Hydrobiologia (2005) 546:323–331
A. Herzig, R.D. Gulati, C.D. Jersabek & L. May (eds.) Rotifera X: Rotifer Research: Trends, New Tools and Recent Advances
DOI 10.1007/s10750-005-4241-z
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The development of a bdelloid egg: a contribution after 100 years

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Key words: Bdelloid rotifers, *Macrotrachela quadricornifera*, embryo development, cytoskeleton, mastax, confocal microscopy

Abstract

Rotifer development has received very little attention: studies date back to the 19th century and to the first half of 20th century, and very limited contributions have been added in recent times. All information we have on rotifer embryology is mostly based on *in vivo* observation of developing embryos by light microscopy, and only in a minor way by classical histology. The study of rotifer embryogenesis is approached here using *in vivo* observation and laser confocal microscopy. We revealed cytoskeletal components (filamentous actin and tubulin) and nuclear DNA of the embryos to draw the pattern of the early development of *Macrotrachela quadricornifera*. Our results were then compared to the literature data, to determine a development pattern that can be generalized to the whole rotifer group. On the whole, our results agree with the general description provided by previous authors, i.e. the holoblastic unequal segmentation, the transverse furrow of the first division, the typical 16-cell stage, and the early gastrulation by epiboly. A peculiar pattern could also be seen that was interpreted as the formation of the mastax; it seemed to start from a mould of actin, visible by confocal only. The present study provides a preliminary contribution to a too-long-neglected aspect of rotifer biology.

Introduction

Like several lower invertebrates, rotifers possess a determinative spiral cleavage, meaning that the fate of the cells is established very early in development. Moreover, they are eutelic (e.g. Clément & Wurdak, 1991): throughout all life rotifers possess a constant number of cells, or better, of nuclei, because several tissues are syncytial. Consequently, cell divisions occur during embryogenesis only, when the egg divides several times and gives rise to a fixed cell number that is species-specific. At the end of development, the newborn rotifer possesses the same number of cells as the adult, and the cells will only increase in size during life. In eutelic animals, tissues and organs of the animal are thus formed during embryogenesis and will not be modified during the animal's life; and any anomaly could have consequences for the integrity of the adult. Development is therefore a very delicate process, and its knowledge is very important.

Unfortunately, the study of rotifer development received very little attention: studies date back to the 19th century and to first half of 20th century (e.g. Tessin, 1886; Zelinka, 1892; Jennings, 1896; Tannreuther, 1920; Nachtwey, 1925; Remane, 1929–1933; de Beauchamp, 1956; Pray, 1965; Lechner, 1966). In recent times very limited contributions appeared (e.g. Plasota & Plasota, 1980; Castellano Paez et al., 1988). Rotifer embryology was approached through *in vivo* observation of living specimens by light microscopy or through classical histology. Only Lechner

(1966) followed a different approach, exposing embryos at various stages of development to UV microbeams. He intended to destroy selected blastomeres and to analyse later embryos, with a view to reconstructing the cell lineage. These two approaches, the descriptive and the experimental, produced all the information we have at present. However, the use of new techniques developed in the last decades provide the opportunity to explore in greater detail the developmental process.

Outline of developmental pattern

The steps through which the zygote becomes a complete pluricellular animal are typically 3: cleavage, gastrulation and organogenesis (e.g. Wolpert, 2002). During cleavage, the zygote gives rise to smaller and smaller cells, called blastomeres, that do not grow after each mitosis. The egg cytoplasm, which is not homogeneous because of morphogenetic determinants (molecules that will contribute to the different fate of the blastomeres), segregates differently in the blastomeres. After the undifferentiated mass of cells, called blastula, is formed, gastrulation starts and the three germ layers (ectoderm, endoderm and mesoderm) are defined. Organogenesis consists of the differentiation of each germ layer to form the definitive tissues and organs. In different taxa, different patterns of development are recognized. Cleavage of the rotifers can be ascribed to a modified spiral type (as summarised by Gilbert, 1989).

