Early development in the annual fish *Cynolebias viarius*

M. J. Arezo*, L. Pereiro and N. Berois

Biología Celular, Facultad de Ciencias, Universidad de la República, Iguá 4225, Montevideo (11400), Uruguay

The detailed characteristics and timing of early development in *Cynolebias viarius*, an annual fish endemic to Uruguay, were obtained from *in vivo* observations of embryos cultured under standardized laboratory conditions. The chorion ultrastructure is reported for this species for the first time. Comparison with previous data from other annual fishes showed that *C. viarius* exhibited the longest early development including cleavage, blastula formation and epiboly. © 2005 The Fisheries Society of the British Isles

Key words: annual fishes; *Cynolebias*; developmental stages.

INTRODUCTION

Fishes have a basal position in the vertebrate lineage and their embryos share general chordate characters with other vertebrates. In addition, most fish embryos develop externally from transparent eggs. These characteristics made them a special model for the study of vertebrate embryogenesis (Langeland & Kimmel, 1997).

The zebrafish *Danio rerio* (Hamilton) has become a widely used model organism because its general developmental pattern is similar to all osteichthyans (Metscher & Ahlberg, 1999). Thus, its embryological development is considered representative for teleosts (Westerfield, 2000).

In annual fishes (order *Cyprinodontiformes*) a different developmental pattern occurs (Myers, 1952). Between cleavage and embryogenesis, the blastomeres undergo dispersion followed by an aggregation phase. Furthermore, the embryos show reversible arrests (diapauses) at different stages (Wourms, 1972*a*, *b*, *c*; Ballard, 1981). This developmental strategy is closely related with their life cycle. These fishes inhabit temporary pools that undergo drying during summer when adults die. The embryos remain buried in the bottom mud and are resistant to desiccation. In the subsequent rainy season, they hatch a few hours after the pool is flooded and a new reproductive cycle begins (Wourms, 1964, 1967, 1972*c*).

*Author to whom correspondence should be addressed. Tel.: +598 2 5258618 /145; fax: +598 2 5258617; email: maui@fcien.edu.uy

The genus *Cynolebias* occurs from north-eastern Brazil to north-eastern and southern Argentina, Uruguay and Paraguay (Costa, 1995). The more relevant available data on *Cynolebias* include: ecology and ethology (Vaz Ferreira *et al.*, 1964; Vaz Ferreira & Sierra, 1973), morphology (Loureiro & de Sá, 1996, 1998), systematic and phylogeny (Costa, 1995, 1996, 1998) and cytogenetic and molecular phylogeny (Máspoli & García, 1988; García *et al.*, 1993, 1995, 2000, 2001, 2002). Developmental studies, including cell behaviour during early development, were reported for three species: *Cynolebias constanciae* Myers, *Cynolebias whitei* Myers and *Cynolebias nigripinnis* Regan (Wourms, 1972*b*; Carter & Wourms, 1990, 1991). Since this genus is very specious, more data are needed to perform comparative studies.

Cynolebias viarius Vaz-Ferreira, Sierra de Soriano & Scaglia de Paulete is an endemic species from Uruguay (Vaz Ferreira *et al.*, 1964) and the adults easily adapt to and reproduce in laboratory conditions. Moreover, in spite of this genus being the subject of systematic controversy (Costa, 1998; García *et al.*, 2000, 2002) members of this species are taxonomically well characterized. These features make it a useful model to study early development.

Interpretation of experimental studies requires the structural and temporal framework provided by morphology and duration of developmental stages (Long & Ballard, 2001). Thus, as an essential first step, the morphological characteristics and timing of *C. viarius*'s early development in standardized laboratory conditions were determined.

