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Stereological analysis of mitochondria in embryos of *Rana temporaria* and *Bufo bufo* during cleavage

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Abstract: Total numbers of mitochondria and their morphology have been quantitatively determined in mature oocytes and in cleaving embryos of two anuran species *Rana temporaria* and *Bufo bufo* using stereological methods. Surface densities of inner mitochondrial membranes for both studied species during cleavage ranged from 5.43 m^2/cm^3 to 7.53 m^2/cm^3, whereas volume densities of mitochondria did not exceed 1.65%. Since values of these parameters were low, thus embryos during cleavage may be considered as metabolically "silent". Transition of ultrastructural morphology of mitochondria towards that characterising actively respiring organelles occurs at stage 9 for *R. temporaria* and at stage 8 for *B. bufo*, correlated with blastula-gastrula and mid-blastula transition, respectively. The total numbers of mitochondria N(c) in mature oocytes are as high as 114.8 and 107.2 millions for *R. temporaria* and *B. bufo*, respectively, and during cleavage at late blastula stages they increase to 300 millions for both species under study. We suggest that an undefined mechanism might eliminate during cleavage those amphibian embryos which contain small number of mitochondria and low levels of nutrient substances.

Key words: Mitochondria - Embryo - Cleavage - *Rana* - *Bufo* - Stereology

Introduction

In amphibians eggs, large amount of materials necessary for further development and growth are stored during oogenesis, therefore the eggs may reach up to 2 mm in diameter for *Rana temporaria* and *Bufo bufo* oocytes [21]. The material accumulated in the oocyte cytoplasm includes different forms of the yolk, enzymes, precursors for DNA, RNA and protein synthesis, mRNAs, tRNAs, structural proteins and ribosomes as well as mitochondria [6, 11]. It has been suggested that the number of mitochondria in the mature amphibian oocyte is of the order of 10^5 as estimated by measurements of the total mitochondrial DNA [28]. Despite this common opinion and by using appropriate stereological methods it was proved that there is enormous number of mitochondria in the amphibians oocytes: 16 millions per *Xenopus laevis* ovulated oocyte [13] and above 100 millions in *R. temporaria*. In the oocytes, mitochondria are not dispersed at random but their distribution pattern evolves during oogenesis [24, 31]. Arrangement of mitochondria possesses axial symmetry along the animal-vegetal axis (*a-v* axis) until fertilization [15]. There exists a mitochondrial distribution gradient in the amphibian oocytes that starts in the animal hemisphere with large number of mitochondria and ends at the vegetal pole with very small number of mitochondria. Immediately after fertilization, rotation of the egg cortex modifies this initial symmetry to create the embryonic dorsal-ventral axes [24, 32], and then, during cleavage, further rearrangements proceed [15].

Fertilization initializes cleavage, which is characterized by high speed and frequency of the cell divisions. This high mitotic activity leads to a fragmentation of the egg into an evergrowing number of blastomeres, hence the population of mitochondria is divided into numerous, increasingly smaller fractions. In amphibians, at the beginning of the cleavage the mitoses are synchronous, then the cell divisions lose their synchrony, the new genes become expressed, cleavages become slower and cells become motile [3, 6]. This mid-blastula transition for *R. temporaria* and *B. bufo* occurs at stage 8 [14].

Following fertilization, different metabolic processes start at different time during cleavage in amphibians embryos. A few minutes after insemination protein synthesis and amino acid transport are activated [16, 30], but the main process is an intensive DNA synthesis in a series of quick, mitotic divisions of blastomeres [6]. The whole genome is replicated in less than 10 minutes [3]. It is possible because the machinery for DNA synthesis

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is ready in the embryo cytoplasm. The level of protein synthesis is initially low, but steadily increases during cleavage. However, even at the blastula stage, 85% of overall protein synthesis is still supported by maternal mRNAs. Initially after fertilization, the total RNA synthesis is weak in comparison to later stages of cleavage. In amphibians, the synthesis of mRNAs starts during early cleavage, at the 16-cell stage and continues at a low and constant rate throughout the cleavage [6]. The synthesis of the small RNAs, such as 5S RNA and tRNAs starts at the mid-blastula transition but there is no measurable synthesis of other rRNA nor formation of new ribosomes during this period of development. Thus, embryos are using the ribosomes that had been produced during oogenesis in amphibian embryo [6]. Later, at the blastula-gastrula transition, the embryo starts synthesis of all rRNAs and assembly of new ribosomes. At that time, mRNA synthesis also greatly increases [3].

All the processes that occur during cleavage regime cause progressive increase in oxygen consumption in amphibian eggs [12, 20]. This increase starts almost immediately, one minute after insemination, becomes faster from mid-blastula transition [20] and is controlled by the concentration of ADP in the egg. Thus, consumption of ATP in cleaving embryo must increase as well. In somatic cells, alteration of energy consumption leads to changes of mitochondrial volume and membrane surface [26]. Frequently, the number of mitochondria also increases. However, it is known that mitochondria do not replicate during cleavage in amphibian embryos [4]. Thus the questions arises in what way mitochondria may modify their structure to meet the new requirements of the growing embryo. The aim of the present study was to analyze stereological parameters of mitochondria , *i.e.* numerical and volume densities as well as surface densities of the inner and outer mitochondrial membranes.