Review of the literature

Very few authors studied rotifer embryology; major contributions concerned monogonont species and are very old (see Gilbert, 1989). In addition, the different studies dealt with different species, making any comparison difficult (Table 1). The bdelloids were studied by Zelinka (1892) in detail and, subsequently, by Plasota & Plasota (1980), only.

Authors agree on the description of the rotifer egg as oligolecitic, i.e. with a moderate amount of yolk, that does not interfere with cleavage pattern (Remane, 1929–1933). The egg is laid unsegmented, and extrudes the polar body (two polar bodies only in Hsu, 1956a, b) before undergoing the first cleavage. Cleavage is holoblastic (total) and cell divisions are unequal: the blastomeres differ in size and in amount of cytoplasm. Development is determinative: each blastomere will form only a given part of the adult and cannot change its fate (Lechner, 1966).

The different authors used different nomenclature when referring to a same blastomere. When possible, the different nomenclature is here unified to present a common pattern that should serve as

Table 1. List of studies on rotifer embryo development

Author	Year	Studied species
Tessin	1886	*Brachionus urceolaris* O.F. Müller, 1773
		Eosphora najas (as *E. digitata*) Ehrenberg, 1830
		Rotaria rotatoria (as *Rotifer vulgaris*) (Pallas, 1766)
Zelinka	1892	*Mniobia russeola* (as *Callidina russeola*) (Zelinka, 1891)
Jennings	1896	*Asplanchna herrickii* de Guerne, 1888
Tannreuther	1920	*Asplanchna sieboldii* (as *A. ebbesbornii*) (Leydig, 1854)
Nachtwey	1925	*Asplanchna priodonta* Gosse, 1850
Remane	1929–1933	Review
Hyman	1951	Review
de Beauchamp	1956	*Ploesoma hudsoni* (Imhof, 1891)
Pray	1965	*Lecane cornuta* (as *Monostyla cornuta*) (O.F. Müller, 1786)
Lechner	1966	*Asplanchna girodi* de Guerne, 1888
Plasota & Plasota	1980	*Habrotrocha rosa* Donner, 1949
Castellano Paez et al.	1988	*Brachionus plicatilis* O.F. Müller, 1786
Gilbert	1989	Review

background for further comparison. We shall follow the nomenclature used by Jennings (1896), Nachtwey (1925) and Lechner (1966) (hereafter JNL), that is similar to that currently used in modern embryology. For precision, we shall report two more ‚names’ within brackets; these refer to the nomenclature of Zelinka (1892) and of Tannreuther (1920), respectively. Thus, for example, the first 2 blastomeres are respectively AB2 and CD2 according to JNL, but A and I by Zelinka (1892), AB and CD by Tannreuther (1920). These are here reported as AB2 (A, AB) and CD2 (I, CD), respectively (Fig. 1). Other authors do not name each blastomere after second cleavage.

The first two cleavage divisions are unequal and the produced cells differ in size. The first cleavage produces a small AB2 (A, AB) and a large CD2 (I, CD). de Beauchamp (1956) and Pray (1965) found that the yolk granules are mostly attributed to AB2 (A, AB), and a little amount is maintained by CD2 (I, CD). The cleavage furrow is commonly reported as transverse to the major axis of the egg. Only Zelinka (1892) found that the furrow is initially almost longitudinal, and becomes transverse later on.

