

Developmental stages in diapausing eggs: an investigation across monogonont rotifer species

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Abstract Monogononts reproduce by parthenogenesis punctuated by events of mixis that generate ‘resting eggs’, which actually are embryos whose development is arrested. We document the nuclei number and position of the resting eggs of nine rotifer species (*Brachionus plicatilis*, *B. calyciflorus*, *B. manjavacas*, *Plationus patulus*, *Epiphanes senta*, *E. chihuahuaensis*, *Rhinoglena frontalis*, *Lecane bulla* and *Sinantherina socialis*) in the attempt to assess the stage at which dormant embryos are arrested. The morphology of the entire embryos was reconstructed

visualising nuclear DNA using confocal microscopy, and their developmental stage identified. Two groups of species were identified: dormant embryos of one group possessed on average less than 30 nuclei, with low variation within and between species, and the stage may correspond to early gastrula; embryos in the second group contain relatively more nuclei, averaging around 40–60, with higher variation within species. The two groups of species seem not to reflect any phylogenetic relationship. Consequences of the dormant embryo stages are discussed.

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Introduction

Rotifera is a taxon of small metazoans comprising two major free-living groups, Bdelloidea and Monogononta. Both these groups are able to survive unfavourable conditions through two different forms of dormancy: quiescence and diapause, respectively (Ricci, 2001). Bdelloids can enter dormancy any time during their lives: when relative humidity decreases in the environment (e.g. for drying or freezing), the rotifer retracts head and foot and contracts into a compact shape halting metabolic expenditures (Ricci et al., 2003, 2007, 2008). In this condition, the dormant animal can resist severe stresses (Ricci et al.,

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2005, 2007). Conversely, monogononts do not tolerate desiccation at any age, but are resistant to unfavourable conditions at a specific developmental stage, the resting egg. Commonly, monogonont populations consist of ‘amictic’ females that reproduce by apomictic thelytokous parthenogenesis (production of female offspring through ameiotic parthenogenesis). In response to specific environmental cues and/or genetic traits, mictic females are produced that are morphologically similar to the amictic ones, but undergo meiosis and produce haploid eggs (reviewed in Gilbert, 1974; Pourriot & Snell, 1983; Wallace et al., 2006). If unfertilised, these eggs develop into haploid males that fertilise other haploid eggs giving rise to a diploid zygote that is encased in a composite shell made of two or three layers (Wurdak et al., 1978), and starts cleavage till some stage, when it arrests development and becomes dormant. The so-called ‘resting egg’ is therefore a diapausing embryo that will remain dormant for variable periods (days to years) surviving stressful conditions like drought or freezing, and will resume and complete its development till hatching after being activated by external (e.g. illumination, temperature, salinity) or/and internal stimuli (Gilbert, 1974; Pourriot & Snell, 1983 for reviews; García-Roger et al., 2006a; Snell & DesRosiers, 2008). Capacity to hatch is affected by several conditions, including the age of the resting egg. For instance, the hatchability of *Brachionus plicatilis* resting eggs was found to vary with egg appearance (‘healthy-looking’ or deteriorate) and depth in the sediment, and the time needed to hatch ranged between 36 and 50 h, with few late hatchlings (García-Roger et al., 2006b). However, the time likely depends on the stage at which the diapausing embryo is arrested.

The development stage at which the dormant embryo is arrested is not fully known and the information available is sometime confusing. Mrázek (1897) first described the resting egg of *Asplanchna herricki* as consisting of an outer layer with several small nuclei and an inner layer of about 30 large nuclei. He could not recognise cell membranes and described the resting egg as composed of internal and external syncytial masses. A similar appearance of the resting egg was also described by Wurdak et al. (1978) in *A. sieboldii* and in *Brachionus calyciflorus*, but no nuclei number is recorded. A more recent study on *B. plicatilis* reports that a dormant embryo is

composed of 39 nuclei (Hagiwara et al., 1995), but no cell membranes were identified (A. Hagiwara, pers. communication). Differently, the resting egg of *Lacinularia socialis* was described to contain about 300–350 nuclei (Bogoslavsky, 1929 in Gilbert, 1974). Other authors stated that dormant rotifer eggs had already completed mitotic divisions and will differentiate tissues and organs upon activation (Lange, 1913 in Gilbert, 1974; Remane, 1929–1933).

