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Article *in* Developmental Biology · July 2018
DOI: 10.1016/j.ydbio.2018.07.015

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Developmental Biology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Developmental Biology

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Dispersion/reaggregation in early development of annual killifishes: Phylogenetic distribution and evolutionary significance of a unique feature

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ARTICLE INFO

Keywords:
Killifish
Nothobranchius
Austrofundulus
Dispersion/reaggregation
Annual fish development

ABSTRACT

Annual killifishes are members of the Aplocheiloidea and live in ephemeral habitats that desiccate regularly during the dry season and refill during the rainy season. Populations of these fishes survive the dry season by producing drought-resistant diapausing eggs that are buried in the substrate. When the pool refills during the rainy season the juveniles hatch, grow rapidly and reproduce until the pool desiccates again during the next dry season. The association with such unpredictable habitats has led to the evolution to a variety of developmental adaptations such as a dispersed/reaggregation phase of the deep blastomeres, three possible diapause stages, extreme tolerance to high salinity and anoxia, an efficient DNA repair system and an extremely short life span.

Here, we review the course of the dispersed/reaggregation phase, its evolution and phylogenetic distribution and diversity within the Aplocheiloidea. The phenomenon of blastomere dispersion/reaggregation in these fishes was first described in the 1960s and 70s. Blastomeres of most teleost fishes segregate into three groups that give rise to the enveloping cell layer, the yolk syncytial layer and the deep blastomeres that will form the embryo itself. When epiboly commences, the deep blastomeres form a more or less coherent cell sheet with a so called embryonic shield at it marginal zone marking the area where gastrulation takes place. In annual killifishes, the deep blastomeres segregate when epiboly starts and disperse when epiboly commences. After epiboly has been completed, the deep blastomeres are randomly distributed and migrate all over the enveloping cell layer. After several days they start to reaggregate and form the actual embryo that starts gastrulation. The evolutionary origin and mechanism behind this peculiar developmental pathway have puzzled developmental biologists for almost 50 years. However, several of these annual killifishes (*Nothobranchius furzeri*, *Austrofundulus limnaeus*, *Austrolebias charrua and Austrolebias bellottii*) have become model organisms in studies on developmental physiology, aging and stress tolerance. This has led to the establishment of modern genetic techniques such as transgenesis and cell fate mapping that are now used to tackle questions about the origin and mechanisms behind the dispersal/reaggregation phase.

1. Introduction

Annual killifishes (Aplocheiloidea) are a group of cyprinodont fishes that occupy unpredictable habitats that are often inhospitable for other teleosts (Cellerino et al., 2016). They inhabit temporary water bodies in the savannahs of Africa (family Nothobranchiidae) and the Neotropics (family Rivulidae) that desiccate annually during the dry season and refill during the wet season (Berois et al., 2016; Cellerino et al., 2016). The life cycle of annual killifishes is greatly bound to the ephemeral and unfavorable conditions of such habitats (Berois et al., 2012, 2016) (Fig. 1). Juvenile fishes hatch from drought-resistant eggs, when their pool is filled with water, grow rapidly and become sexually mature within a few weeks (Wourms, 1972a). Adult fishes reproduce continuously and, when the pool desiccates, perish during the dry season. The whole population survives as diapausing eggs, buried in the dry soil until the pool is refilled during the next wet season and juveniles start to hatch (Furness, 2016; Furness et al., 2015; Wourms, 1972a). Many peculiar developmental adaptations like a unique dispersed/reaggregation phase of the deep blastomeres, three potential drought-resistant diapause stages, a high hypoxia (or even anoxia) and high salinity tolerance and a short life span evolved in concert with this annual life cycle. Despite the gross similarities of these adaptations in the different aplocheiloid families it is still unclear if annualism is a synapomorphy of the Aplocheiloidea and has been lost in some taxa

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https://doi.org/10.1016/j.ydbio.2018.07.015
Received 18 May 2018; Received in revised form 17 July 2018; Accepted 17 July 2018

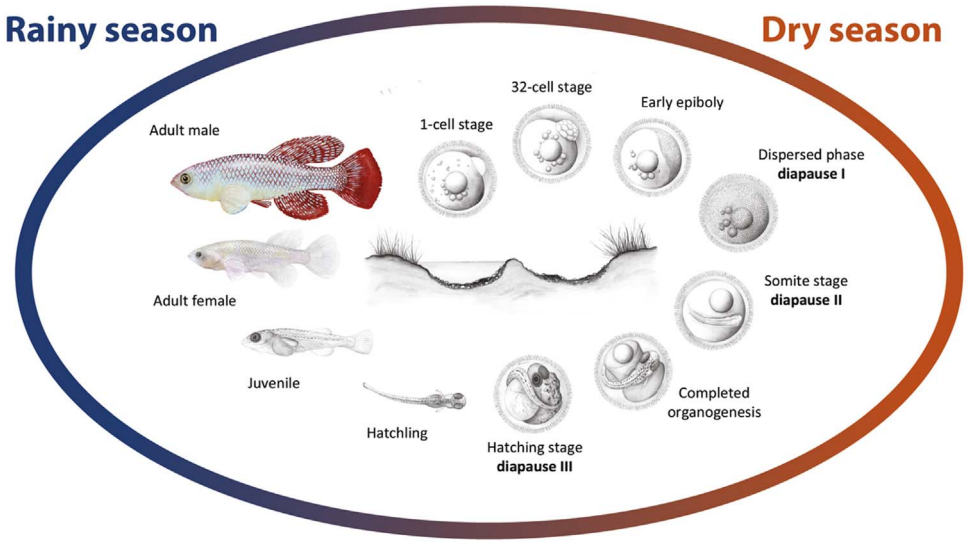

Fig. 1. Life cycle of *Nothobranchius furzeri* as an example for a typical annual killifish life cycle. At the beginning of the rainy season, *N. furzeri* hatch and grow rapidly. After four to five weeks they reach sexual maturity and reproduce almost daily. Depending on the conditions, embryos can develop completely, hatch and reproduce in the same season or enter diapauses at three possible stages of development (dispersed phase, somite stage, hatching stage). Diapausing eggs can survive the dry season in the substrate and hatch when the pond is filled again during the next rainy season. The Figure is modified from Platzer and Englert, 2016. Original source: FLV© Alexander Schmidt, Atelier Symbiota.

(Murphy and Collier, 1997) or if it has evolved several times independently within this group (Furness, 2016; Hrbek and Larson, 1999). Some species of annual killifishes like *Nothobranchius furzeri* (Nothobranchiidae) and *Austrofundulus limnaeus* (Rivulidae) have recently become model organisms in age research and studies on developmental stress resistance, aging and genome adaptation (Berois et al., 2016; Cellerino et al., 2016; Podrabsky et al., 2016; Terzibasi et al., 2007; Wagner et al., 2018). Most research is focused on the physiology of diapause stages and the early aging phenotype connected to the short life span (Hartmann et al., 2009; Machado and Podrabsky, 2007; Muck et al., 2018; Platzer and Englert, 2016; Podrabsky et al., 2010a, 2007; Podrabsky and Hand, 1999; Terzibasi et al., 2009, 2008, 2007; Valdesalici and Cellerino, 2003). What has received less interest but recently came into focus again is the unique dispersed/reaggregation phase of the deep blastomeres (Pereiro et al., 2017; Reig et al., 2017). This developmental phase is of special interest since it is the time when diapause I can occur (Peters, 1963; Wourms, 1972a, 1972b, 1972c). In annual killfishes there are three distinct diapause stages at which development can be arrested (Wourms, 1972a, 1972b, 1972c). Diapause I occurs during the dispersed phase, diapause II at early somite stages and diapause III shortly before hatching (Wourms, 1972a). During diapause development arrests completely and the metabolic activity of embryonic cells is extremely low (Podrabsky and Hand, 1999; Podrabsky et al., 2017). Concerning diapause I during the dispersed phase, blastomeres can remain in this stage for several days or even months depending on the environmental conditions (cold temperatures, hypoxia, high salt concentrations, drought, etc.) (Martin and Podrabsky, 2017; Podrabsky et al., 2010b). Studying the dispersion/reaggregation phase has the potential to elucidate questions regarding coordinated cell movement, cell aggregation, the ability of the vertebrate embryo to deal with the loss of cells due to disturbance events during early development and the evolution of complex developmental traits such as diapause sages.