Except Pray (1965), all authors report that the second cleavage division occurs in the two cells asynchronously, starting in CD2. This gives a larger D3 (I, D) and a smaller C3 (II, C). Cell AB2 then divides into A3 (a, A) and B3 (b, B), of similar sizes. Thus the cells A3, B3, and C3 have same size, while D3 is larger. At first, the four blastomeres are arranged asymmetrically, and become symmetrical very shortly (e.g. Zelinka, 1892). According to de Beauchamp (1956) and Pray

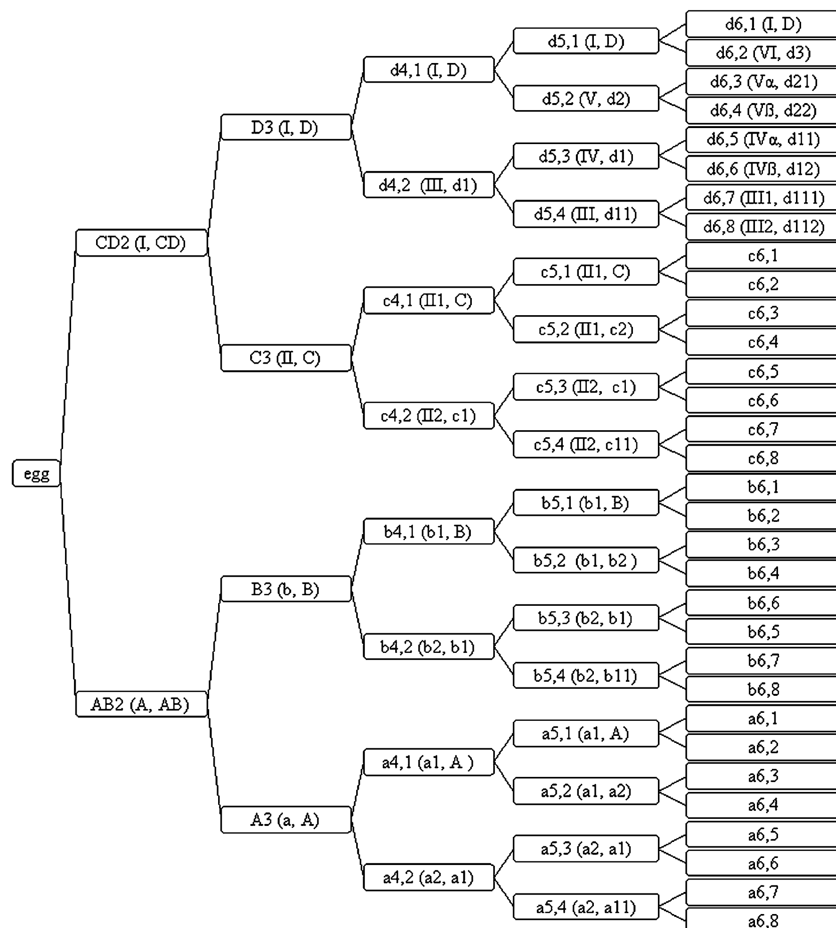

Figure 1. Scheme presenting the cleavage divisions of rotifer embryos. See text for names of blastomeres.

(1965), most yolk granules are segregated in micromeres A3, B3, and C3. The polar body is recognized at the contact between the four cells.

The third cleavage also is not synchronous in the 4 cells, and produces one larger cell (macromere), called d4,1 (I, D), and 7 smaller ones (micromeres). Immediately after division, the cells are not symmetrically arranged, but become symmetrical soon after (e.g. Zelinka, 1892).

Then the macromere d4,1 (I, D) divides into a large d5,1 (I, D) and a small d5,2 (V, d2). The other micromeres divide asynchronously and produce the 16-cell stage.

This stage is typical of all rotifers: four rows of four cells each are easily recognized (i.e. Tessin, 1886; Zelinka, 1892; Jennings, 1896; Tannreuther, 1920; de Beauchamp, 1956; Pray, 1965; Lechner, 1966). Each row is produced by one cell at the 4-cell stage; the row derived from D3 (I, D) contains larger cells. Very minor differences about this stage are present among the different studies. Some discrepancies concern the way this stage is attained, and the number of mitoses required (see Remane, 1929–1933). According to de Beauchamp (1956) and Pray (1965), the yolk granules are now concentrated in one cell for each row; this produces a ring of yolk-rich dark cells that surround the large blastomere d5,1 (I, D).