Indeed, the data on nuclei number and developmental stage of rotifer resting eggs are contrasting, results often differ, and the discrepancy might be due either to different techniques of investigation or to actual differences among species. In this article, we document the cell number and structure of the resting eggs of nine monogonont taxa using fluorescent molecules and observing by confocal microscopy. On the basis of nuclei number, the developmental stage of the resting egg is determined in rotifer embryos of different species by comparison (cf. Boschetti et al., 2005) to assess whether diapausing embryos of different taxa are arrested at a similar developmental stage. In other words, is the stage of the resting egg referable to a known blastula or gastrula or more advanced stage, and is the stage species-specific or common to different species?

Materials and methods

We analysed resting eggs of nine species of monogonont rotifers from three ploumid and one flosculariid families. List of species, families and number of eggs investigated are given in Table 1.

The resting eggs came from different areas: *Brachionus plicatilis* strain CU1 from Spain, *B. manjavacas* from Russia (formerly *B. plicatilis* strain RUS, Gómez et al., 2002; Fontaneto et al., 2007), *B. calyciflorus*, *Plationus patulus*, *Epiphanes chihuahuaensis* and *Sinantherina socialis* from USA, and *Epiphanes senta*, *Rhinoglena frontalis* and *Lecane bulla* from Italy. Dry resting eggs were first re-hydrated shortly in deionised water; eggs of all taxa followed a same procedure but were processed separately to avoid cross-contamination. Eggs were fixed in 4% paraformaldehyde in PBS (phosphate-buffered saline, 110 mM, pH 7.4) for 1 h at room temperature, rinsed in PBS, dehydrated at increasing ethanol concentrations and kept in 100% ethanol at

Table 1 List of the analysed species with descriptive statistics of the number of nuclei per embryo

Order	Family	Species	#	Mean \pm SD	Median	Interval	
Ploima	Brachionidae	<i>B. plicatilis</i>	21	59 \pm 30	52	26–160	
		<i>B. calyciflorus</i>	5	24 \pm 2	24	22–26	
		<i>B. manjavacas</i>	5	45 \pm 8	46	34–53	
		<i>P. patulus</i>	50	25 \pm 3	25	20–30	
	Epiphanidae	<i>E. senta</i>	3	68 \pm 26	73	39–91	
		<i>E. chihuahuaensis</i>	4	25 \pm 4	25.5	21–30	
		<i>R. frontalis</i>	4	24 \pm 4	24.5	18–27	
	Lecanidae	<i>L. bulla</i>	1	–	–	63	
	Flosculariacea	Flosculariidae	<i>S. socialis</i>	3	21 \pm 3	20	19–24
	General			96	35 \pm 21	26	18–160

–20°C overnight. Eggs were then rehydrated at decreasing ethanol concentrations, rinsed in PBST (PBS with 1% Triton X-100) and kept in 3% Triton X-100 and 1% Tween-20 in PBS for 30 min at room temperature to improve permeability of the membranes. Nuclear DNA staining was performed by incubating eggs for 20 min at room temperature in 0.5 μ g/ μ l DAPI (4',6-diamidino-2-phenylindole dihydrochloride, Sigma) in PBS, then rinsing in PBS. Eggs were then mounted on microscope slides with DABCO (Aldrich) and MOWIOL 4–88 (Calbiochem) and observed using a Leica TCSNT Confocal Laser Scanning Microscope (CLSM), exciting the DAPI with a UV laser (361–365 nm). For each embryo, series of optical sections were recorded and three dimensional projections were made using Leica software.

Images from CLSM were analysed with Image-Pro Plus that allows to count the nuclei in each optical section and prevents errors when a same nucleus is present in more than one optical section. Statistical analysis was carried out with SPSS 17 for Mac OS X. Normal distribution of dataset was assessed with Kolmogorov–Smirnov test, followed by non-parametric Kruskal–Wallis test and pair-wise comparisons by Mann–Whitney *U* test to evidence significant differences among taxa.