2. Cell dispersion and reaggregation in the early development in annual killifishes

Dispersion and aggregation seem to be central biological processes that appear in many developmental systems (Wourms, 1972b). It is involved in pathological processes such as metastasis formation in cancer cells (Wourms, 1972b), in regenerative processes such as blastema formation (Wourms, 1972b) or neoblast interactions in planarian regeneration (Betachaku, 1967) and in some developmental processes such as migratory neural crest cells (Weston, 1970) and the mesoblast of the chicken (Hay, 1968). The phenomenon of blastomere dispersion and reaggregation in annual killifishes was first described in the 1960s and 70s by by Nicolaus Peters in the german literature (Peters, 1963) and by John P. Wourms in the english literature (Wourms, 1965, 1967, 1972a, 1972b, 1972c). The early development starts, as in non-annual teleosts, with the segregation of the blastomeres into three different populations (Fig. 2A). The ventral peripheral blastomeres (Fig. 2B, YCs) that are in contact with the yolk will fuse and form the external and internal yolk syncytial layer (Fig. 2C). The remaining peripheral blastomeres (Fig. 2B, PBs) also fuse and form the enveloping cell layer (also see Fig. 3C). The central mass of blastomeres, called deep blastomeres, are the cells that will give rise to the embryo itself (Fig. 2B and C, DBs) (Wourms, 1972b). During epiboly, deep blastomeres start to disperse from the central mass and migrate ventrally guided by the cell-cell borders of the enveloping cell layer (Fig. 2D, E) (Carter and Wourms, 1991; Reig et al., 2017). When epiboly is complete, deep blastomeres are dispersed all over the egg. The dispersed phase of annual killifish blastomeres can last a few days (Wourms, 1972a, 1972b; Furness, 2016), commonly three to four in most of the examined species when embryos do not enter Diapause I (Wourms, 1972b). Thereafter the deep blastomeres start to aggregate at the vegetal pole of the egg attracted by a granular mass of unknown composition (Wourms, 1972b) (Fig. 2F). This aggregate will give rise to the actual embryo.

3. The enveloping cell layer

The enveloping cell layer (ECL) is an epithelial layer that encloses the deep blastomeres and the yolk syncytial layer (Carter and Wourms, 1990). The ECL originates from the peripheral cells of the early blastula (Fig. 3A to D; light blue). During development, the cells of the ECL start to flatten and separate from the central (or deep) population of blastomeres (Podrabsky et al., 2017; van Haarlem, 1983; Wourms, 1972a, 1972b). The emerging space between the ECL and the deep blastomeres is called the segmentation cavity (Wourms, 1972b). During this separation process, cells of the ECL form transient extensions (lobopodia) into the segmentation cavity in *Fundulopanchax arnoldi (Aphyosemion arnoldi* in Wourms, 1972b),

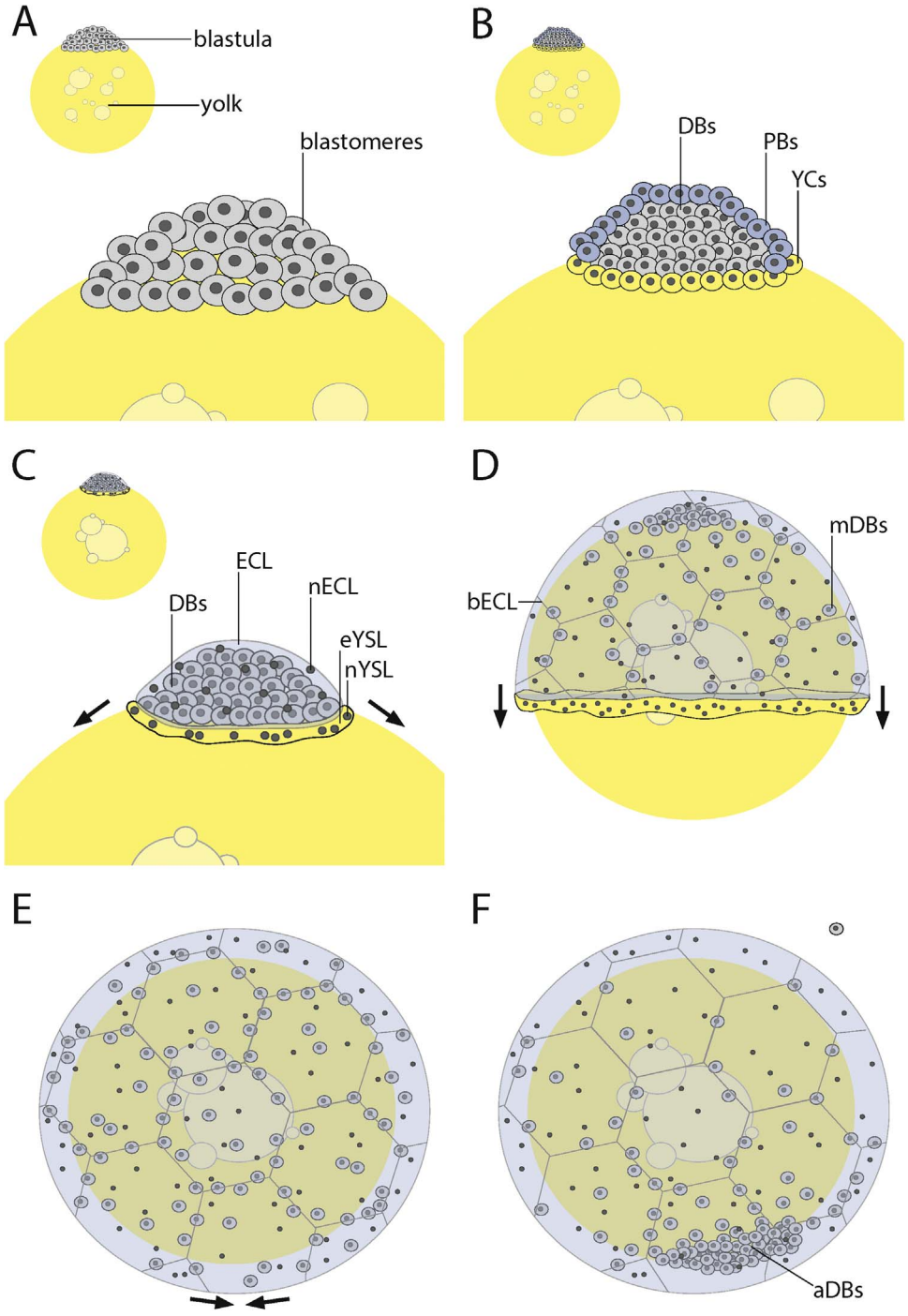

Fig. 2. Schematic illustration of the dispersed/reaggregation phase in the development of annual killifishes. The illustration is based on descriptions and images from *Austrolebias nigripinnis* (Reig et al., 2017) but is consistent with description in other species (Carter and Wourms, 1990, 1991; van Haarlem, 1983; Wourms, 1972a, 1972b, 1972c). A, early blastula stage with blastomeres on top of the yolk. B, blastomeres segregate in three groups. The ventral peripheral blastomeres become yolk cells (yellow), the other peripheral blastomeres (blue) will give rise to the enveloping cell layer and the deep blastomeres (grey) will form the embryo. C, at the onset of epiboly (black arrows), the yolk cells fuse and form the external and internal yolk syncytial layer. The peripheral blastomeres become first bi- and then multinucleated and form the enveloping cell layer. The deep blastomeres are organized in a central mass at the animal pole of the egg. D, at 50% epiboly (black arrows), deep blastomeres start to disperse from the central mass and migrate ventrally guided by the cell-cell borders of the enveloping cell layer. E, when epiboly is complete (black arrows), deep blastomeres are dispersed all over the egg. Migration of the deep blastomeres can last for several days. There is also the possibility to enter Diapause I at this stage. F, after a few days the deep blastomeres start to aggregate at the vegetal pole of the egg. This aggregate will give rise to the embryo. aDBs, aggregating deep blastomeres; bECL, cell-cell border of the enveloping cell layer; DBs, deep blastomeres; ECL, enveloping cell layer; eYSL, external yolk syncytial layer, mDBs, migrating deep blastomeres; nECL, nucleus of the enveloping cell layer; nYSL, nucleus of the yolk syncytial layer; YCs, yolk cells.