Gastrulation is assumed to start as early as the 16-cell stage is attained (Zelinka, 1892; Nachtwey, 1925; de Beauchamp, 1956; Pray, 1965; Lechner, 1966; Gilbert, 1989). Only Tannreuther (1920) recognized gastrulation at a later stage (32–64 cells). Gastrulation consists of movements of the cells that first encircle the large blastomere (d5,1) through epiboly and then invaginate.

According to Zelinka (1892) the blastomere d5,1 (I, D) divides into d6,1 (I, D) and to d6,2 (VI, d3). The blastomere d6,1 produces, through 3 subsequent divisions, eight cells. Of them, the two anterior ones, d9,1 (E1) and d9,3 (E2), are responsible for mid gut, the two central ones, d9,2 (ϵ1) and d9,4 (ϵ2), are the origin of the reproductive system (germarium and vitellarium), the four posterior cells, d9,5 (eo1), d9,6 (eo2), d9,7 (eu1) and d9,8 (eu2), will give the posterior gut. Gastrulation concerns also the three cells a5,1 (a1, A), b5,1 (b1, B) and c5,1 (II1, C), that invaginate at the blastopore giving rise to the stomodeum (mouth, pharynx and then mastax). Therefore Zelinka (1892) reported the common origin of the digestive and reproductive system from the cell d5,1 (I, D).

The other authors gave similar descriptions of the gastrulation process, but discrepancies concerned the fate of the cells. Some authors (Jennings, 1886; Tannreuther, 1920) stated the common origin of the digestive and reproductive system from the cell d5,1 or its derivatives, in accord with Zelinka (1892). Other authors (Nachtwey, 1925; de Beauchamp, 1956; Pray, 1965; Lechner, 1966) reported that the large cell d5,1, when invaginating, gives rise to the reproductive system only. The digestive system is formed by other small cells produced by cell d4,2 (III, d1) and by other quadrants (cells stemmed from blastomeres A3, B3 and C3). Lechner (1966) described gastrulation as a two-stage process. The first is the epibolic growth of the blastoderm cell (little cells coming from the A, B and C quadrants) around the d5,1 cell, that will be pushed into the embryo. The second stage is a further epibolic growth and the involution of the blastoderm to form the blastopore. During this process the cells that migrate inward form the endoderm and, later, the digestive system.

Also the position of the blastopore and the origin of the mouth are doubtful. The blastopore does not originate directly from the stomodaeum: it will be formed either near (de Beauchamp, 1956; Pray, 1965) or at the opposite side of the embryo (Tannreuther, 1920). In contrast Zelinka (1892), Nachtwey (1925) and Lechner (1966) reported that the blastopore gives rise to the stomodaeum and the mouth, as expected in protostomes.

Organogenesis has not been studied in great detail, and there are discrepancies on this stage also. The main difference concerns the different origin of digestive and reproductive systems. Gilbert (1989) reports that ectoderm gives rise to stomodaeum and pharynx, nervous system, but also excretory system and muscles. The mesoderm gives rise to the reproductive system, germarium and vitellarium only. Endoderm gives the digestive system.

Present contribution to rotifer embryology

Using a confocal laser microscope, we observed the morphology of early embryo stages of a

bdelloid species. Here we present the pattern of the early development and compare our results with literature data with a view to finding a development pattern that can be generalized to the whole rotifer group.

Material and methods

The experimental model is a bdelloid rotifer, *Macrotrachela quadricornifera* Milne, 1886. For many years it has been reared under laboratory conditions, at 22 °C (details in Ricci et al., 1999).

Eggs at various stages of development were collected and observed on a Leica TCSNT confocal microscope equipped with Argon–Krypton laser (CLSM). To visualize cytoskeletal elements, actin and tubulin of embryos at different stages of development were stained using fluorescent markers.