Materials and methods

Animals. Mature males and females of frog (*R. temporaria*) and toad (*B. bufo*) were collected 30 km south of Cracow, Poland, during one mating season (the second half of March for frog and the first half of April for toad) and used to obtain oocytes and embryos. Nine stages of development (St) [14] were analysed: St.1 - mature oocyte, St.2 - fertilized eggs, 2 hr after fertilization, St.3, 4 and 5 - two, four and eight blastomeres, respectively, St.6 - morula, St. 7, 8 and 9 - early, medium and late blastula, respectively. Mature oocytes (St.1) were isolated from the oviducts. Embryos at each stage of development during cleavage (from St.2 to St.9) were chosen from the set of *in vitro* fertilized eggs [14, 23].

Light and transmission electron microscopy. Oocytes and embryos were decapsulated and fixed for 24 h at 4˚C with a mixture of 3% glutaraldehyde, 2% formaldehyde and 2% dimethylsulphoxide (DMSO) buffered with 0.1 M cacodylate buffer (pH 7.4). The material was then rinsed in 0.1 M cacodylate buffer at 4˚C for 24 h, oriented in 2% agar and postfixed at 4˚C for 24 h in 1% OsO_4 buffered with 0.1 M cacodylate buffer (pH 7.4). Samples were subsequently dehydrated in ethanol using a standard dehydration schedule and finally embedded in epoxy resin (Agar 100 Resin, Agar Scientific Ltd). Technical details were presented in previous articles on embryo orientation in the epoxy resin block [21, 22].

Blocks with oocytes or embryos were trimmed to an accuracy of 20 μm by means of a wood lathe equipped with a stereoscopic microscope MSt130 (PZO, Poland) and an eyepiece micrometer to obtain axial sections (*AS*), which contain *a-v* axis of embryos (Fig. 1a). Semi-thin sections, 1 μm thick, were cut from *AS* using Porter-Blum ultramicrotome (Servall Inc. Norwalk Comp, USA) and stained with mixture of 1% methylene blue and azure II. Subsequently, sections were examined with the Biolar (PZO, Poland) light microscope equipped with an eyepiece micrometer to evaluate total volume of cytoplasm of oocytes and embryos V(c) for each developmental stage. Then, to reduce the size of the sections, *AS* were trimmed by hand to an accuracy of 25 μm using razor blade and MSt130 stereoscopic microscope with an eyepiece micrometer, [21, 22] (Fig. 1a). Trimmed and oriented blocks were cut into ultrathin sections; sections were placed on 400 mesh grids and double stained with uranyl acetate and lead citrate for TEM. Ultrathin sections were then analysed in the Jeol 100SX (JEOL, Japan) transmission electron microscope at 80 kV. To calibrate magnifications, Fullam grid (E.F. Fullam, Schenectady, N.Y. No 321) was used.

Stereology. The following stereological parameters were estimated for mature oocytes and for each stage of development: numerical densities of mitochondria per unit volume of cytoplasm N_v(m,c), volume densities of mitochondria per unit volume of cytoplasm V_v(m,c) and surface densities of outer and inner mitochondrial membranes per unit volume of mitochondria: S_v(ext,m) and S_v(in,m) respectively. Methods of unbiased estimation of the above stereological parameters for oocytes and embryos from *AS* were previously described in detail [21, 22]. In brief, nine embryos for each stage from St.1 to St.9 were selected and one *AS* randomly oriented around *a*-v axis was trimmed per each oocyte or embryo (Fig.1a). Then, from the set of nine *AS* for each St, three animal - A, three vegetal - V and three equatorial - E subareas of *AS* were trimmed and analysed (one subarea A, V or E per *AS*). Sizes of A, V and E are shown in Figure 1a. Thus, nine ultrathin "axial" sections sampled the entire volume of oocyte or embryo randomly (Fig.1b). Then each subarea (A, V, E) was sampled by the n_i randomly chosen meshes of the electron microscope grids [22]. Index *i* is the number of subarea for each stage (i = 1, ..., 9) and n_i is the number of chosen meshes for *i*th subarea, usually n_i = 9 but if the area of cytoplasm was small, *e.g.* in A for stages with a large blastocel, then n_i = 18 or 27.