MATERIALS AND METHODS

Adult females and males of *C. viarius* were collected during the rainy season in temporary ponds from Depto Rocha, Uruguay (Fig. 1). They were kept in the laboratory in 30 l aquarium, filled with continuously aerated, dechlorinated tap water (pH 7–7·5), and exposed to natural light for *c.* 14 h daily. Mean water temperature was *c.* 19° C, and water was partially changed every 5 days. Specimens were fed once a day with live
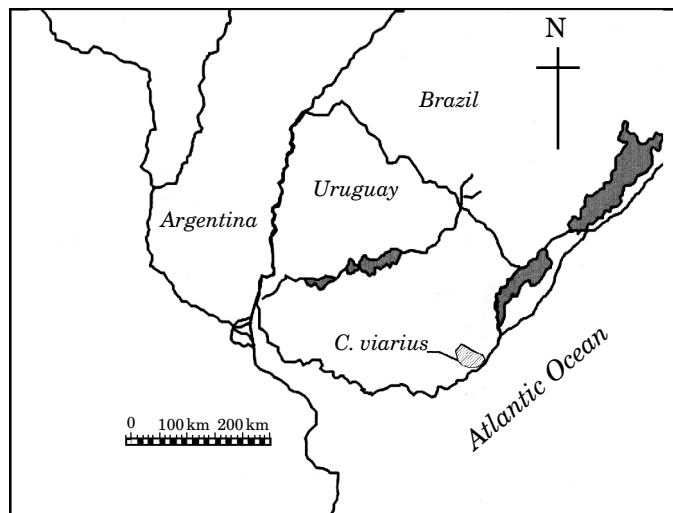

FIG. 1. Map of Uruguay showing *Cynolebias viarius* collection area (▨).

Tubifex sp. (bloodworms). Spawning was obtained naturally from fish pairs or groups of one male to two females isolated in an aquarium that had a container with peat moss on the bottom. Eggs were collected with a pipette and they were cleaned by rolling with a soft paintbrush on filter paper moistened with aquarium water.

EMBRYO CULTURE

A total of 1344 fertilized eggs from 30 matings were collected. Eggs were placed in Nunclon 24 well plates (1 ml per well working volume) and incubated in darkness at 25° C in Yamamoto's solution (NaCl, 0·75%, KCl, 0·02%, $CaCl_2$, 0·02%; Yamamoto, 1967) plus 100 U ml^{-1} penicillin G (Sigma) following Wourms (1972a). Embryos were checked daily by placing each egg, with a drop of Yamamoto solution, in a 1·7 mm deep-chamber carved in tape layers over a microscopic slide. A coverglass, gently placed over the chamber, touched the top of the egg allowing rotation and observation under a light microscope. Live embryos were examined and photographed using an Olympus Vanox microscope.

ULTRASTRUCTURAL ANALYSIS

Ripe oocytes obtained from ovaries were fixed in 2·5% glutaraldehyde/0·1 M phosphate buffer, pH 7.4, for 2 h. After fixation the oocytes were washed three times (15 min each) in 0·1 M phosphate buffer and dehydrated in increasing concentrations of acetone (15 to 100%). Subsequently, they were dried at the critical point of CO_2, mounted on holders and sputtered with gold in a Denton Vacuum LLC Desk II Cold/ETCH UNI sputter apparatus. Specimens were examined and photographed with a JEOL JMS 59000 LV scanning electron microscope (SEM).

RESULTS

Cynolebias viarius embryos were successfully cultured in Yamamoto's solution. In these conditions it was possible to follow the stages in a large mass of embryos with a good survival rate. It was possible to maintain live embryos without any mould contamination even if dead embryos were present in the same well.

Developmental stages are described below, and the corresponding timing is summarized in Table I.

DEVELOPMENTAL STAGES

Oocyte

The oocyte is a hyaline sphere *c*. 1·7 mm in diameter surrounded by a thick acellular chorion. Analysis by SEM reveals the chorion has a rough outer surface ornamented by dense hairlike projections [Fig. 2(a)]. Two kinds of hair-like filaments, that differ in thickness, are found. Thick filaments, that appear more rigid, are 2–2·3 μm in diameter at their base, whereas thinner and more flexible filaments are 0·7–1·2 μm in diameter. Both filaments have an overall cone-shaped morphology. The micropyle, the single site of sperm entry, marks the animal pole [Fig. 2(b)]. The micropyle apparatus does not show any special features. It is a funnel-shaped structure with an external aperture of *c*. 3·6 μm and the nucleus of the oocyte lies immediately below the micropyle (unpubl. obs.). The yolk is homogeneous in appearance except for