The shell of *M. quadricornifera* egg is transparent, very resistant and impermeable to many molecules. It was therefore necessary to make the eggshell permeable to the large molecules used to stain the cytoskeleton. After several attempts with different chemicals, e.g. NaOCl, NaOH, HCl, we used a mixture of 1% thioglycolic acid and 0.05% pronase in TRIS buffer (Tris(hydroxymethil)aminomethane, 200 mM, pH8.5) for 90′. The permeabilized embryos were then fixed with 4% paraformaldehyde in PBS (phosphate-buffered saline, 110 mM, pH 7.4) for 1 h, permeabilised with 0.25% Triton X−100 and 0.1% Tween in PBS for 20′, and then processed for visualizing actin (microfilaments) and tubulin (microtubules). Actin was resolved by staining the embryos overnight at +4°C with phalloidin conjugated with rodamin (Sigma, 0.5 μg/ml in PBS). To resolve microtubules, the embryos were incubated with the primary antibody anti α-tubulin (Sigma, clone n. B−5–1–2, 1:500 in PBS) overnight at +4°C, treated again with 0.25% Triton X−100, and exposed to the secondary antibody (fluorescein conjugated antibody anti-mouse IgG, Molecular Probes, 1:50 in PBS) overnight at +4°C. After rinsing in PBS, embryos were stained with DAPI (Sigma, 0.5 μg/ml in PBS) for 20′ to visualize DNA, mounted on microscope slides with DABCO (Aldrich) and MOWIOL 4–88 (Calbiochem) and observed by CLSM.

Results

The egg of *M. quadricornifera* is laid at one-cell stage (Fig. 2a). At 22 °C, after about one hour, before undergoing first cleavage, it extrudes one polar body, recognizable as a nucleus close to the shell (Fig. 2b). The first cleavage occurs about 3 h later. Its furrow is transverse to the major axis of the egg. The cell divides asymmetrically into a small blastomere, AB2, and a large blastomere, CD2, and the polar body remains along the furrow (Fig. 2c). About 90′ later, the second cleavage produces four cells. The blastomere CD2 divides into a small C3 and a large D3. Subsequently, AB2 divides into two cells of equal size: A3 and B3. At the 4-cell stage (Fig. 2d), three small cells of the same size (A3, B3 and C3,) and one large cell, D3, are recognizable. About 75′ later, the third cleavage follows. Similar to previous cleavage, the divisions are not synchronous. Initially the large D3 divides into large d4,1 and small d4,2. Then C3 follows, and subsequently both B3 and A3 divide. The fourth cleavage produces the 16-cell stage (Fig. 2e, f). Blastomeres keep dividing, but the cells resulting from each blastomere cannot be distinguished. At this time, the cells start moving. The blastomeres derived from the D3 (called D quadrant) divide and migrate inward, and the small superficial cells wrap them (Fig. 2g). Judging from the cell displacement, the embryo is starting gastrulation. All cells continue divisions, and become smaller and smaller. Many small cells form a thin layer at one pole of the egg, and wrap the large internal cells (Fig. 2h).

Cells keep dividing, but it is very difficult to track each single stage. Tissues starts organizing but single structures are hardly distinguishable, except for the invagination of the cells that will form the pharyngeal pouch. In the middle of the embryo a double ring of cells is recognisable, because of the concentration of actin filaments. These are mostly visible between the two cell rings, along the cell membranes of the outer ring (Fig. 3a). Some hours later, in the same region, embryos often present the gross design of a mastax (Fig. 3b) where the teeth are clearly visible if stained with phalloidin (Fig. 3c). It must be stressed that by light microscopy the same embryos do not reveal any structure (Fig. 3d). In a later embryo (Fig. 3e), the mastax structure also is visible by light microscopy (Fig. 3f). In embryos at this stage also