For each *ij*th chosen mesh, N_{vij}(m,c), V_{vij}(m,c), S_{vij}(ext,m) and S_{vij}(in,m) were measured. Index *j* is the number of the mesh chosen on the subarea number *i*. In the geometrical centre of each chosen mesh, one micrograph of cytoplasm at ×2800 magnification (to evaluate V_{vij}(m,c)) and one micrograph of mitochondria at ×10000 magnification (to evaluate S_{vij}(ext,m) and S_{vij}(in,m)) were taken. The volume densities V_{vij}(m,c) were measured using point counting method [29] and B100 double square lattice system with the number of test points P_t =400. To estimate surface densities: S_{vij}(ext,m) and S_{vij}(in,m), B100 lattice was used too, but with total test line length L_T=2280 mm. Thus:

$$S_{V,ij}\,(ext,m) = 2\,\frac{P_{ext,ij}}{L_T}\,\frac{P_t}{P_{m,ij}}\,M$$

and

$$S_{V,ij}\,(in,m) = 2\,\frac{P_{in,ij}}{L_T}\,\frac{P_t}{P_{m,ij}}\,M$$

where M is the magnification of micrographs (M = ×10000), $P_{ext,ij}$ and $P_{in,ij}$ are the numbers of intersections between test lines and external or internal mitochondrial membranes, respectively. $P_{m,ij}$ is the number of test points on mitochondria.

To estimate numerical densities of mitochondria N_{vij}(m,c), Weibel-Cruz-Orive method was used [5, 29]. Then:

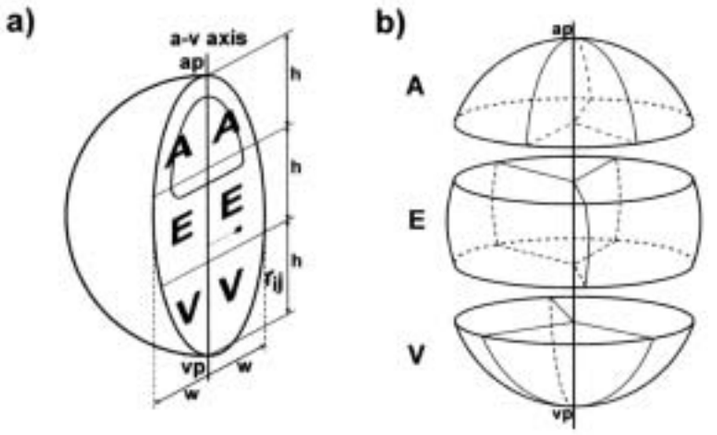

Fig. 1. a. Axial section (AS) of embryo isotropically oriented about *a-v* axis. Animal (A), equatorial (E) and vegetal (V) subareas of AS are shown. *ap*: animal pole, *vp*: vegetal pole. $w = r_i$ and $h = \frac{2}{3} r_i$ are width and height of subareas, respectively. r_i is diameter of *i*th embryo and r_{ij} is distance between *a-v* axis and *GCM*. **b**. Isotropic sampling of embryo around *a-v* axis by three subareas A, E and V.

$$N_{V,ij}(m,o) = \frac{2\, N_{A,ij}\,(m,c) \sum_{k=1}^{n} m_k^{-1}\, q_k\,(w_k^2 \mid 0,0,0)}{\pi\, n}$$

where

$$w_k^2 = 1 - (\frac{m_k}{M_k})^2$$

$N_{A,ij}(m,c)$ are numerical densities of mitochondrial profiles per unit area for *ij*th mesh. Numbers of mitochondrial profiles per unit area were counted at ×56000 magnification using square test area [7] and ten randomly chosen fields of view of TEM per each chosen mesh. $q_k(w^2|0,0,0)$ are auxiliary coefficients [29]. M_k and m_k are lengths of major and minor principal axes of *k*th recorded profile of mitochondria on section number *i* and *n* is the number of measured mitochondrial profiles. M_k and m_k were measured on micrographs of mitochondria taken at ×10000 magnification.

To estimate the unbiased mean of $N_v(m,c)$, $V_v(m,c)$, $S_v(ext,m)$ and $S_v(in,m)$ in relation to the whole volume of cytoplasm of the oocyte or embryo for each stage, corrections factors were used [22]:

$$X_x = \frac{\sum \frac{r_{ij}}{n_j} X_{x,ij}}{\frac{r_{ij}}{n_j}} \qquad i = 1, ..., 9;\ j = 1, ..., n_i$$

where $X_x = N_v, V_v$, or S_v and $X_{xij} = N_{vij}$, V_{vi}j or S_{vij}; r_{ij} is the distance between *a-v* axis and geometrical centre of the *j*th mesh chosen in the *i*th subarea. r_{ij} were measured to an accuracy of 16 μm using the assumed coordinate system based on the location of the ultrathin sections on the 400 mesh grids. Grids were previously measured with Biolar light microscope equipped with an ocular micrometer.

To evaluate the mean, total volume of cytoplasm of embryos at each stage, the stereological method called "nucleator" was used [8, 21]. In each of nine semi-thin section of *AS*, five intercepts were drawn from *ap* to the surface of the embryo. Thus, for each stage the set of 45 intercepts isotropically oriented in space were measured and mean, total volume of cytoplasm of embryos V(c) was derived using the formula:

$$V(c) = \frac{4}{3}\,\pi\,(\bar{l}_n)^3$$

where l_n is the summarised length of all fractions of the *n*th intercepts, which were contained in the cytoplasm and $\bar{l}_n$ is mean of l_n. Finally, total number of mitochondria per embryo was calculated:

$N(m) = V(c) \cdot N_v(m,c)$.