TABLE I. Comparison of approximate post-fertilization duration of early developmental stages among annual fishes: *Cynolebias viarius*, *Austrofundulus myersi* (Wourms, 1972a), *Nothobranchius korthausae* (Van Haarlem, 1983), *Cynolebias whitei*, *Cynolebias nigripinnis* and *Cynolebias constanciae* (Carter & Wourms, 1991) and two non annual fishes species: *Danio rerio* (Kimmel *et al.*, 1995) and *Fundulus heteroclitus* (Armstrong & Child, 1965)

| Stage	Annual fishes				Non annual fishes	
	C. viarius (25° C)	*A. myersi* (25° C)	*N. korthausae* (25° C)	*C. whitei, C. nigripinnis* and *C. constanciae* (28° C)	*D. rerio* (28° C)	*F. heteroclitus* (20° C)
Cleavage	1 day	7 h	15 h	9 h	2 h	10 h
Blastula formation	3 days	20 h	24 h	20–22 h	5 h	24 h
Epiboly	7 days	44–48 h	51 h	44–48 h	11 h	40 h
Reaggregation	12 days	9 days	3 days	9 days		
Embryonic axis	13 days	9·5 days	4 days	9·5 days		37 h
Somatogenesis (0–10 pairs)	16 days	11 days	6 days	11 days	14 h	56 h
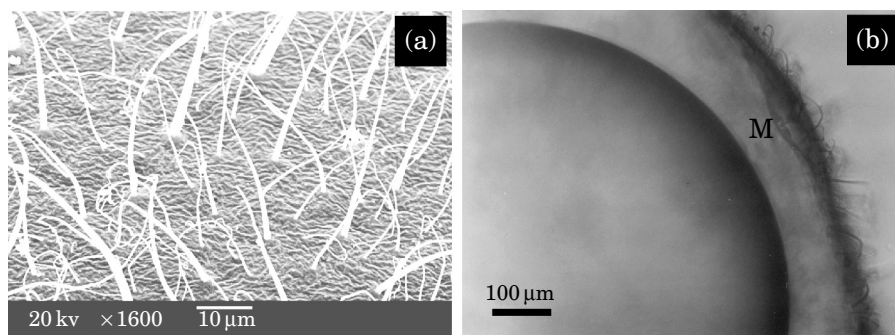
Fig. 2. Some features of *Cynolebias viarius* oocytes: (a) chorion surface (SEM) and (b) animal pole of oocyte (light microscopy). M, micropyle.

several lipid droplets. The amount and size of the droplets varies among individual eggs.

One cell stage

After fertilization, a perivitelline space develops between the chorion and the cell surface of the zygote. At this stage, an accumulation of cytoplasm in the animal pole is observed. The blastodisc is easily distinguishable from the yolk mass. The process of amphimixis is shown in Figs 3(a) and 4(a).

Two cell stage [Fig. 3(b)]

The first mitotic division is meridional and divides the blastodisc into two cells of equal size. There is no cell membrane between the cytoplasm of the blastomeres and the yolk cytoplasmic layer.

Four and eight cell stage

The planes of the second and third cleavages are also meridional. Eight blastomeres of approximately equal size are generated and all of them are in contact with the yolk.

Sixteen cell stage [Fig. 3(c)]

At this stage, the animal pole consists of a solid cluster of cells. There are one or two centrally located blastomeres that are completely surrounded by the others.

Thirty-two – 64 cell stage [Fig. 3(d)]

The hemispherical blastoderm consists of two kinds of cells: an outer layer of peripheral blastomeres that gradually flatten and a population of rounded and centrally–located blastomeres called deep cells. Cleavage becomes asynchronous at this stage.