Austrofundulus, *Austrolebias* (*Cynolebias*), *Pterolebias* and *Rachovia* (Carter and Wourms, 1991; Wourms, 1972a, 1972b) (Fig. 3C, black arrow). In *Nothobranchius*, such extensions of the ECL into the segmentation cavity have not been observed (Wourms, 1972b). The uni-nucleated cells of the ECL start to flatten and become first bi- and later multi-nucleated (Fig. 3B to D) (Carter and Wourms, 1990, 1991; Wourms, 1972a, 1972b, 1972c). The outer-most cells of the ECL, which are still uni-nucleated, continue to divide and produce daughter cells which are released into the segmentation cavity and contribute to the population of deep blastomeres (Wourms, 1972b). Cells of the ECL

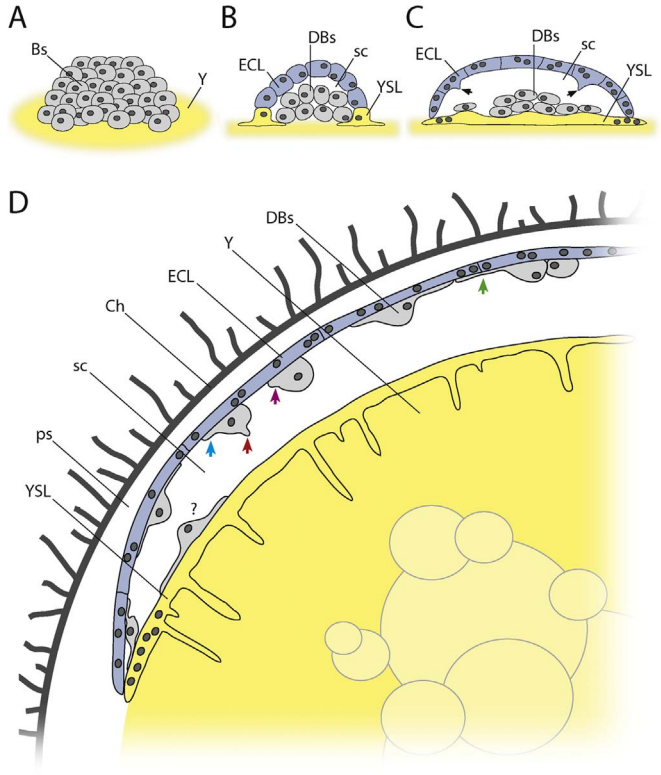

Fig. 3. Schematic sections through an annual killifish embryo at different developmental stages. The illustration is based on similar descriptions and images from different species of annual killifishes (Carter and Wourms, 1990; Wourms, 1972b). A, early blastula stage. B, late blastula stage. The blastomeres have segregated into the enveloping cell layer (blue), the yolk syncytial layer (yellow) and the deep blastomeres (grey). Cells of the enveloping cell layer are still uni-nucleated. The segmentation cavity starts to form due to the separation of the enveloping cell layer from the deep blastomeres. C, the onset of epiboly. The cells of the enveloping cell layer have become bi-nucleated and form transient protrusion into the segmentation cavity (black arrows). The central mass of deep blastomeres starts to become loose. D, early epiboly. Deep blastomeres migrate around in the segmentation cavity on the inner surface of the enveloping cell layer. At the marginal zone, where the enveloping cell layer meets the yolk syncytial layer they migrate on both. Carter and Wourms (1991) reported that some deep blastomeres are also found to migrate on the surface of the yolk syncytial layer alone (?). A bleb is indicated by a lilac arrow, a lobopodium by a blue arrow, a filopodium by a green arrow and a ruffle by a red arrow. Bs, blastomeres; Ch, chorion; DBs, deep blastomeres; ECL, enveloping cell layer; ps, perivitelline space; sc, segmentation cavity; Y, yolk; YSL, yolk syncytial layer.

that have already become bi- or multi-nucleated do not contribute daughter cells to the deep blastomere population anymore (Wourms, 1972b). The syncytial condition of the ECL can be established in two ways. In *Austrofundulus*, the formation of this syncytial cell layer is due to continued nuclear divisions and a stop of the division of cell somata (Wourms, 1972b). In *Nothobranchius*, cells of the ECL stop cell division relatively early. Instead cells of the ECL fuse to become syncytial (van Haarlem, 1983). Cell fusion of the ECL begins late in *Nothobranchius*, at the time when reaggregation of the deep blastomeres has already started (*Nothobranchius* stage 23 in van Haarlem, 1983). In contrast, in *Austrofundulus* multi-nucleation of the ECL starts at the early flat hollow blastula stage (stage 12 in Wourms, 1972a). In *Austrolebias nigripinnes, Nematolebias whitei* and *Simpsonichthys constanciae*, bi-nucleated cells of the ECL are first observed at the late flat blastoderm stage (stage 13 in Carter and Wourms, 1991). The same was observed for Austrolebias varius (Arezo et al., 2005). When epiboly commences, the syncytial ECL extends ventrally until it encases the whole egg (Wourms, 1972a, 1972b).

As pointed out by van Haarlem (1983) one of the major questions of epiboly concerns the mechanism driving the spread of the ECL over the yolk. It was shown that passive spreading driven by cell divisions play no role in this movement in *Nothobranchius* (van Haarlem, 1983). Another possibility could be an active migration of the marginal zone of the ECL. However, structures involved in active cell movement such as lobopodia or filopodia have not been observed (van Haarlem, 1983). The most likely possibility is that the yolk cells are the active motile force driving epiboly (Hernández-Vega et al., 2016; Trinkaus, 1976; van Haarlem, 1983). After epiboly has been completed the ECL tends to become increasingly thinner and more syncytial (Carter and Wourms, 1990, 1991).

The ECL is thought to serve as an osmotic barrier between the external perivitelline space and the internal parts of the egg (Carter and Wourms, 1990; Furness, 2016; Machado and Podrabsky, 2007). This view is supported by apical tight junctions, pits in the apical plasma membrane, a well-developed Golgi complex and the presence of coated vesicles at the basal region of cells of the ECL (Carter and Wourms, 1991). It has been hypothesized, that these features are involved in recycling and replacing the apical plasma membrane and its associated glycocalyx (Carter and Wourms, 1991). Additionally, the ECL seems to serve as the main substratum for the migrating deep blastomeres during the dispersed stage (Carter and Wourms, 1990, 1991; Van Haarlem, 1979; van Haarlem, 1983; Wourms, 1972a, 1972b).