Results

The staining protocol was successful for eggs of most taxa, but not for *L. bulla* in which only one egg could

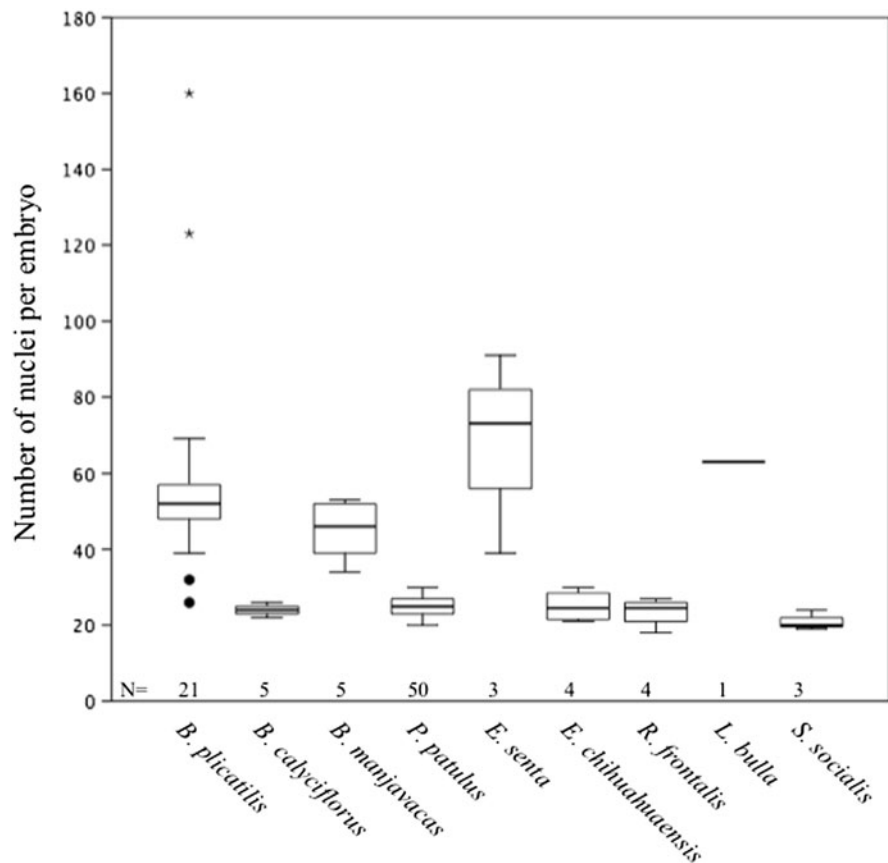
be stained successfully. This contained 63 nuclei and was not included in the statistical analysis.

The mean and standard deviation of the number of nuclei recorded in the resting egg of each taxon is given in Table 1, together with sample size, median and interval (minimum and maximum) of the number of nuclei. The number of nuclei in resting eggs varies considerably, from a minimum of 18 to a maximum of 160. Four values are true outliers, and all concern *B. plicatilis* resting eggs. The box plot of resting eggs nuclei number (Fig. 1) shows that two groups of taxa can be distinguished. One group, consisting of *B. calyciflorus*, *P. patulus*, *E. chihuahuaensis*, *R. frontalis* and *S. socialis*, possesses about 20–25 nuclei per resting egg, with irrelevant differences among taxa and low standard deviation. The second group, made of *B. plicatilis*, *B. manjavacas*, *E. senta* and, possibly, *L. bulla*, possesses an average nuclei number spanning between 45 and 68, with a rather high variation both within and among taxa.

Data are not normally distributed (Kolmogorov–Smirnov test, $D(95) = 0.293$, $P = 0.000$), and non-parametric Kruskal–Wallis test shows that there are significantly different groups in the data-set ($H(7) = 60.545$, $P = 0.000$), and pair-wise comparisons between groups with Mann–Whitney *U* tests confirm that *B. plicatilis*, *B. manjavacas* and *E. senta* differ significantly from the other taxa.

Irrespective of the number, the position of the nuclei is relatively similar in all the observed resting eggs: in general, a central group of large nuclei (up to ~ 10 μ m in diameter) is surrounded by a peripheral layer of smaller nuclei (up to ~ 5 μ m in diameter) (Fig. 2).