Blastula formation

Several transitional stages are recognized as the blastoderm becomes hollow and flat [Figs 3(e), (f) and 4(b), (c)]. First, the blastoderm expands along and over the yolk mass while a cavity (segmentation cavity or blastocoel) forms in
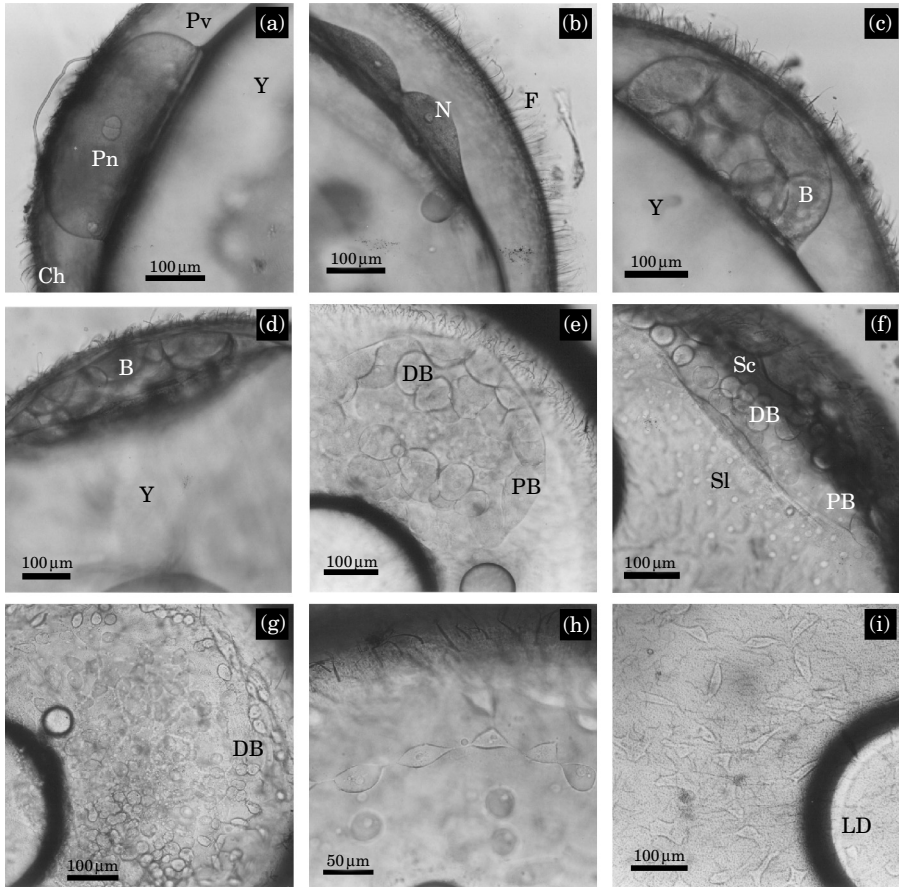
FIG. 3. Development of *Cynolebias viarius* embryo from fertilization to dispersion stage: (a) one cell stage
    embryo (amphimixis process) (Ch, chorion; Pn, pronucleus; Pv, perivitelline space; Y, yolk), (b) two
    cell stage (F, chorion fibrils; N, nucleus), (c) 16 cell stage (B, blastomere; Y, yolk), (d) 32–64 cell
    stage (time post-fertilization: 1 day) (B, blastomere; Y, yolk), (e) early blastula (DB, deep blas-
    tomere; PB, peripheral blastomere), (f) late blastula (time post-fertilization: 3 days) (DB, deep
    blastomere; PB, peripheral blastomere; Sc, segmentation cavity; Sl, syncitial layer), (g) epiboly (DB,
    deep blastomere), (h) deep blastomeres in active dispersal, arranged in short rows with fine
    cytoplasmic extensions and (i) dispersed phase (time post-fertilization: 7 days) (LD, lipid droplet).

its centre. The outer blastomeres form the enveloping cell layer. Initially, they
are mononucleated, then they become binucleate and finally multinucleate. The
segmentation cavity is 'roofed' by the enveloping cell layer. Within the cavity
the spherical blastomeres (deep cells) are loosely arranged and centrally located.
The cell population increases in number and the blastoderm continues to
flatten. At the edge of the expanding blastoderm small nuclei organized in
rows appear in the yolk syncitial layer.