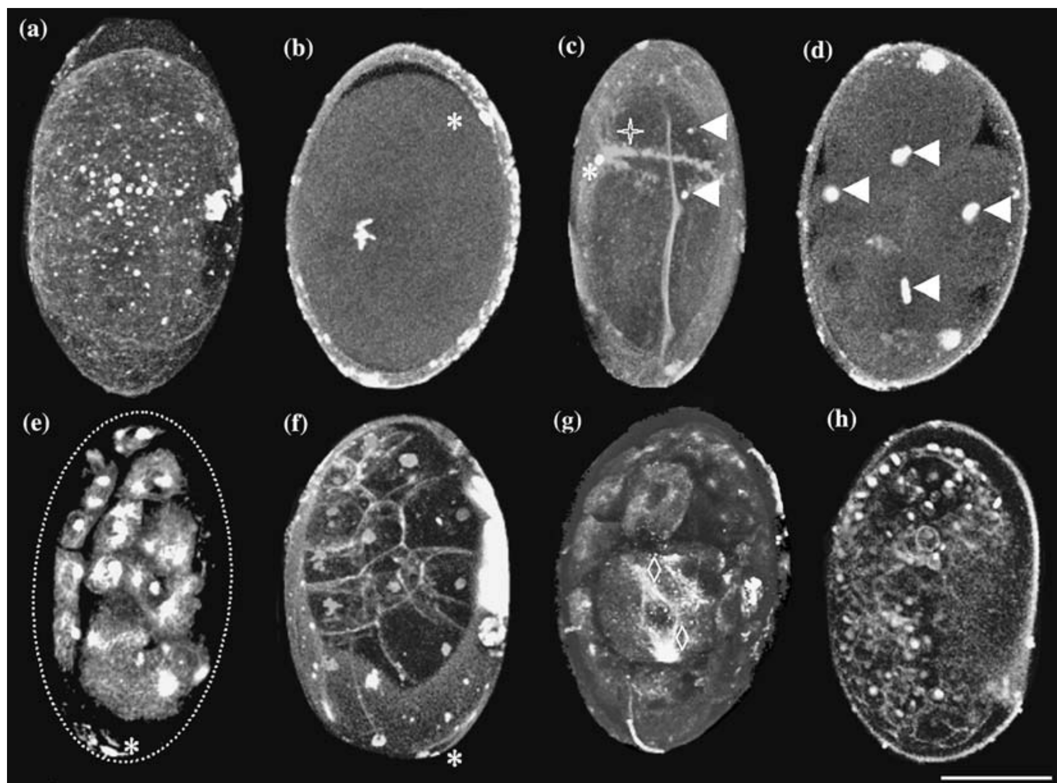

Figure 2. Cleavage and gastrulation process of *M. quadricornifera*'s embryo demonstrated by confocal microscopy. (a) Unsegmented egg. (b) Unsegmented egg and its polar body. (c) First cleavage division: nuclei and actin ring prior to cytodieresis. (d) 4-cell embryo. (e) Embryo between 3rd and 4th cleavage divisions: small blastomeres from A, B and C quadrants on left, large blastomeres derived from D quadrant on right. (f) The 16-cell stage with 4 rows of cells (3 visible). (g) Gastrulation: the mitotic spindle present in d5,1 blastomere. (h) Late gastrulation: small blastomeres externally and large blastomeres internally (one optical section). Scale bar: 40 μm. Arrowhead: nucleus. Fourpoint star: actin ring. Asterisk: polar body. Rhombus: mitotic spindle. a, b, c, d, f, h: rhodaminated phalloidin and DAPI. e: antibody anti-α-tubulin and DAPI. g: antibody anti-α-tubulin.

other structures are recognizable: circular muscles, similar to those present in the adult (Santo, personal communication), are already visible around the embryo body (Fig. 3g). Also the mastax musculature can be distinguished (Fig. 3h), and the trophi are clearly visible (Fig. 3i).

When the embryo is completely formed, it remains in the egg for about 24 h for this species. Structures are complete and fully functional, since it is common to see the trophi pieces moving inside the egg.