Fig. 1 Box-plot of the number of nuclei in the resting eggs of the nine taxa considered. In each ‘box’, the *transversal line* represents the median, the ‘*box*’ indicates the interquartile distance (IQR, the 25% of the values higher or lower than the median), the two *vertical lines* (whiskers) extend outside the box for 1.5 times the IQR, the *circles* represent the outliers (1.5–3 times the IQR) and the *asterisks* represent the extreme outliers (>3 times the IQR). The number of samples analysed for each group (*N*) is shown

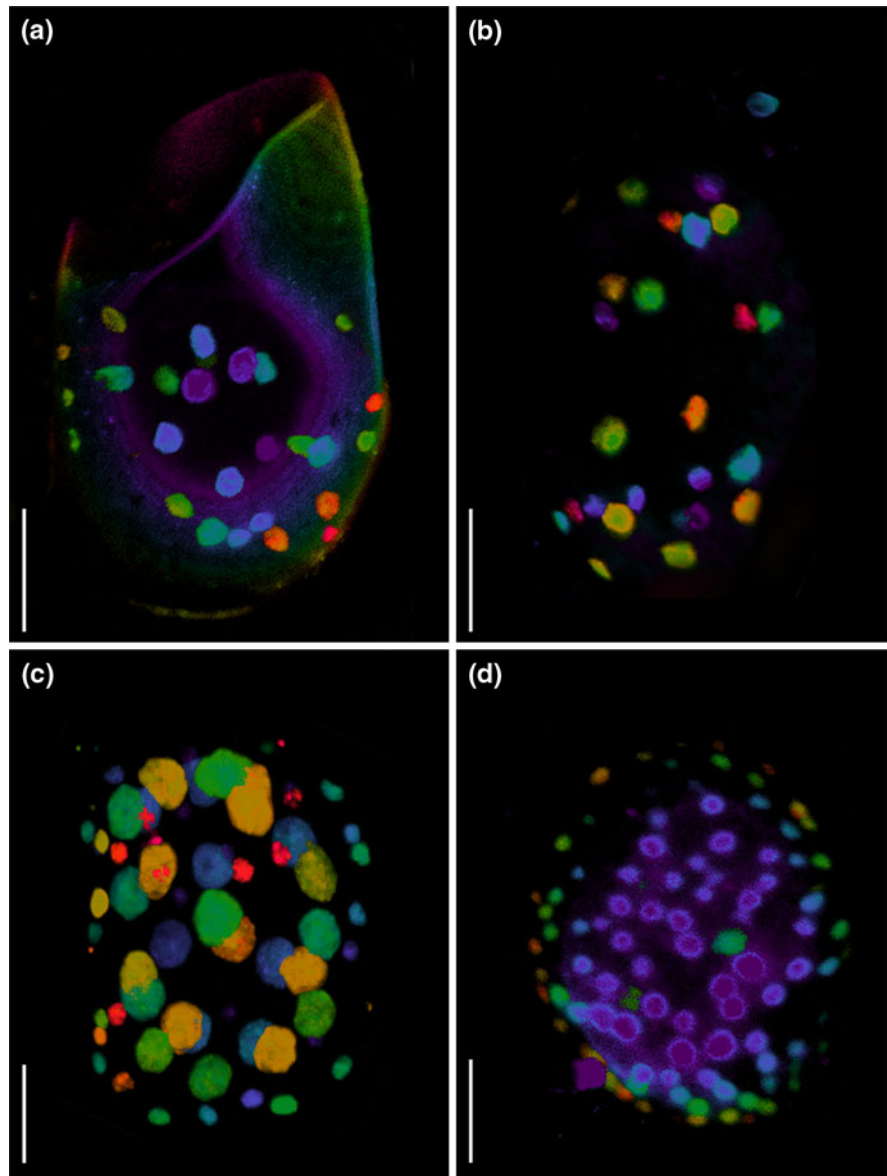


Discussion

On the basis of the number of nuclei in the dormant embryo, two groups of species can be recognised. One group, rather homogeneous, possesses less than 30 nuclei, of which less than one third belong to the inner mass and two-thirds to the outer layer. Similar nuclei outline is reported also in other rotifer species (see Gilbert, 1974 for review). The second group of species has more nuclei and their number varies both among and within species; this is the case of *B. plicatilis*, *B. manjavacas*, *E. senta* and possibly *L. bulla*. To the same group *Lacinularia socialis* (formerly *L. flosculosa*) and *Conochilus* can be assigned on the basis of literature data (Bogoslavsky, 1929 in Gilbert, 1974). It is remarkable that some resting eggs of the very same taxon possess relatively few nuclei (as few as 30–40), while others possess as many as 90 or even more (see outlier values for *B. plicatilis*). We do not have any explanation to offer that can account for the recorded variability. For