Epiboly

The blastoderm expands over the yolk mass towards the vegetal pole
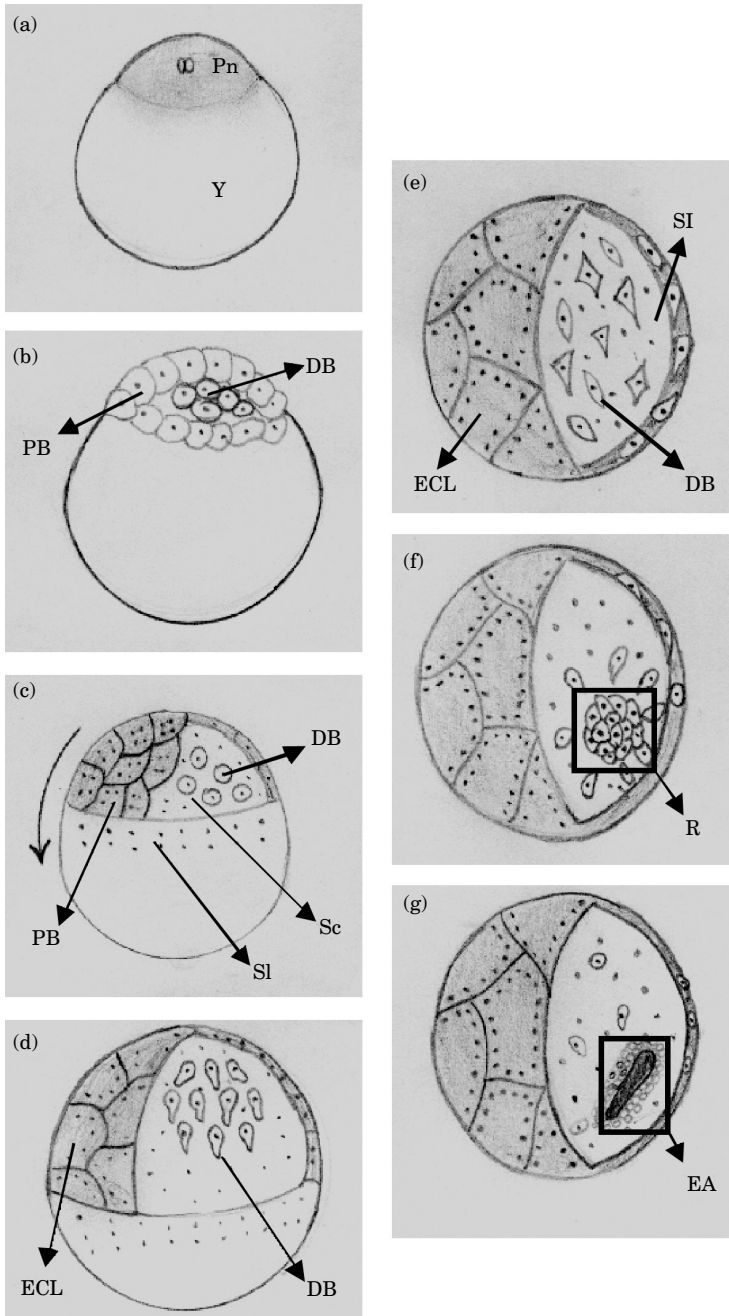[Figs 3(g) and 4(d)]. The nuclei of the enveloping cell layer are located at the

FIG. 4. Schematic representation of main developmental stages in *Cynolebias viarius*: (a) one cell stage (Pn, pronucleus; Y, yolk), (b) early blastula (DB, deep blastomere; PB, peripheral blastomere), (c) late blastula (DB, deep blastomere; PB, peripheral blastomere; Sc, segmentation cavity; Sl, syncitial layer; ↘, movement direction), (d) epiboly (DB, deep blastomere; ECL, enveloping cell layer), (e) dispersed phase (Sl, syncitial layer; DB, deep blastomere; ECL, enveloping cell layer), (f) early reagreggate (highlighted by square) (R, reagreggate) and (g) embryonic axis (highlighted by square). (EA, embryonic axis).

periphery. The advancing edge of the blastoderm includes concentric rows of nuclei belonging to the yolk syncytial layer. The blastomeres inside the segmentation cavity begin to flatten and, at the same time, they migrate individually and lose contact with one another. At about one fourth epiboly, the number of these deep cells is *c*. 100.

Migration takes place, from the animal pole, between the enveloping cell layer and the yolk syncitial layer. As they migrate, the blastomeres either contact the enveloping cell layer or the yolk syncitial layer (unpubl. obs.). During the active dispersal phase, the blastomeres are triangular, elongated or rhomboidal in shape. These cells can be seen isolated or arranged in short rows; fine cytoplasmic extensions connect the cells [Fig. 3(h)].

At the end of epiboly, the yolk mass is covered by the yolk syncytial and the enveloping cell layers. The deep blastomeres lie between these two layers and are dispersed over the egg surface.

Dispersed phase

Blastomeres are completely and apparently randomly dispersed. In some cases, they have filopodia contacting them [Figs 3(i) and 4(e)]. Elongated cells have an average length of 80 μm. The lipid droplets, which are fewer in number than in previous stages, appear in a peripheral position on the yolk. At this stage, the first diapause can occur but in the standardized conditions used in the present study it did not take place. Embryos maintained in peat moss inside the aquaria with adults, however, reached disperse phase and remained at this stage until transferred to Yamamoto solution.