Values $N_v(m,c)$, $V_v(m,c)$, $S_v(ext,m)$ and $S_v(in,m)$ for oocytes and embryos at each stage of development were compared by analysis of variance (ANOVA) and using Tukey's intervals.

Results

Tables 1a and 2a present numerical densities of mitochondria $N_v(m,c)$ and volume densities of mitochondria $V_v(m,c)$ in relation to the whole cytoplasm of the oocyte or embryo for each stage of development during cleavage, from St.1 to St. 9 for *R . temporaria* and *B. bufo*, respectively. Additionally, mean total volumes of embryo cytoplasm V(c) and mean total numbers of mitochondria per embryo during cleavage are presented.

In general, the mean values of $N_v(m,c)$ for *R. temporaria* are higher than those for *B. bufo* at all developmental stages. The lowest values of $N_v(m,c)$ were found for St.1 and the highest for St. 9. In frog embryos, numerical density of mitochondria during cleavage increases from 5.73×10^{-2} μm^{-3} at St.1 to 10.51×10^{-2} μm^{-3} at St. 9 and in toad embryos from 4.03 to 9.74×10^{-2} μm^{-3}. The analysis of variance did not show any statistically significant differences between numerical densities $N_v(m,c)$ from St.1 to St. 6, both in *R. temporaria* and *B. bufo*, respectively, (Tab.1b and 2b). For *B. bufo*, one exception exists: difference between St. 1 and St. 6 is significant. Statistically significant differences occur later, between all means calculated for St. 7, 8 and 9 for both studied species: $N_v(m,c)$ increases as the cleavage goes on. Additionally, values of $N_v(m,c)$ at St. 7, 8 and 9 are significantly higher than those found for stages 1 to 6, (Tab. 1 and 2).

Volume density of mitochondria $V_v(m,c)$ in *R. temporaria* embryos does not change from St. 1 to St. 8 but increases for St. 9 and is significantly higher than at all others stages (Tab.1b). In *B. bufo* embryos, statistically significant differences between the means of $V_v(m,c)$ occur earlier, at St.8 (Tab. 2b).

The total number of mitochondria N(m) for *R. temporaria* and *B. bufo* embryos increases significantly

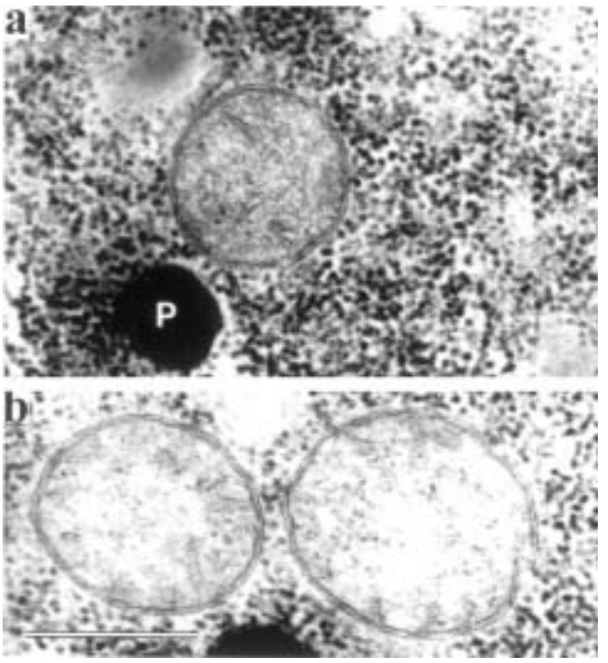

Fig. 2. Mitochondria in morula (St. 6) of *B. bufo* by TEM from: **a** - animal and **b** - vegetal hemisphere of the embryo. The cytoplasm contains many small glycogen granules. p - single pigment granule is seen. Bar = 0.5 μm.

during cleavage (Tab.1a and 2a) and is 2.5 times higher at St. 9 in comparison to N(m) calculated for St. 1. At the late blastula stage, single embryo contains almost 300 millions mitochondria.

Table 3 summarizes results for surface densities of external S_v(ext,m) and internal S_v(in,m) mitochondrial membranes per mitochondrial volume unit. There are no morphological differences, quantitative or qualitative, in structure of mitochondria, in the animal, equatorial and vegetal parts of *R. temporaria* and *B. bufo* embryo during cleavage (Fig. 2). One-way analysis of variance did not show statistically significant differences between means calculated for all stages under study in *B. bufo* embryos, for S_v(ext,m) and S_v(in,m) (Tab. 3a). In *R. temporaria* embryos, surface density of external mitochondrial membranes at St. 1 is significantly lower than S_v(ext,m) for St. 7 and 8, but S_v(in,m) increases at St. 9 only (Tab. 3b).