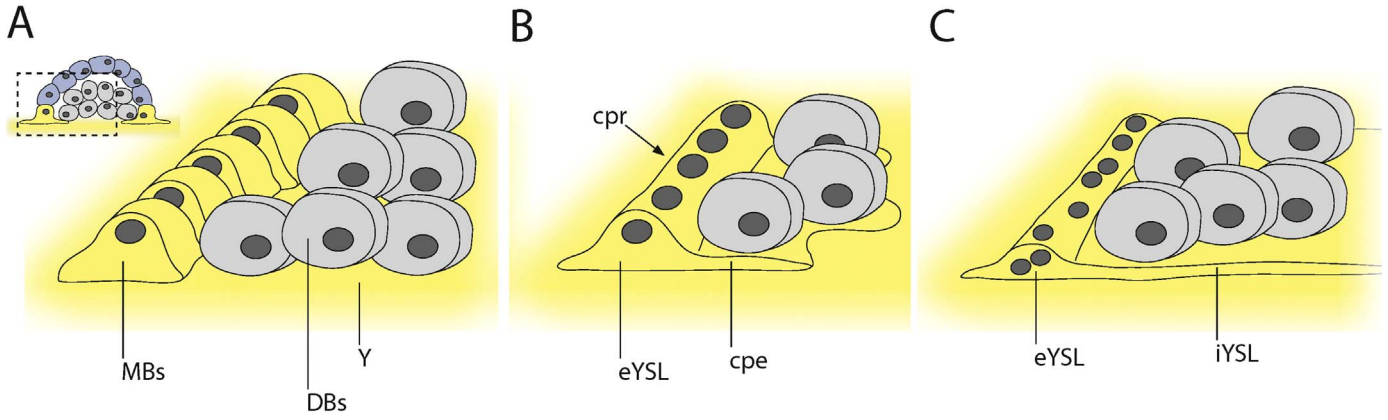

Fig. 4. Schematic illustration of the formation of the yolk syncytial layer. The illustration is based on similar descriptions from different species (Carter and Wourms, 1990; van Haarlem, 1983). A, The dashed square indicates the region of the ventral marginal blastomeres. They are single cells distal to the mass of deep blastomeres. B, single yolk cells start to fuse. The nuclei lay in a structure called the cytoplasmatic ridge. This will become the external yolk syncytial layer. The yolk cells also start to extend beneath the deep blastomeres. C, the cytoplasmatic ridge flattens and nuclei of the external yolk syncytial layer start to divide. The cytoplasmatic extensions have covered the complete yolk surface beneath the deep blastomeres and are called the internal yolk syncytial layer. cpe, cytplasmatic extension; cpr, cytoplasmatic ridge; DBs, deep blastomeres; eYSL, external yolk syncytial layer; iYSL, internal yolk syncytial layer; MBs, marginal blastomeres.

4. The yolk syncytial layer

The formation of the yolk syncytial layer (YSL) has been described in detail in *Nothobranchius* (van Haarlem, 1983). The external YSL develops from the marginal peripheral blastomeres (Fig. 4A). During the later cleavage period the cells of this so-called yolk cellular layer stop dividing and start to fuse to form a cytoplasmatic ridge with one row of nuclei inside (Fig. 4B) (van Haarlem, 1983). This ridge then flattens and the nuclei start to divide. The cytoplasm of the ridge spreads under the new marginal peripheral blastomeres. This nuclei-free part of the YSL has been termed the internal YSL (Fig. 4C) (van Haarlem, 1983). During epiboly, the YSL extends ventrally and might be the driving force for the movement of the ECL (Hernández-Vega et al., 2016; Trinkaus, 1976; van Haarlem, 1983). In the closely related genus *Fundulus*, the internal YSL extends cytoplasmatic projections into the yolk that degrade and transport glycogen. Glycogen is then digested by the YSL and transferred into the segmentation cavity where it serves as an energy source for the blastomeres (Carter and Wourms, 1990; Lentz and Trinkaus, 1967) (Fig. 3D). The presence of glycogen-rich particles, lipid-droplets and vesicular bodies in the YSL of *Austrolebias nigripinnis* and *Simpsonichtys constanciae* suggests that these structures serve as energy support also in aplocheiloid killifishes (Carter and Wourms, 1990). Additionally, Carter and Wourms (1990) describe “villous projections” that contain glycogen and could be involved in nutrient transport from the YSL into the segmentation cavity. It has been proposed that the amount of glycogen in the yolk of *Austrolebias nigripinnis* and *Simpsonichtys constanciae* is larger than in other teleosts such as *Oryzias* (Medaka) and *Fundulus* (Carter and Wourms, 1990). In *Austrofundulus limnaeus* there is no such difference in the amount of stored glycogen compared to other teleosts (Podrabsky et al., 2007). However, it seems that the glycogen reserves provide the energy during the dispersed stage or hypoxia-induced Diapause I and II in both studies (Carter and Wourms, 1990; Podrabsky et al., 2007). A very peculiar feature of the yolk in annual killifishes is the presence of a “granular substance of unknown composition” (Wourms, 1972a, 1972b, 1972c). Wourms hypothesized, that granular material (stored metabolites) accumulate in the ventral egg hemisphere at the interface between the YSL and the yolk maybe in response to gravity (Carter and Wourms, 1991). This granular substance seems to play a crucial role in the initiation of reggression of the dispersed blastomeres (Carter and Wourms, 1987, 1990, 1991; Podrabsky et al., 2017; van Haarlem, 1983; Wourms, 1972a, 1972b, 1972c). However, even after more than 40 years, the composition of the granular substance remains unknown.

5. The deep blastomeres

The deep blastomeres (DBs) are the cell population that gives rise to the embryo itself (Wourms, 1972a). This cell population originates from the central-most (deep) blastomeres of the killifish blastodisc (Fig. 3B) (Wourms, 1972b). When epiboly of the ECL and YSL commences, the DBs first come together as a consolidated mass and then migrate distally as amoeboid cells (Wourms, 1972b). Like the development of ECL and YSL, the dispersion of the DBs has been studied in some species of *Nothobranchius*, *Aphyosemion*, *Fundulopanchax*, *Austrolebias* (*Cynolebias* in Wourms, 1972b), *Cynopoecilus* (*Cynolebias* in Wourms, 1972b), *Callopanchax* (*Roloffia* in Wourms, 1972b), *Simpsonichthys*, *Austrofundulus*, *Pterolebias*, *Rachovia* (Carter and Wourms, 1987, 1990, 1991; Podrabsky et al., 2017; Van Haarlem, 1979; van Haarlem, 1983; Wourms, 1972a, 1972b, 1972c). Wourms (1972b) determined the “coefficient of dispersion” during the different stages of the dispersion/reaggregation process. He found, that the DBs are uniformly distributed when epiboly is complete. Later, DBs are randomly distributed before they start to aggregate at the granular mass of unknown composition in the ventral (vegetal) hemisphere of the egg. In *Austrolebias nigripinnis*, migration of the DBs is first random but the higher tension values at cell-cell borders of the ECL seem to attract DBs on a short range. It is proposed, that this attraction is due to mechanical cues rather than chemical sensing. After a DB has aligned with an ECL cell-cell border migration is no longer random but more or less guided by the cell-cell border (Reig et al., 2017). This “lining-up” along cell-cell borders of the ECL has also been observed in *Nothobranchius neumanni* (Lesseps et al., 1975). A facultative Diapause I may occur when DBs are “randomly” distributed (Wourms, 1972b). The dispersion/reaggregation phase and a Diapause I, which seems to be facultative under laboratory but obligate under natural conditions, exists only in aplocheiloid fishes showing an annual life-style (Domínguez-Castanedo et al., 2013). Closely related non-annual species lack such a phase (Dolfi et al., 2014; Furness et al., 2015; Wourms, 1972b). However, differences exist in the migration patterns of DBs when different annual taxa are compared in detail.

It has been hypothesized by Carter and Wourms (1990, 1991), that cell motility of DBs is facilitated by punctuate regions of adhesion that ensure cell traction. This hypothesis gains support by a recent study in *Austrolebias nigripinnis* showing that cell-cell-contact of the ECL and DBs is mediated by E-Cadherin (Reig et al., 2017). Three types of cell extension have been described in blastomeres of the non-aplocheiloid fish *Fundulus*, namely blebs, lobopodia and filopodia (Hogan and Trinkaus, 1977). Blebs are transient, hemispheric protrusion of the plasma membrane (Fig. 3D, lilac arrow). They contain mitochondria and glycogen and exhibit a cortical layer of microfilaments at their base (Carter and Wourms, 1990; Hogan and Trinkaus, 1977). Blebs can transform into other types of extension such as lobopodia and filopodia (Carter and Wourms, 1990). Lobopodia (Fig. 3D, blue arrow) and Filopodia (Fig. 3D, green arrow) are filled with microfilaments. A lobopodium can stretch and transform into a filopodium. The filopodium adheres to the substratum at its tip and by contraction moves the cell body towards it (Carter and Wourms, 1990). All these types of extensions can also be observed for the migratory DBs of annual aplocheiloid fishes (Carter and Wourms, 1987, 1990, 1991; Van Haarlem, 1979; van Haarlem, 1983; Wourms, 1972a, 1972b). In *Austrolebias nigirpinnes* and *Simpsonichthys constantiae* an additional type of apical cell extension, called ruffles, has been described (Carter and Wourms, 1990) (Fig. 3D, red arrow). Ruffles do not contain organelles and are separated from the cytoplasm of the soma by a mat of microfilaments (Carter and Wourms, 1990). If these ruffles play a role in cell motility or maybe nutrient uptake is not known.