Reaggregation phase

This stage is characterized by a region of increasing cell density (divisions proceed while cells are becoming closer) [Fig. 5(a)]. Deep cells migrate towards this zone forming a loose aggregate that is one cell thick. There is only one site of aggregation per embryo.

As development proceeds the aggregate undergoes some changes. As the area of the cluster increases, the deep cells become rounded again; their size is similar to that shown before dispersion [Figs 4(f) and 5(b)]. Finally, these cells end up tightly packed and smaller in size. First, they form a disc and then an elliptical plate several cell layers thick at its centre [Fig. 5(c)]. This condition is identifiable by simple side observation of the embryo. In all the embryos observed some dispersed cells remained outside the plate.

Embryonic axis

This transitory stage is characterized by a linear arrangement of cells that appears at the midline of the elliptic plate. Two parallel lines of cells following the major axis of the reaggregate are identifiable [Fig. 4(g)].

Neural keel (zero to three somite pairs)

The neural keel extends along the embryonic axis. The embryo's posterior end can be identified by the presence of Kupffer's vesicle whereas the anterior end is wider. [Fig. 5(d)]. The first three pairs of somites can be seen at the mid-way along the antero-posterior axis.
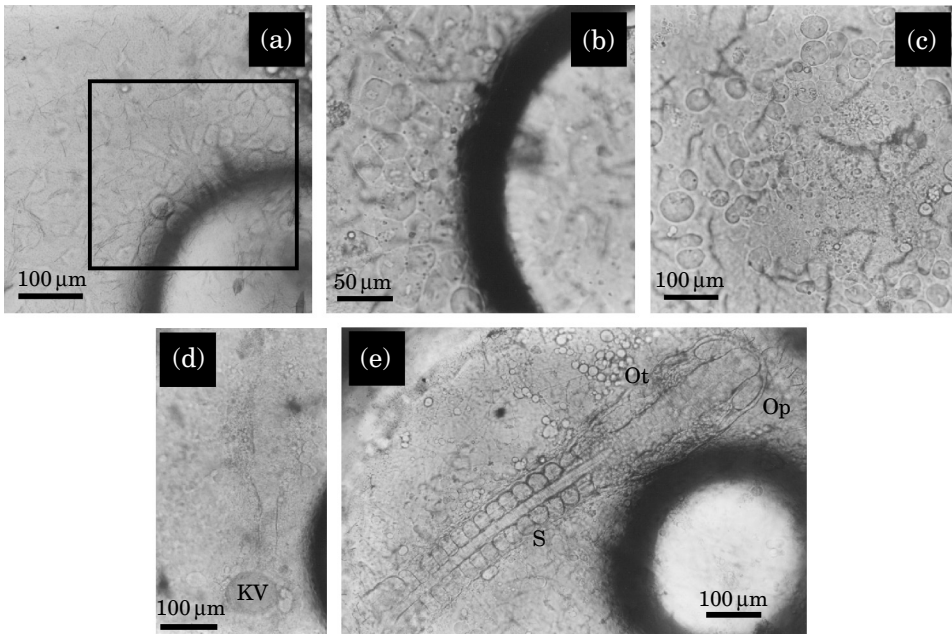
Fɪɢ. 5. Development of *Cynolebias viarius* embryo from reaggregation to 15 somite embryo: (a) early reag-
reggate (highlighted by square), (b) advanced reaggregate, (c) late reaggregate (time post-fertilization:
12 days), (d) neural keel (time post-fertilization: 13 days) (KV, Kupffer's vesicle) and (e) 15 somite
embryo (time post-fertilization: 18 days) (Op, optic vesicle; Ot, otic vesicle; S, somites).

Early somite embryo (three to 15 somite pairs)

Somites form in groups of three pairs per day until the ninth pair, after
which, the rate of formation increases. The cephalic region is characterized by
the presence of the developing brain, optic and otic vesicles. Optic vesicles are
the most anterior ones and they project laterally. Otic vesicles lacking otoliths
can be identified at the level of the cephalic trunk. At this stage the embryo has
no pigmentation [Fig. 5(e)].

DISCUSSION

Annual fishes share with the model zebrafish features such as oviparity, ease
of maintenance in the laboratory, long periods of breeding as well as transpar-
ency of eggs and embryos. Annual fishes, however, have unique stages of
dispersion-reaggregation and diapauses during development. These events
make them a useful model to study cell behaviour and the effect of environ-
mental factors on development.