Discussion

Quantitative analysis of the mitochondrial ultrastructure may provide some clues to estimate the cell metabolic rate. It is obvious when we compare surface densities of inner mitochondrial membranes and energy demands in the different somatic cells: S_v(in,m) significantly increases when metabolism of the cell becomes higher [12]. A similar correlation also exists during changes of the V_v(m,c). Surface densities of inner mitochondrial membranes for *R. temporaria and B. bufo* eggs during the whole period of cleavage ranged from 5.43 m^2/cm^3 to 7.53 m^2/cm^3 whereas V_v(m,c) did not change more than by 1.65% . Thus, both parameters posses low values as compared to those measured in the "standard cells", *e.g.* rat hepatocytes - 40 m^2/cm^3 and 12%, respectively [9]. Additionally, the ratio of inner to outer mitochondrial membrane is very low, about 1 .2, (Fig. 2) whereas for other somatic cells, this ratio is as high as 5. Thus, in the light of the present data, the cleavage period up to midblastula transition may be considered as metabolically silent, and the embryo uses maternally inherited proteins or proteins translated during cleavage from maternally inherited mRNAs. Considering the low values of mitochondrial volume and surface densities, the embryos exhibit lower metabolism than somatic cells.

The shift in ultrastructural morphology of mitochondria towards that characteristic of more actively respiring organelles is seen at St. 9 for *R. temporaria*. In toad embryos, V_v(m,c) significantly increased from St. 8 whereas S_v(in, m) remained constant during the whole period of cleavage. These events can be correlated with the mid-blastula transition, when a faster increase in overall rate of metabolic activity occurs [3, 18, 32]. In the frog embryos, the changes in mitochondrial morphology start at the late blastula stage, *i.e.* near blastula-gastrula transition, at the moment when embryo starts producing new ribosomes [3, 6]. In contrary to the toad, both parameters V_v(m,c) and S_v(in,m) are significantly higher than those calculated for earlier stages of development. Remarkably, the quantitative differences in mitochondrial ultrastructure occur at different stages of embryonic development of *R. temporaria* and *B. bufo*. The possible explanation is that it results from temperature differences of environment in which embryos of both studied species develop, since temperature influences rates of chemical and biological reactions. Indeed, embryos of *R. temporaria* develop during the second half of March when temperature of water is lower than that for *B. bufo* embryos developing during the first half of April.

In the amphibians, dorsal-ventral gradient of different constituents such as mRNAs, ribosomes and mitochondria exist in the embryo, [6, 15, 24, 28, 32]. Thus, it was reasonable to expect that in the amphibian embryos uneven distribution of organelles or biomolecules which take part in different metabolic processes may serve spatial differentiation of energy production following spatial differentiation of mitochondrial ultrastructure [12, 25, 32]. It was surprising that during cleavage in the whole embryo of the studied species mitochondria posses high homogeneity of their ultrastructure. It is likely that spatial differentiation of energy demand is so small that structural changes of these organelles are not significant.

Table 1a. Numerical densities $N_V(m,c)$, volume densities $V_V(m,c)$ and total number of mitochondria N(m) per *R. temporaria* embryos. St: stage of development, V(c): total volume of embryo cytoplasm.

Rana temporaria				
St.	$N_V(m,c)$ ±SD $[\mu m^{-3}] \times 10^{-2}$	$V_V(m,c)$ ±SD [%]	V(c) ±SD $[mm^3]$	N(m) ±SD $\times 10^6$
1	5.73 ± 0.15	0.86 ± 0.11	2.00 ± 0.14	114.8 ± 8.5
2	6.23 ± 0.68	0.94 ± 0.08	2.17 ± 0.16	135.3 ± 17.6
3	5.84 ± 0.02	0.96 ± 0.12	2.14 ± 0.15	125.0 ± 8.5
4	5.46 ± 0.25	0.76 ± 0.09	2.25 ± 0.27	122.7 ± 15.9
5	6.25 ± 0.22	0.86 ± 0.03	2.37 ± 0.45	147.9 ± 28.3
6	6.86 ± 0.41	0.92 ± 0.02	2.40 ± 0.21	164.0 ± 19.7
7	9.42 ± 0.76	1.01 ± 0.05	2.43 ± 0.53	228.5 ± 53.3
8	9.52 ± 0.54	1.11 ± 0.10	2.42 ± 0.38	230.2 ± 38.1
9	10.51 ± 0.48	1.34 ± 0.13	2.78 ± 0.53	292.4 ± 57.6
F	27.23	5.34	–	–
TUK	1.85	0.37	–	–

F: value of F-distribution, TUK: length of the Tukey's confidence interval, SD: standard deviation. $F_{\alpha,f1,f2} = 2.51$: critical value of F where: $\alpha = 0.05$, $f_1 = 8$ and $f_2 = 18$.

Table 2a. Numerical densities $N_V(m,c)$, volume densities $V_V(m,c)$ and total number of mitochondria N(m) per *B.bufo* embryos. St: stage of development, V(c): total volume of embryo cytoplasm.