Data about the velocities, by which DBs move above the ECL are scarce. They have been studied in detail in *Austrolebias* and *Simpsonichthys* (Carter and Wourms, 1987). Carter and Wourms (1987) subdivided the dispersed phase and reaggregation phase in two sub-phases each (DI and DII; RI and RII). During DII, BDs move with a mean velocity of 3,48 (+/−0,91) micrometers per minute (μm/min). When reaggregation starts they slow down and move with 1,28 (+/−0,46) μm/min during RI and 1,31 (+/−0,31) μm/min during RII (Carter and Wourms, 1987). In *Austrofundulus*, a mean velocity of 12 μm/min has been measured without data on the exact sub-phase of dispersion (Wourms, 1972b). In the closely related, non-aplocheiloid genus *Fundulus* a mean velocity of 10–15 μm/min has been measured during epiboly (Trinkaus and Erickson, 1981, 1983). In *Oryzias* (Medaka) a mean velocity of 2–3 μm/min has been measured (Kageyama, 1977). It is not known if the velocities of DBs changes when they switch from a random migration to a “lined-up” migration at ECL cell borders.

Different migration types of DBs can be distinguished on the basis of the studies by Wourms (1972a, 1972b), van Haarlem (1983) and Carter and Wourms (1991). The DBs may migrate as single amoeboid cells (Fig. 5A). Cell-cell-contact via protrusions and/or the soma may occur sometimes but is only transient (van Haarlem, 1983; Wourms, 1972b). This type can be found in some species of *Austrofundulus*, *Pterolebias*, *Rachovia* and *Nothobranchius kirki*, *N. lourensi*, *N.*

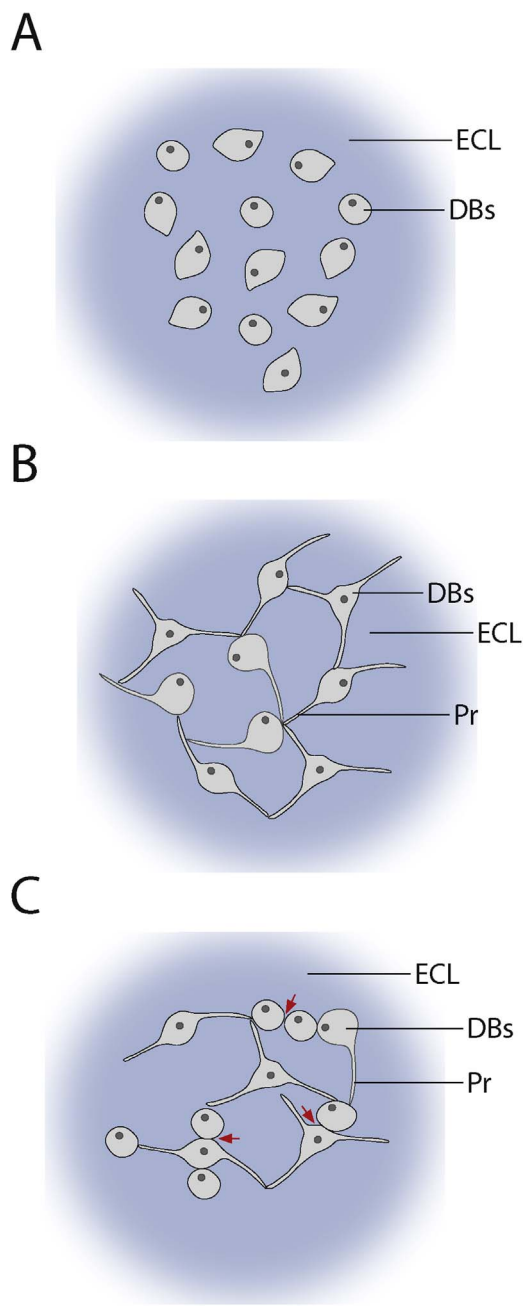

Fig. 5. Schematic illustration of the different migration types of deep blastomeres. A, deep blastomeres migrate as single cells and make only random contact. B, deep blastomeres are in contact via protrusions. C, deep blastomeres are in contact via protrusions and somata (red arrows). DBs, deep blastomeres; ECL, enveloping cell layer; Pr, protrusions.

rachovii and maybe *N. guentheri* (Carter and Wourms, 1991; van Haarlem, 1983; Wourms, 1972b). Furthermore, migratory DBs can be found alone but also establish stable contact via protrusions (Fig. 5B), e.g. in species of *Austrolebias* and *Cynolebias* and in *N. guentheri* (van Haarlem, 1983; Wourms, 1972b). There are also species in which all DBs are always connected via their protrusions and more-or-less forming some kind of "cell net", covering the yolk. Such a type has been described in *N. taeniopygus*, *N. palmqvisti* and *N. korthausae* (van Haarlem, 1983). The DBs can also be in stable contact via their cell somata in addition to protrusion contact (Fig. 5C). This is sometimes found in *N. steinforti*, *N. taeniopygus*, *N. neumanni*, *N. palmqvisti*, *N. melanospilus* (van Haarlem, 1983). It seems that those patterns are also not connected to the different numbers of DBs in different *Nothobranchius* species (Fig. 6). It is therefore unclear how the migration types of DBs are determined and if they have a developmental significance.

It has been mentioned by Wourms (1972b), that annual killifishes seem to have less DBs than non-annual killifishes of related teleosts. When epiboly commences, the number of DBs ranges from 50 to 350 in *Nothobranchius* (van Haarlem, 1983), less than 300 in *Pterolebias* and *Rachovia*, 100–300 in *Austrolebias* (Arezo et al., 2005; Wourms, 1972b) and 200–300 in *Cynopoecilus* (Wourms, 1972b). An exception are some species of *Austrofundulus* with 2.000–2.500 blastomeres in *A. myersi*, *A. transilis*, *A. dolichopterus* (Wourms, 1972a, 1972b) and up to 8.000 in *A. limnaeus* (Podrabsky et al., 2017). This is in contrast to many other teleosts where the number of deep blastomeres ranges from 4.000 in *Danio* (Marrable, 1965), 27.500 in *Fundulus* (Richards and Porter, 1935) up to 40.000 in *Coregonus* (Richards and Shumacher, 1935). A special case can be seen in the genera *Aphyosemion* and *Fundulopanchax* (treated as one genus, *Aphyosemion*, by Wourms, 1972b). *Aphyosemion* and *Fundulopanchax* include non-annual species (no dispersed phase), annual species (dispersed phase) and "transient" species ("intermediate type" of a dispersed phase) (Fig. 7) (Wourms, 1972b). As stated by Wourms (1972b) it seems that non-annual *Aphyosemion* and *Fundulopanchax* species have more DBs than annual species of this genus. "Transient" species appear to have and intermediate number of cells (Wourms, 1972b). However, this can only be regarded as a trend since no discrete numbers are given by Wourms (1972b). Recently, a similar case has been reported in *Laimoseion* a former subgenus of the non-annual genus *Rivulus*. Some species of *Laimosemion* show a very short dispersed phase of around 24 h (transitional type *sensu* Wourms?) while other species stay in the dispersed phase of up to one week (Furness et al., 2018). However, the *Laimosemion* species investigated did not enter a diapause I phase which allows speculating that they exhibit some "transitional" state in the evolution of the dispersed/reaggregation phase and diapause I. A wider, comprehensive approach including more species of different aplocheiloid genera is needed to confirm these trends.