Discussion

The study of bdelloid developmental pattern seems very important and in need of much more attention, as is the study of embryology of the rotifers in general. The whole field has been silent for a very long time (the last detailed study was published by Lechner in 1966) and it is unbelievable that the new techniques developed in recent times, from electron microscopy to molecular studies, were never applied to rotifer embryology. Reasons can be found in the difficulty of the material, the resistance of the eggshell, whose chemical composition is almost unknown (Depoortere & Magis, 1967; Piavaux, 1970; Piavaux & Magis, 1970), the unfamiliarity of rotifer scientists with embryology, and of embryologists with rotifers. Nevertheless, the parthenogenetic reproduction of most rotifers, both monogononts and bdelloids, gives the opportunity to investigate the mechanisms responsible for zygote activation and embryo orientation, in the absence of fertilization.

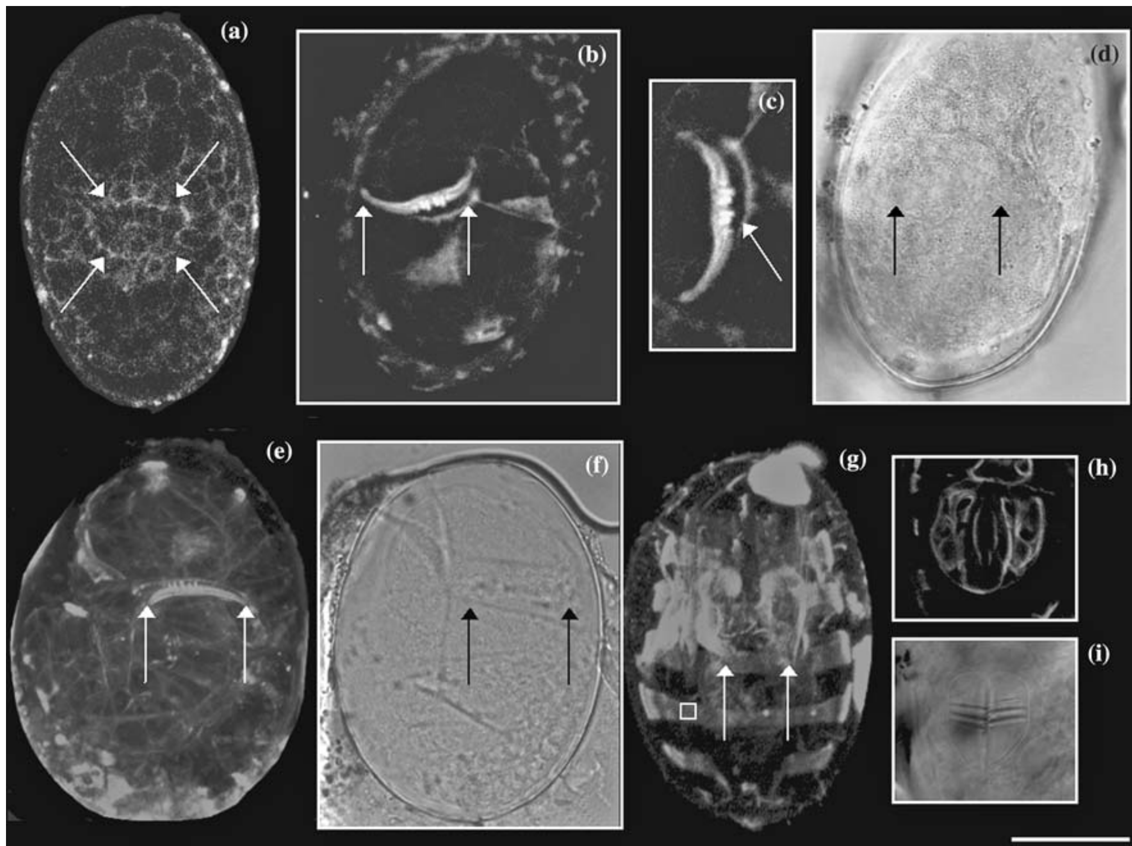

Figure 3. Early organogenesis of *M. quadricornifera*'s embryo demonstrated by confocal microscopy. (a) Actin filaments preliminary to mastax mould formation (see text for explanation). (b) Mould of mastax. (c) 2× magnification of b. (d) Same as b under light microscopy. (e) Mastax in a later embryo. (f) Same as e under light microscopy. (g) Muscles of a developed embryo. (h) Single section of mastax musculature. (i) Same as h under light microscopy. a, e, f, g, h, i: scale bar 40 μm. b, d: scale bar 35 μm. c: scale bar 20 μm. a, b, c, e, g, h rhodaminated phalloidin. d, f, i light microscopy. Arrow: mastax. Square: circular body muscle.