Bufo bufo				
St.	$N_V(m,c)$ ±SD $[\mu m^{-3}] \times 10^{-2}$	$V_V(m,c)$ ±SD [%]	V(c) ±SD $[mm^3]$	N(m) ±SD $\times 10^6$
1	4.03 ± 0.21	0.64 ± 0.05	2.66 ± 0.45	107.2 ± 18.8
2	4.72 ± 0.41	0.75 ± 0.05	2.71 ± 0.24	128.1 ± 15.9
3	5.14 ± 0.28	0.84 ± 0.06	2.86 ± 0.26	147.1 ± 15.6
4	4.53 ± 0.59	0.92 ± 0.04	3.02 ± 0.29	136.7 ± 22.2
5	5.55 ± 0.57	1.18 ± 0.10	2.87 ± 0.34	159.1 ± 24.9
6	6.28 ± 0.52	1.31 ± 0.13	2.91 ± 0.34	182.5 ± 26.1
7	7.24 ± 0.84	1.14 ± 0.07	2.85 ± 0.32	206.4 ± 33.4
8	8.47 ± 0.78	1.60 ± 0.08	2.78 ± 0.36	235.5 ± 37.3
9	9.74 ± 0.36	1.65 ± 0.10	2.81 ± 0.31	273.8 ± 31.6
F	18.85	29.81	–	–
TUK	2.21	0.33	–	–

F: value of F-distribution, TUK: length of the Tukey's confidence interval, SD: standard deviation. $F_{\alpha,f1,f2} = 2.51$: critical value of F where: $\alpha = 0.05$, $f_1 = 8$ and $f_2 = 18$.

Table 1b. Comparison of the mean values $N_V(m,c)$ and $V_V(m,c)$ calculated for all *R. temporaria* stages of development under study. Character "+" indicates that differences between the stages possess statistical significance.

Rana temporaria

$N_V(m,c)$									
St	1	2	3	4	5	6	7	8	9
1	-								
2	-	-							
3	-	-	-						
4	-	-	-	-					
5	-	-	-	-	-				
6	-	-	-	-	-	-			
7	+	+	+	+	+	-	-		
8	+	+	+	+	+	-	-	-	
9	+	+	+	+	+	+	-	-	-

$V_V(m,c)$									
St	1	2	3	4	5	6	7	8	9
1	-								
2	-	-							
3	-	-	-						
4	-	-	-	-					
5	-	-	-	-	-				
6	-	-	-	-	-	-			
7	-	-	-	-	-	-	-		
8	-	-	-	-	-	-	-	-	
9	+	+	+	+	+	+	-	-	-

Table 2b. Comparison of the mean values NV(m,c) and VV(m,c) calculated for all *B. bufo* stages of development under study. Character "+" indicates that differences between the stages possess statistical significance.

Bufo bufo

$N_V(m,c)$									
St	1	2	3	4	5	6	7	8	9
1	-								
2	-	-							
3	-	-	-						
4	-	-	-	-					
5	-	-	-	-	-				
6	+	-	-	-	-	-			
7	+	+	-	+	-	-	-		
8	+	+	+	+	+	-	-	-	
9	+	+	+	+	+	+	+	-	-

NV(m,c)									
St	1	2	3	4	5	6	7	8	9
1	-								
2	-	-							
3	-	-	-						
4	-	-	-	-					
5	+	+	-	-	-				
6	+	+	+	+	-	-			
7	+	+	-	-	-	-	-		
8	+	+	+	+	+	-	+	-	
9	+	+	+	+	+	+	+	-	-

Table 3a. Surface densities of external S_V(ext,c) and internal S_V(in,c) mitochondrial membranes for *R. temporaria* and *B. bufo* embryos. St: stage of development.

	Rana temporaria		*Bufo bufo*	
St.	S_V(ext,m) ±SD [m^2/cm^3]	S_V(in,m) ±SD [m^2/cm^3]	S_V(ext,m) ±SD [m^2/cm^3]	S_V(in,m) ±SD [μ^2/cm^3]
1	5.03 ± 0.29	6.58 ± 0.49	5.43 ± 0.28	7.08 ± 0.34
2	5.61 ± 0.18	6.60 ± 0.26	5.46 ± 0.23	7.07 ± 0.33
3	5.49 ± 0.31	6.60 ± 0.36	5.08 ± 0.19	6.78 ± 0.35
4	5.39 ± 0.24	6.62 ± 0.36	5.41 ± 0.17	7.17 ± 0.28
5	5.46 ± 0.16	6.66 ± 0.18	5.20 ± 0.13	6.93 ± 0.31
6	5.67 ± 0.15	6.76 ± 0.28	5.02 ± 0.17	6.38 ± 0.29
7	6.10 ± 0.25	7.24 ± 0.44	5.16 ± 0.16	6.64 ± 0.27
8	6.19 ± 0.12	7.31 ± 0.30	4.94 ± 0.13	6.14 ± 0.23
9	5.81 ± 0.14	7.53 ± 0.32	5.20 ± 0.13	6.80 ± 0.36
F	4.53	4.07	0.59	1.24
TUK	1.06	0.88	–	–

F: value of F-distribution, TUK: length of the Tukey's confidence interval, SD: standard deviation. $F_{\alpha,f1,f2} = 3.89$: critical value of F where: $\alpha = 0.01$, $f_1 = 8$ and $f_2 = 18$.

Table 3b. Comparison of the mean values S_V(ext,m) and S_V(in,m) calculated for all *R. temporaria* stages of development under study. Character "+" indicates that differences between the stages possess statistical significance.