A recent study of *Austrolebias* species has shed light on the initiation and early steps of the reaggregation process of the DBs (Pereiro et al., 2017). In *A. charrua* and *A. bellottii* the reaggregation is first recognizable as a small central group of rounded cells (5–10) surrounded by many migratory blastomeres (Pereiro et al., 2017). Progressively, more and more of the migratory blastomeres are added to the central group, forming a monolayer. When the aggregate consists of around 100 cells some of the cells start to adopt a deeper position within the aggregate. When the aggregate consists of around 200 cells it can be divided in a central, multi-layered area of small cells (around 15 µm) and a peripheral, mono-layered area of bigger cells (25–35 µm). Initially, the central area makes up only 10% of the complete aggregate but progressively increases to up to 70% in later stages (Pereiro et al., 2017). Shortly after, the first signs of axis formation are recognizable in the central area of the aggregate. Using a photoconvertible protein the authors of the same study showed that cells from early stages of the reaggregate do not participate in the formation of the embryo but can be found in the peripheral area of the aggregate. This suggests that the blastomeres giving rise to the embryo do not participate in the early reaggregation process (Pereiro et al., 2017).

6. The phylogenetic distribution of the dispersed phase

Information about the cell behavior and migration patterns of the DBs of the 48 described killifish genera (785 species) is scarce (Berois et al., 2016). From these 48 genera, 18 are categorized as non-annual, showing the same developmental pattern as other non-annual teleosts, while the remaining 30 are categorized as annual and are considered to have a dispersion/reaggregation phase (Table 1). It has been explicitly mentioned in only 14 out of the total 48 genera if a dispersion phase is

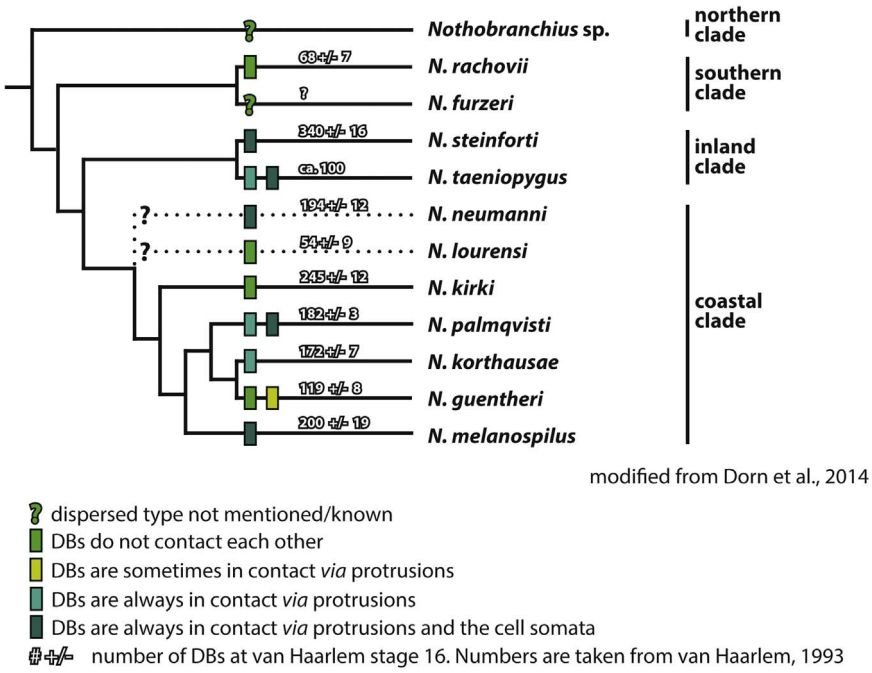

Fig. 6. Phylogenetic relationship of different *Nothobranchius* species from which data about the migration types of deep blastomeres are available (van Haarlem, 1983). The cladogram is a simplified version from Dorn et al. (2014). *N. neumanni* and *N. lourensi* are not included in the study of Dorn et al. (2014). They were placed in an uncertain position in the "coastal clade" due to their distribution in this area. The number of deep blastomeres seems to not correspond to a specific migration type.

present/absent. When available data are plotted onto a pre-existing phylogeny (Furness et al., 2015), the dispersed cell phase appears to be evolutionarily labile (Fig. 8). This is not surprising since it has been proposed that the annual life style, which seems to be a prerequisite for a dispersed phase, has evolved several times independently within aplocheiloid killifishes (Furness, 2016). Phylogenetic patterns become apparent if the migration types of dispersed DBs are taken into account. In the two closely related rivulid genera *Austrolebias* and *Cynopoecilus* (considered as Cynolebias by Wourms, 1972b) DBs migrate as single cells and only make brief random contact with other blastomeres (Wourms, 1972b). In the closely related genera *Pterolebias*, *Rachovia* and *Austrofundulus* (also Rivulidae) some of the DBs seem to frequently make contact with neighboring blastomeres during the dispersed phase (Wourms, 1972b). However, this trend doesn't hold if data on the migratory behavior of the DBs in different species of the African genus *Nothobranchius* are considered. As stated before, species of *Nothobranchius* exhibit a high diversity in the migratory patterns of their deep blastomeres (van Haarlem, 1983). No discernible phylogenetic trends are observed when migratory patterns are plotted on a simplified pre-existing cladogram of different *Nothobranchius* species (Fig. 6) (Dorn et al., 2014). Therefore it is unclear why DB migratory patterns appear to be conserved among some related genera in the family Rivulidae but show so much diversity within the genus *Nothobranchius*. A systematic survey of the migration behavior of DBs in more aplocheiloid species has to be carried out to clarify these questions.

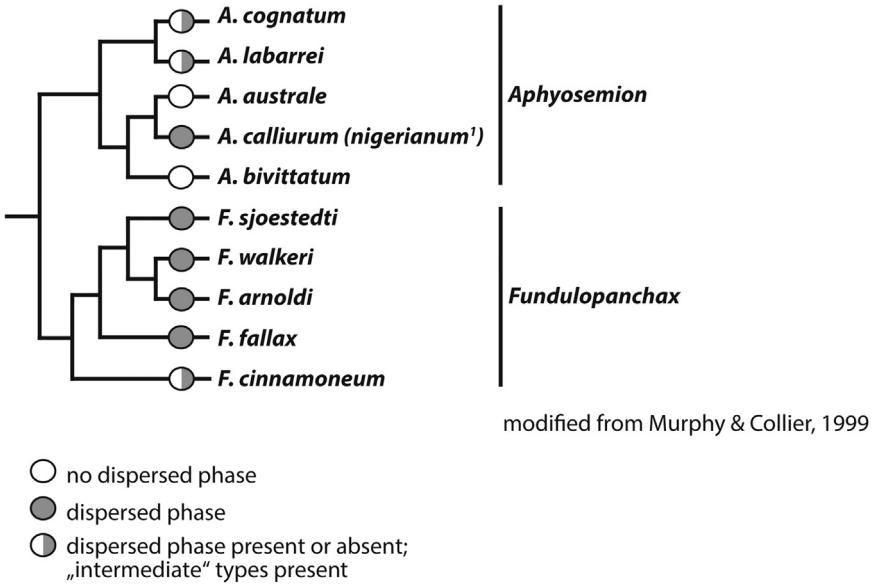

Fig. 7. Phylogenetic relationship of different *Aphyosemion* and *Fundulopanchax* species from which data about the presence/absence of a dispersed phase are available (Wourms, 1972b). The two generea were grouped together as one genus, *Aphyosemion*, in Wourms (1972a, 1972b, 1972c). The cladogram is a simplified version from Murphy and Collier (1999). [1]*A. calliurum* of Murphy and Collier (1999) is *A. nigerianum* of Wourms (1972b).