B. plicatilis, a large overlapping of cryptic species is well known (e.g. Gómez et al., 2002; Suatoni et al., 2006), but the fact that the relevant variance could be related to genetic differences seems hard to assess. Apparently, the resting embryos of the second species group reach a slightly more advanced developmental stage before becoming dormant. Remarkably, the difference between the embryos of two species groups may reflect a single mitosis event. The fact that some embryos are clearly arrested at a later developmental stage might imply faster hatching, or differences in egg ‘appearance’ and perhaps viability, as found by other authors (García-Roger et al., 2005, 2006a, b). Viability of the resting eggs was not tested by us, but some correlation between embryo developmental stage and viability cannot be ruled out. However, at this point we cannot say whether embryos with a higher number of nuclei could be faster to hatch, or be more viable after diapause, or if the opposite is true, *i.e.* embryos at an earlier developmental stage are more robust and therefore

Fig. 2 Three-dimensional reconstruction of the resting eggs of different species representative of the two groups. (a) *B. calyciflorus* and (b) *R. frontalis* with less than 30 nuclei (25 nuclei each); (c) *B. plicatilis* and (d) *E. senta* with more than 40 nuclei (69 and 91 nuclei, respectively). Nuclei are stained with DAPI and observed at CLSM; different colours indicate different depth of the focal plane in the embryo. Scale bars 25 μ m



have a higher survival rate after diapause. Studies in this direction will shed further light on the significance of the developmental stage for diapause survival.

The number of nuclei of the resting eggs, coupled to the morphological appearance, seems to correspond to the outcome of epibolic movement of outer cells around larger cells that are pushed inward, a condition present during gastrulation of amictic eggs of both monogonont and bdelloid rotifers (see Gilbert, 1974; Boschetti et al., 2005, and references

therein). All resting eggs seem to be composed of a central core of large nuclei surrounded by an external layer of small nuclei; this pattern is common to all the investigated taxa and agrees with previous findings (Mrázek, 1897; de Beauchamp, 1965 in Gilbert, 1974; Gilbert & Wurdak, 1978; Wurdak et al., 1978; Hagiwara et al., 1995). The evidence therefore shows that resting eggs of the first group of species are paused at some stage of gastrulation, possibly at early gastrulation, while the resting eggs of the second group might correspond to a late gastrula. Anyway,

both species groups, with a higher or lower number of nuclei, contradict earlier observations stating that the final nuclei number had already been attained before entering diapause (see Lange, 1913 in Gilbert, 1974; Remane, 1929–1933).

Most studies point out that the cell membranes are not discernible either in the inner or in the outer masses, while Gilbert (1989) already noticed that the mictic embryo is composed of well-defined blastomeres rather than by a syncytium during cleavage, like the amictic egg and the bdelloid egg are during their entire development. On the other hand, while adult rotifers possess many syncytia, several tissues are clearly made of single cells. We did not succeed in evidencing membranes, because treatments with phalloidin after permeabilisation failed in the resting egg; therefore if, why and how the syncytia will revert into cells on reactivation after diapause, as expected because of the cellular tissues of the animal, remains an open question. As pointed out by Gilbert (1989), there might be some functional significance for the presence of syncytia instead of single blastomeres, and the post-diapause development has never been investigated.

If gastrulation is the stage of the resting egg in a number of monogonont species, as it is in crustaceans (Benesch, 1969; Alekseev et al., 2007 for a recent review), then it is also possible that these rotifer embryos use stored maternal RNA till diapause, and start transcribing their own RNA on reactivation, *i.e.* after gastrulation has occurred (Gilbert, 2003). The resting egg in its ‘life time’ encounters two very different environments: a ‘pre-dormancy’ environment that is common to its mother, and a ‘post-dormancy’ environment, that reasonably will differ from the previous one, when the resting egg will complete the development and hatch. That the ‘dual life’ of the resting eggs requires different gene expression might be reasonable, but this issue has never been addressed. It is still not known whether maternal RNA is used at the beginning of development or whether the embryo starts transcribing as soon as cleavage occurs, but recent advances in rotifer transcriptomics (Suga et al., 2007; Boell & Bucher, 2008; Denekamp et al., 2009) allow hoping for a fast answer to this interesting question.

On the whole, more detailed studies are required to better understand the biology of rotifers, and in particular monogononts and their resting eggs, but

happily this seems to become easier by the day, thanks to the recent advances in biology and the access to cheaper molecular techniques for genomics and transcriptomics.

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