EMBRYO CULTURE CONDITIONS

A great number of healthy embryos could be obtained repeatedly in the
culture conditions established in the present study. Wourms (1972*c*) reported
that diapause I is facultative for the *Cynolebias* genus. This is supported here
because in standardized laboratory conditions the embryos of *C. viarius* did not

exhibit this arrest. Inglima *et al.* (1981) showed that embryos of an other annual fish, *Nothobranchius guentheri* (Pfeffer), cultured in aquarium water where adults were maintained, stopped in diapause I. The same effect was observed here in embryos of *C. viarius*. It has been suggested that a hypothetical diffusible factor secreted by adults could be responsible for this developmental arrest (Inglima *et al.*, 1981).

Temperature strongly influences the duration of development. After different culture temperatures had been tried, 25° C was selected for optimal embryo survival and to allow a comparison of these results with the available data on early development of *Austrofundulus limnaeus* Shultz (Wourms, 1972a) and *Nothobranchius* sp. (Van Haarlem, 1983).

OOCYTE CHARACTERISTICS

The amount and appearance of the *C. viarius* oocyte yolk is similar to that of other telolecithal demersal eggs. The pattern of fibrils found in the *C. viarius* chorion is described for the first time. Loureiro & de Sá (1996) analysed the ultrastructural pattern (SEM) of chorion surface in 13 *Cynolebias* species. The hair-like filaments are similar in shape to these reported for *Cynolebias adloffi* Ahl. Two kinds of chorion filaments, however, were identified for *C. viarius* based on their thickness and regular organization while Loureiro & de Sá (1996) reported only one type. It has been suggested for freshwater fishes in general (Johnson & Werner, 1986), and for annual fishes in particular (Wourms & Sheldon, 1976), that surface ornamentation of the chorion is species-specific.

DEVELOPMENT

Development can be characterized by relating the time post-fertilization to discrete embryonic stages. Teleostean embryos can be studied following this criterion even though asynchrony has been reported in zebrafish embryos belonging to one female and *in vitro* fertilized at the same moment (Westerfield, 2000). Timing differences are more striking in annual fishes. Variation in duration of developmental stages and hatching time among genera and species within a same genus have been reported (Wourms, 1972a; Lesseps *et al.*, 1975; Van Haarlem, 1983). Sibling eggs can produce individuals that develop in different times (Wourms, 1972c). In addition, facultative arrest in diapauses increase asynchrony, for example *A. limnaeus* can hatch at different times ranging from 40 days to *c.* 320 days post-fertilization (Wourms, 1972c).

Under the present culture conditions, *C. viarius* showed the longest early development time (16 days to reach 10 somite pairs) among the annual species studied (Wourms, 1972a; Van Haarlem, 1983; Carter & Wourms, 1991). The analysis of the different stages through the early development showed that cleavage, blastula and epiboly are longer in *C. viarius* than the same stages when compared with *Nothobranchius* sp. (Van Haarlem, 1983), *A. limnaeus* (Wourms, 1972a) and *C. constanciae* and *C. whitei* (Carter & Wourms, 1991).

Temperature influences development duration. In *N. guentheri*, an increase of 5° C in temperature resulted in a reduction of 10 days in development (Markofsky & Matías, 1977). It is therefore necessary to take in account that

temperature variation could explain time differences between early development in *C. viarius* (embryos cultured at 25° C) and *C. whitei, C. nigripinnis* and *C. constanciae* (embryos cultured at 28° C) (Carter & Wourms, 1991). Compared to non-annual fishes, it appears that *C. viarius* early development time is almost 30 times longer than zebrafish (Kimmel *et al.*, 1995) and seven times longer than that of *Fundulus heteroclitus* (L.) (Armstrong & Child, 1965) another cyprinodontiform (Table I).

In *C. viarius*, two cellular populations were identified during cleavage at the beginning of 32–64 cells stage. Peripheral blastomeres will give rise to the enveloping cell and yolk syncitial layers, while deep cells will undergo dispersion. At the beginning of blastula formation, the enveloping cell layer and a segmentation cavity can be easily identified, as reported for all other annual fishes (Wourms 1972a, b; Van Haarlem, 1983; Carter & Wourms, 1991). In *D. rerio*, this stage is named stereoblastula because a segmentation cavity is absent (Kimmel *et al.*, 1995). At this stage, a second important event has been reported for *F. heteroclitus* (Trinkaus, 1992) and zebrafish embryos (Kane & Kimmel, 1993): the mid-blastula transition (cell cycles become longer and asynchronic, zygotic genes and cell motility activation) (Kane & Kimmel, 1993). A mid-blastula transition should occur in *C. viarius* but it is necessary to establish the properties that identify it.