Table 1
Genera included in the Aplocheiloidea and their life style (annual or non-annual) (Berois et al., 2016). Data for *Laimosemion* are from Furness et al. (2018). Data of *Aphyosemion* and *Fundulopanchax* are from Wourms (1972b). Monotypic genera are included with the complete species name.

family/genus	annual/non-annual
Aplocheilidae	
Aplocheilus	non-annual
Pachypanchax	non-annual
Nothobranchiidae	
Epiplatys	non-annual
Nimbapanchax	non-annual
Archiaphysemion guineense	non-annual
Callopanchax	annual
Scriptaphyosemion	non-annual
Fenerbahce	non-annual
Pronothobranchius	annual
Nothobranchius	annual
Foerschichthys flavipinnis	non-annual
Aphyosemion	non-annual, annual and transitional
Fundulopanchax	non-annual, annual and transitional
Rivulidae	
Kryptolebias	non-annual
Rivulus	non-annual
Laimosemion	non-annual (and transitional and annual?)
Plesiolebias	annual
Papiliolebias	annual
Stenolebias	annual
Pituna	annual
Melanorivulus	non-annual
Cynodonichthys	non-annual
Anablepsoides	non-annual
Atlantirivulus	non-annual
Moema	annual
Trigonectes	annual
Neofundulus	annual
Pterolebias	annual
Renova oscari	annual
Micromoema xiphophora	annual
Terranatos dolichopterus	annual
Gnatholebias	annual
Llanolebias stellifer	annual
Rachovia	annual
Millerichthys robustus	annual
Nematolebias	annual
Xenurolebias	annual
Ophthalmolebias	annual
Simpsonichthys	annual
Spectrolebias	annual
Hypsolebias	annual
Cynolebias	annual
Austrolebias	annual
Notholebias	annual
Mucurilebias leitaoi	annual
Leptolebias	annual
Campellolebias	annual
Cynopoecilus	annual

7. Hypotheses on the developmental significance of the dispersed phase

Aplocheiloid killifishes have colonized many different aquatic habitats that are roughly grouped in three different types, permanent, marginal and seasonal (Fig. 8) (Furness, 2016).

Embryos of non-annual species are typically deposited in permanent water bodies (Furness, 2016). In such habitats, environmental conditions are normally stable and there is no demand for special developmental adaptations to deal with unpredictable changes. In marginal and seasonal habitats, the situation is different. Marginal habitats are always at the risk of desiccation. However, desiccation does not happen for sure and not in discrete cycles. The environmental conditions in such habitats are relatively unpredictable (Furness, 2016). Embryos deposited in marginal habitats (small brooks, forest pools, flood plains, swamps) may have to deal with poor water quality. They may be exposed to hypoxia/anoxia, unfavorable osmotic conditions and short periods of desiccation and increased UV radiation (Furness, 2016). Seasonal habitats in contrast fall dry and re-fill according to dry and rain seasons. Although environmental conditions show the most extreme variations in this habitat type they are somewhat predictable over the long-term. Embryos deposited in seasonal habitats will suffer from desiccation, anoxia and increased doses of UV radiation (Furness, 2016; Wagner and Podrabsky, 2015; Wourms, 1972b).

(1) The first hypothesis for the evolution of a dispersed phase in annual killifishes is based on the advantage of an increased developmental time before the onset of organogenesis (Wagner and Podrabsky, 2015; Wourms, 1972b). It has been shown that early developmental processes such as gastrulation, axis formation, neurulation and early morphogenesis are particularly susceptible to interference from physical or chemical factors (Blaxter, 1969; Wourms, 1972b). The introduction of the dispersed phase extends the developmental time between early cleavage and gastrulation and axis formation by three to five days (Wourms, 1972b). During these days the embryo is able to respond to unfavorable conditions by entering the diapause I phase and arrests development until environmental conditions become more favorable (Wourms, 1972b, 1972c). The evolutionary origin of the diapause I stage in annual killifishes is still unclear. However, it seems to be somehow connected to the origin dispersed phase since both are mostly present in a combined manner (Wourms, 1972b). A recent study describes the presence of a very short dispersed phase in the rivulid genus *Laimosemion* (Furness et al., 2018). Species of *Laimosemion* show a diapause II and III but no diapause I. This allows speculating that a diapause I can only occur if the dispersed phase last over a specific time. Arresting development at the dispersed phase could be advantageous since this is a phase where we expect little cell-cell signaling that governs developmental processes such as gastrulation, axis formation or the onset of organogenesis. Therefore, the dispersed phase seems to be involved in a developmental "switch" that postpone critical developmental processes and ensures long-term survival under lasting unfavorable conditions (Wourms, 1972b).

(2) A second hypothesis of the evolution of a dispersed phase is based on a "critical mass model" (Wourms, 1972b). Wourms, based on chance observations, reported a prolonged dispersed phase in annual killifish embryos lacking the usual number of deep blastomeres. In these embryos the mitotic activity of the deep blastomeres increased and led to a normal cell number with subsequent aggregation and normal embryogenesis (Wourms, 1972b). Therefore, an additional advantage of the dispersed phase might be to re-store cell number and ensure normal embryonic development after short disturbance events. This hypothesis is weakened by the observation, that dispersed blastomeres of different annual killifish species are arrested in the G1-phase of the cell cycle and do not undergo mitosis (Dolfi et al., 2014). However, these cells might still be able to enter the cell cycle again and become mitotic if the number of cells has been decreased by a disturbance event.

(3) A third hypothesis is based on studies on the extreme tolerance and buffering of UV-C induced DNA damage in *A. limnaeus* (Podrabsky et al., 2017; Wagner and Podrabsky, 2015) and *Nothobranchius furzeri* embryos (own observations). It states that during the dispersed phase, prolonging developmental time before axis formation, the DNA damage repair system is able to remove many of the DNA damages and ensure survival of the cells harboring the damage. Aquatic embryos developing in small and shallow ponds are often exposed to UV-radiation and successful progression in ontogeny depends on the removal of DNA damage

