudal fin underwent restructuration but also did the anal, first and second dorsal fins. Fin fold reabsorption was the last clear morphologic change observed and then it could be considered as the end of the metamorphic process in pejerrey (from 42 to 56 dph; 20.1 to 23 mm TL).

In this species, as in any ectotherm organism, larval growth rate, both in length and weight, was affected by temperature (Angilletta *et al.*, 2004). Larvae of the same age were longer and heavier and had more caudal fin-ray segments when they were raised at higher temperatures. Temperature also affected the moment of definitive caudal fin shape acquisition. It must be noted that, in agreement with Fuiman *et al.* (1998), this thermal dependence of a time related processes diminished when the developmental changes were considered in terms of body length. Thus, size could be useful indicators of the degree of development.

In pejerrey, male producing temperature, 29ºC, is close to the lethal temperature (32ºC) reported for juveniles, it is higher than rearing temperatures usually considered appropriate for this species (Gómez *et al.*, 2007; Somoza *et al.*, 2008), and can induce partial (numerically or temporarily) or even permanent sterility in larvae, juveniles and adults of both sexes in pejerrey (Strüssmann *et al.*, 2010). Even when deformities were not observed at 29ºC in this work, they were reported by Strüssmann *et al.* (1997) in similar experiments. However, it is important to know that growth at MPT was achieved with an allometric index greater than 3 indicating that the condition in larvae rearing on this temperature range is physiologically suitable.

In summary, we provided a brief morphological and quantitative description of embryonic and larval period of *O. bonariensis* and showed clear effects of temperature on larval growth and condition. The present data showed that, in pejerrey, the degree of development can be inferred from size, as already stated in other fish species (Sæle & Pittman, 2010). The juvenile phenotype is acquired when the fin fold is reabsorbed and the caudal fin acquires the definitive forked homocercal structure. These data will allow performing new studies taking the present development description as a reference point in the framework of studies related to TSD and chronologies of sexual determination and differentiation.

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