Inspite of the great variation that fishes present as the most diverse vertebrate group, teleost gastrulation follow the basic vertebrate pattern (Ho, 1992). It involves movement of deep cells to congregate and form the embryo and at the same time epiboly of the blastoderm and yolk syncitial layer. Epiboly proceeds independently of deep cell movements and depends on expansion of the enveloping cell layer and yolk syncitial layer (Trinkaus, 1984). Annual fish embryos are unique among vertebrates because epiboly is temporally and spatially separated from organogenesis. During epiboly, deep blastomeres disperse from the blastoderm over the entire syncitial layer (Wourms, 1972a, b).

The number of deep cells varies among fish species. Non annual fishes have thousands of blastomeres at the start of epiboly, *e.g.* 4000 in *D. rerio* (Kimmel *et al.*, 1995) and 27 500 in *F. heteroclitus* (Armstrong & Child, 1965). Annual fishes possess fewer cells (*Nothobranchius* sp. between 54 and 340 among the 10 species studied, Van Haarlem, 1983; *Cynolebias* sp. 250 and *Austrofundulus* sp. 2500, Carter & Wourms, 1991). *Cynolebias viarius* has *c.* 100 blastomeres at early epiboly, the lowest amount reported previously for the genus.

The low number of deep cells could explain their dispersed arrangement preventing their movement as a layer but rather as individual cells (M. Allende, pers. comm.). In zebrafish epiboly, the blastomeres are loosely but closely arranged while moving over the yolk (Warga & Kimmel, 1990). Thus, it could be possible that the disperse phase is the result of the low number of deep cells and their kinetics.

The dispersing cells in *C. viarius* are round before migration but become flat and exhibit cellular extensions as migration proceeds. Triangular, elongated and rhomboidal dispersing cells were found in a similar proportion during epiboly as reported by Carter & Wourms (1991) for other *Cynolebias*. In *A. limnaeus* the blastomeres are amoeboid (Wourms, 1972a, b). In *Nothobranchius* sp. dispersing cells have a variable shape which changes from spherical to triangular and

rhomboidal. Each species of this genus has one predominant form (Van Haarlem, 1983). In a similar way to those in *Nothobranchius neumanni* (Hilgendorf) (Lesseps *et al.*, 1975) *C. viarius* cells often move in rows maintaining contact among each other through cytoplasmic filopodia.

Teleost gastrulation is morphologically characterized by the presence of a germ ring. It consists of two cell layers: the outermost or epiblast and the innermost or hypoblast. In *D. rerio* (Warga & Kimmel, 1990) and *Puntus conchonius* (Hamilton) (Wood & Timmermans, 1988) the formation of the hypoblast has been described to occur by involution. Referring to *F. heteroclitus*, Trinkaus (1984, 1996) has proposed ingression (cells delaminate from their original superficial position to a deeper place) as the responsible mechanism. In *Oncorhynchus mykiss* (Walbaum) however, it has been reported that neither involution nor ingression occurs (Ballard, 1966). Collazo *et al.* (1994) interpreted that the inability to find involution in *Salmo* sp. (Ballard, 1966) and *F. heteroclitus* (Trinkaus, 1984) might be due to technical limitations believing involution to be the mechanism common to all teleosts. *Cynolebias viarius* does not possess a germ ring as in all annual fishes. Gastrulation of deep cells seems to occur in the reaggregated cell plate since the embryo axis appears in the middle of this structure. In *Cynolebias* sp., as in all annual fishes studied, the mechanism by which this process takes place remains to be determined.

Cynolebias viarius showed the longest early development time among the annual fish species reported. As in all annual fishes, embryos possess an identifiable blastocoel. In early epiboly, *C. viarius* has *c.* 100 deep cells, the lowest amount reported previously for the genus. They show triangular, elongated or rhomboidal morphology during the dispersing phase. Diapause I did not occur in *C. viarius* embryos cultured in standardized laboratory conditions. Putative presumed germ ring and embryonic shield are not distinguishable morphologically.

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