The detailed contributions by early authors on the developmental processes of rotifers give an excellent background to study rotifer development through a more experimental approach. However several difficulties have to be overcome. First, data coming from different studies have to be compared, and nomenclature used by each author have to be unified. Our interpretation of the names of blastomeres comes from a detailed analysis of the literature, which was not always successful. Second, old results need to be confirmed, and a general pattern must be found that can be extended to the rotifers as a group. Alternatively, differences in development between species should be checked and understood. At present, it seems impossible to state if the differences in the literature are between the species or between the authors or both, because the studies were run by different authors on different species (see Table 1).

In this paper we report the study of embryology of the bdelloids using a very informative instrument, the confocal microscope. It permits study of optical sections, avoids treatment artifacts, and allows reconstruction of the whole embryo into 3-dimensional images. We dealt with the early process of bdelloid development through a morphological approach only.

We documented that in *M. quadricornifera* the extrusion of one polar body occurs after laying, right before the first cleavage division. Hsu (1956a, b) reported two equational divisions prior to the formation of the egg, with the extrusion of two polar bodies by *Habrotrocha tridens* (Milne, 1886) and *Philodina roseola* Ehrenberg, 1832.

In contrast, all other authors, including Zelinka (1892), mentioned a single polar body. On the other hand, for monogononts authors report the extrusion of a single polar body during the formation of amictic eggs (Gilbert, 1989). The presence of one or two polar bodies in the apomictic parthenogenesis of bdelloids can be relevant for understanding the oogenesis process itself. Whether the bdelloids differ from the monogononts for the number of polar bodies is unclear. We cannot contribute on this point, except for the fact that before the first cleavage division only one polar body is visible in the laid egg. Whether the one we observed is the second one or the only one, is impossible to state at this stage, and deserves further investigation. Another possibility, that we consider quite remote, is that *M. quadricornifera* differs from *H. tridens* and *P. roseola* on this aspect.

Worthy of note is the result on the mould of the mastax. It must be recalled that the newborn rotifer already possesses its definitive mastax structure and that trophi pieces of bdelloids do not change their size during life (Fontaneto & Melone, this volume). The ‚mould of trophi teeth' is visible by fluorescence observation but disappears by light microscopy. Phalloidin resolves filamentous actin, that is present in muscles, but also in cytoskeleton elements. We have no evidence to state that the mastax muscles, at formation, have such a shape as to make teeth visible, or that a sort of mould of actin for trophi pieces is prepared during development. In analogy, a mould of actin very similar in shape to the definitive hard structure, was found during the formation of a nematode cuticle (Costa et al., 1997). In any case, the trophi and the mastax in general are among the first structures that can be recognized during embryo development.

On the whole, our results conform to the general pattern as outlined by the previous authors (Tessin, 1886; Zelinka, 1892; Jennings, 1896; Tannreuther, 1920; Nachtwey, 1925; Remane, 1929–1933; de Beauchamp, 1956; Pray, 1965; Lechner, 1966), i.e. the holoblastic unequal cleavage, the transverse furrow of the first division, the typical 16-cell stage, and the early gastrulation by epiboly. However, there are still points that remain unclear and require further resolution; among them cell-lineage, apoptosis and gene expressions.

Acknowledgements

Nadia Santo and Manuela Caprioli helped at different stages of present research. Russ Shiel corrected the English style. Financial support came from ASI grant to C.R.

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