Rana temporaria

S_V(ext,m)									
St	1	2	3	4	5	6	7	8	9
1	-								
2	-	-							
3	-	-	-						
4	-	-	-	-					
5	-	-	-	-	-				
6	-	-	-	-	-	-			
7	+	-	-	-	-	-	-		
8	+	-	-	-	-	-	-	-	
9	-	-	-	-	-	-	-	-	-

S_V(in,m)									
St	1	2	3	4	5	6	7	8	9
1	-								
2	-	-							
3	-	-	-						
4	-	-	-	-					
5	-	-	-	-	-				
6	-	-	-	-	-	-			
7	-	-	-	-	-	-	-		
8	-	-	-	-	-	-	-	-	
9	+	+	+	+	-	-	-	-	-

The estimated number of mitochondria is not less than 114.8 and 107.2 millions per mature oocyte of *R. temporaria* and *B. bufo*, respectively. These values are surprisingly high but comparable with data for *X. laevis* [13] and *R. temporaria* oocytes during oogenesis. Stereological results concerning the number of mitochondria are reliable in comparison to those obtained from measurements of mitochondrial DNA content in the oocytes [4].

The principal finding of the present study is that numerical densities of mitochondria N_v(m, c) for both studied species remain unchanged up to St. 6 and then progressively increase with statistical significance throughout St. 7, 8 and 9. Furthermore, the total number of mitochondria N(m) as well as total volume of embryo cytoplasm V(c) increase during cleavage. At St. 9, N(m) rises to 292.4 millions and 273.8 millions of mitochondria in *R. temporaria* and B. *bufo* embryos, respectively. Hence, our data contradict the common opinion [3, 6] that during cleavage the number of mitochondria does not change.

We know that the content of mitochondrial DNA remains constant during cleavage in amphibian embryos, as previously determined by hybridization of mtDNA with radioactive cDNA probes [4]. Moreover, in *X. laevis*, specific DNA-binding proteins are known to inhibit mitochondrial transcription [1]. Additionally, mitochondrial rRNA synthesis starts late at gastrula stage (St. 10) [32], whereas levels of other mitochondrial RNAs [4, 27] and mitochondrial DNA-encoded proteins [32] are unaltered during early development of amphibian embryos. In spite of the above facts it is possible that embryos use maternally inherited mitochondrial components to build new organelles. The total level of mtDNA could remain unaltered, while the number of mitochondria increases, since oocyte mitochondria are highly polyploid, containing over 100 genomes for each mitochondrion [2]. Thus, replication of mitochondria is likely if mitochondrial polyploidy decreases during the cleavage period. Another argument which can support the above thesis is the analysis of surface densities of external mitochondrial membranes S_v(ext, m). This parameter increases during St. 7 and 8 in the *R. temporaria* embryos but in *B. bufo* embryos it remains constant during cleavage. S_v(ext, m) increases when mitochondria become smaller, what could result from their replication.

On the other hand we did not observe any mitochondrion under division in the embryos of both species, at any stage during cleavage. Hence, we propose another interpretation of the increase in total number of mitochondria in the cleaving amphibian embryo. An increase in the number of mitochondria may not necessarily result from their division, *i.e.* production of new organelles. In many species, over 50% of oocytes population die by apoptosis before birth of females [17]. Later on, postnatal loss of oocytes is continued. Krakauer and Mirra [10]

have interpreted this phenomenon as removal of oocytes carrying mutant mitochondria. Indeed, it was proved that mitochondria can actually influence oocyte fate. Injection of small number of mitochondria, *i.e.* 5% of total mitochondrial pool per oocyte in FVB female mice caused a decrease in apoptosis rate from 70% for oocytes microinjected with buffer alone to 36% for those microinjected with purified mitochondria [19]. During cleavage, the situation cold be similar: embryos with higher number of mitochondria may reach later stage of development, *i.e.* do not die earlier. Thus, when we collected embryos at different stages of development, those at more advanced stages were larger in size and contained higher total number of mitochondria. In the present study the same pattern was observed for total volume of cytoplasm V(c) during cleavage, although it is known that in most species, especially in amphibians, there is no net increase in embryonic volume during cleavage [6].

These results suggest that in amphibian embryos during cleavage there exists a mechanism, which eliminates defective embryos containing insufficient number of mitochondria and nutrient substances that are necessary for the maintance of the embryonic development. If this is true, the percentage of surviving embryos should decrease progressively as cleavages proceed. Indeed, we observed that a progressively smaller fraction of developing embryos reached more and more advanced stages.