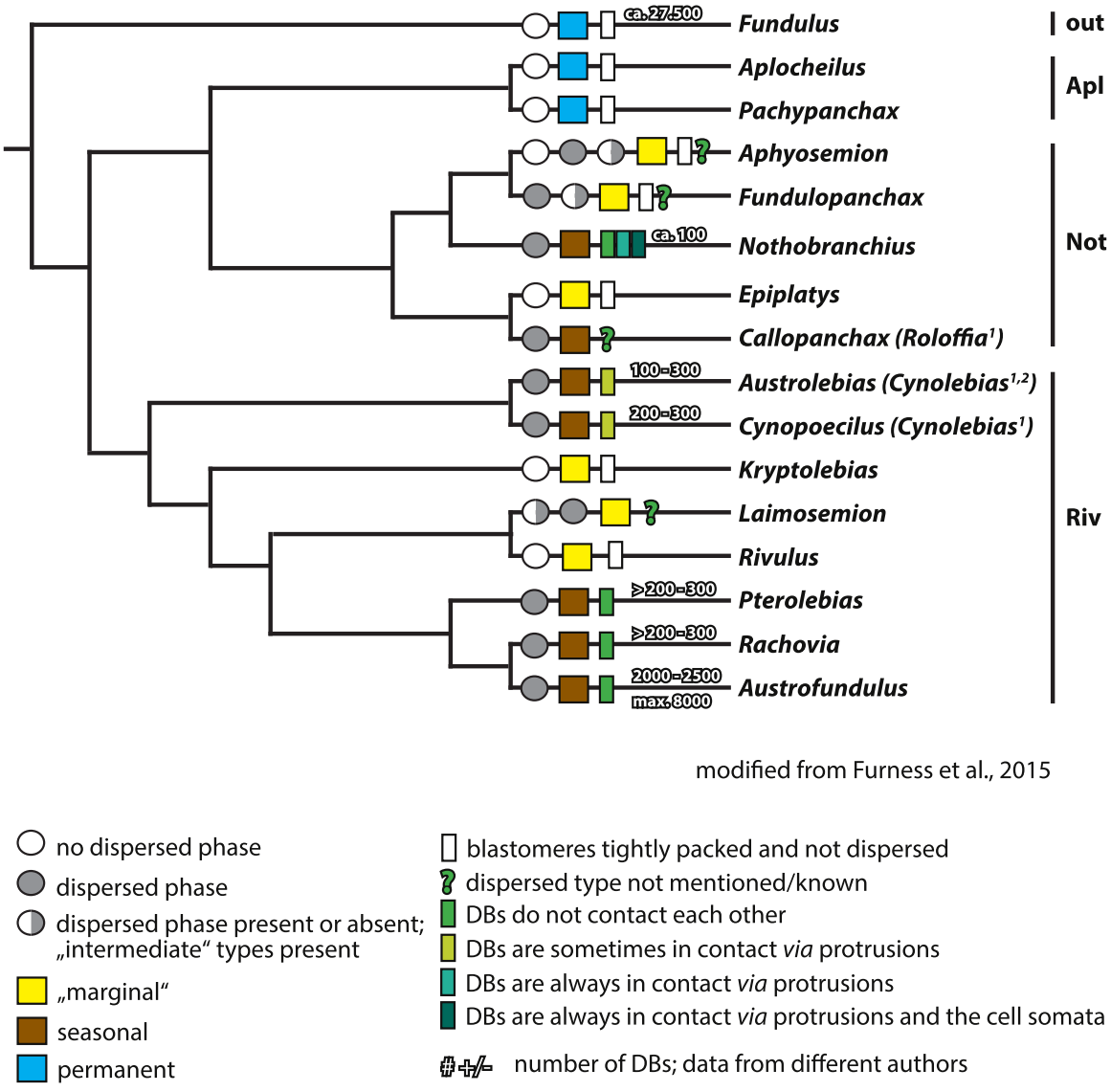

Fig. 8. Phylogenetic relationship of different genera of aplocheiloid killifishes from which data about the presence/absence of a dispersed phase, the migration type of the deep blastomeres, the habitat type and deep blastomere number are available. Information about the presence/absence of a dispersed phase and the migration type are from Wourms (1972a, 1972b), Carter and Wourms (1991) and van Haarlem (1983). Information about the habitat is from Furness (2016). The cladogram is a simplified version from Furness et al. (2015). Numbers of deep blastomeres are from different authors: *Nothobranchius* (Wourms, 1972b), *Austrolebias*, *Cynopoecilus*, *Pterolebias*, *Rachovia* (Carter and Wourms, 1991; Wourms, 1972b); *Austrofundulus* (Carter and Wourms, 1991; Podrabsky et al., 2017). [1]Wourms, 1972b; [2]Carter and Wourms, 1991. Apl, Aplocheilidae; Not, Nothobranchiidae; out, out group; Riv, Rivulidae.

caused by this radiation (Wagner and Podrabsky, 2015). *Austrofundulus limnaeus*, and maybe other killifishes too, seem to have an extremely competent DNA photo repair system (Wagner and Podrabsky, 2015). The assumption, that survival of *A. limnaeus* embryos after UV-damage is due to such a competent repair system gains support by the observation that the number of apoptotic cells does not increase in early embryos after exposure to UV-radiation (Wagner and Podrabsky, 2015).

However which of the hypotheses is assumed to be right, the dispersed phase always acts as a kind of developmental buffer, as proposed by Wourms (1972b) and Wagner and Podrabsky (2015), While the germ cell line has already segregated from the somatic cell line (Berois et al., 2016), the dispersed phase could keep somatic cells in a more-or-less undifferentiated state. This delays sensitive differentiation processes such as axis formation and organogenesis until environmental conditions become more favorable or DNA damage, produced during unfavorable conditions, is repaired or to restore cell number after the loss of blastomeres due to disturbance events (Wagner and Podrabsky, 2015).

8. The evolutionary origin of the dispersed phase

Explaining the origin of complex developmental changes is one of the grand challenges in evolutionary biology (Furness, 2016). Wourms (1972b), by comparing annual, non-annual and “transitional” species developed a hypothesis about the evolution of the dispersed/reaggregation phase. Wourms (1972b) pointed out that the DBs of non-annual teleosts also go through a phase of loose packing and decreased cell-cell adhesion. Cell motility of the DBs also occurs in non-annual teleosts such as the zebrafish, *Danio rerio* (Bruce, 2016). However, they are thought be more or less passive and only dictated by external factors (Kimmel et al., 1995; Morita and Heisenberg, 2013). For annual killifishes Wourms proposed that (1) the reduction of the number of DBs during epiboly was one of the first steps towards the evolution of a dispersed phase. This can be seen in species of *Aphyosemion* and

Fundulopanchax, where non-annual species exhibit the highest number, “transitional” species a lower number and annual species the lowest number of DBs at the beginning of epiboly (Wourms, 1972b). This reduction in cell number would increase the free space on the substrate and offer more free space for potentially migratory cells. Furthermore, he pointed out that in non-aplocheiloid fishes (e.g. *Salmo*, *Fundulus*, *Oryzias*), cellular adhesion leading to the aggregation of the DBs precedes epiboly and embryogenesis. Therefore, (2) a delay of the onset of cell adhesion would allow the DBs of annual killifishes to move into the free space produced by the decrease in DB number (Wourms, 1972b). This can be seen in “transitional” species of *Aphyosemion* and *Fundulopanchax*, where (3) DBs migrate ventrally together with the ECL and YSL as a loosely packed cell mass. They start to “aggregate” and form the embryo when epiboly has already been completed. This is in contrast to non-annual *Aphyosemion* and *Fundulopanchax* (and other non-annual fishes) where DBs migrate ventrally as a cell sheet and start to form the embryo when epiboly is still in progress (Wourms, 1972b). It therefore seems, that (4) the spatial and temporal decoupling of epiboly and later embryogenetic events (axis formation, neurulation, somitogenesis, etc.) must have been indispensable for the origin of a complete dispersed phase (Wourms, 1972b). Wourms (1972b) also mentioned the existence of “proto-annual” species of *Aphyosemion* and *Fundulopanchax* which already show (5) completely separated DBs and a short dispersed phase of around 24 h. Unfortunately he did not state which species he considered to be “proto-annual” and if these species have the possibility to enter the Diapause I stage. Recently, such "proto-annual" species showing a dispersed phase of around 24 hours have been described in the genus *Laimosemion* (Furness et al., 2018). In completely annual species of *Aphyosemion* and *Fundulopanchax*, and their sister taxon *Nothobranchius*, a reduced cell number and a prolonged dispersed phase with the possibility to enter the Diapause I stage show the typical annual fish developmental pattern (Wourms, 1972b).

9. Conclusions

The unique dispersion/reaggregation phase of annual killifishes offers the opportunity to study a remarkable modification of the conserved process of early vertebrate embryogenesis. Examining this developmental phenomenon can lead to new insights into the evolutionary origin of developmental modifications and mechanisms of cell migration and aggregation in vertebrates. Despite the renewed interest in this phenomenon (Dolfi et al., 2014; Pereiro et al., 2017; Reig et al., 2017) there are still many unanswered questions.

What is the biological significance of the dispersed/reaggregation phase? How did it evolve from the non-annual teleost developmental pattern? Are there “new” genes involved that govern this process? Are there more variations of the migratory behavior of the DBs in unstudied species/genera? Did the dispersed/reaggregation phase evolve only once or several times independently? What is the “granular substance of unknown composition” (Wourms, 1972b)? Is reaggregation initiated by a chemical sensing of the “granular substance”?

The use of *Nothobranchius furzeri*, *Austrofundulus limnaeus* and *Austrolebias charrua* as model species has led to a variety of powerful molecular tools to study gene expression, cell fate determination and the migratory behavior of DBs. This will help to answer the questions raised and to better understand the evolution and developmental mechanisms behind the dispersed/reaggregation phase.

Acknowledgements

We thank the two anonymous reviewers for critical reading and helpful suggestions on earlier versions of this manuscript.

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