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References

[1] Allen JF (1996) Separate sexes and the mitochondrial theory of ageing. J Theor Biol 180: 135-140
[2] Billett MA (1979) Oocyte mitochondria. In: Maternal effects in development, Newth DR, Balls M [Eds], Cambridge University Press, London, pp 144-166
[3] Brachet J, Alexandre H (1986) Introduction to molecular embryology. Springer-Verlag, Berlin, Heidelberg, New York, London, Paris, Tokyo, pp 87-105
[4] Chase JW, Dawd IB (1972) Biogenesis of mitochondria during *Xenopus laevis* development. Dev Biol 27: 504-518
[5] Cruz-Orive LM (1978) Stereological analysis of spheroidal particles of variable size and shape. Pract Metallogr 8: 250-259
[6] Gilbert SF (1997) Developmental Biology. Sinauer Associates Inc, Massachusetts, pp 167-203
[7] Gundersen HJG (1977) Notes on the estimation of the numerical density of arbitrary profiles: the edge effect. J Microsc 111: 219-223
[8] Gundersen HJG (1988) The nucleator. J Microsc 151: 3-21
[9] Klyszejko-Stefanowicz L (1998) Cytobiochemia (in Polish). PWN Warszawa
[10] Krakauer DC, Mira C (1999) A mitochondria and germ-cell death. Nature 400: 125-126
[11] Laskey RA (1979) Biochemical processes in early development. In: Companion to Biochemistry 2, Bul AT, Thomsen JO, Tipton KF [Eds], Longman, London, pp 137-160
[12] Lovtrup RH, Nelson L (1982) Changes in mitochondrial respiration during the development of *Xenopus laevis.* Cell Differ 11: 125-133
[13] Marinos E (1985) The number of mitochondria in *Xenopus laevis* ovulated oocytes. Cell Differ 16: 139-143
[14] Michniewska-Predygier Z, Pigoń A (1957) Early developmental stages in *Rana temporaria* L., *Rana terrestris* ANDRZ., *Rana esculenta* L. and *Bufo bufo* L (in Polish). Stud Soc Sci Torunensis, Sect E (Zool) 3: 1-24
[15] Mignote F, Tourte M, Mounulou JC (1987) Segregation of mitochondria in the cytoplasm of *Xenopus* vitellogenic oocytes. Biol Cell 60: 97-102
[16] Mohri T, Ivonnet PI, Chambers EI (1995) Effect on sperm-induced activation current and increase of cytosolic Ca^{2+} by agents that modify the mobilization of $[Ca^{2+}]$. I. Heparin and pentosan polysulfate. Dev Biol 172: 139-157
[17] Morita Y, Tilly JL (1999) Oocyte apoptosis: like sand through an hourglass. Dev Biol. 213: 1-17
[18] Newport J, Kirschner M (1982) A major developmental transition in early *Xenopus* embryos. I. Characterization and timing of cellular changes of the midblastula stages. Cell 30: 675-686
[19] Perez GI, Trbovich AM, Gosden RG, Tilly JL (2000) Mitochondria and the death of oocytes. Nature 403: 500-501
[20] Raddatz E, Lovtrup RH (1986) Changes in activity of the regulatory glycolytic enzymes and the pyruvate-dehydrogenase complex during the development of *Xenopus laevis*. Exp Cell Biol 54: 53-60
[21] Romek M (1998) Spatial distribution of yolk platelets and fat droplets in oocytes and cleaving embryos of the common frog (*Rana temporaria*) and toad (*Bufo bufo*). Folia Histochem Cytobiol 36: 87-96
[22] Romek M, Krzysztofowicz E (2000) Utilization of the yolk platelets during early embryonic development of *Rana temporaria* and *Bufo bufo*. Folia Histochem Cytobiol 39: 283-291
[23] Rugh R (1962) Experimental embryology - techniques and procedures. Burges Publishing Company, Mineapolis
[24] Sardet C, McDougall A, Houliston E (1994) Cytoplasmic domains in eggs. Trends Cell Biol 4:166-172
[25] Thoman M, Gerhart JC (1979) Absence of dorsal-ventral differences in energy metabolism in early embryos of *Xenopus laevis.* Dev Biol 68: 191-202
[26] Vergonet G, Hommes F, Molenaar I (1970) A morphometric and biochemical study of fetal and adult rat liver cells, with special reference to energy metabolism. Biol Neonate 16: 297-305
[27] Webb AC, LaMarca MJ, Smith LD (1975) Synthesis of mitochondrial RNA by full-grown and maturing oocytes of *Rana pipiens* and *Xenopus laevis*. Dev Biol 45: 44-55
[28] Webb AC, Smith LD (1977) Accumulation of mitochondrial DNA during oogenesis in *Xenopus laevis*. Dev Biol 56: 219-225
[29] Weibel ER (1980) Stereological Methods. Vol 2: Theoretical Foundations. Academic Press, New York, Toronto, Sydney, San Francisco
[30] Whitaker M, Steinhardt R (1982) Ionic regulation of egg activation. Q Rev Biophys 15: 593-666
[31] Wyllie CC, Holwill S, O'Driscol M, Snape A, Haesman J (1985) Germ plasm and germ cell determination in *Xenopus laevis* as studied by cell transplantation analysis. Cold Spring Harbour Symp Quant Biol 50: 37-43
[32] Yost HJ, Philips CR, Boore JL, Bertman J, Whalon B, Danilchik MV (1995) Relocation of mitochondria to the prospective dorsal marginal zone during *Xenopus* embryogenesis. Dev Biol 170: 83-90

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