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Lipids during *Bufo arenarum* oogenesis

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Summary

The content and composition of phospholipids and triacylglycerols (TAGs) in *Bufo arenarum* oocytes in stages III and IV of their oogenesis were studied. The total amount of phospholipids in stage IV oocytes is 0.5-fold higher than in stage III oocytes. In both cases, the main phospholipids are phosphatidylcholine (PC) and phosphatidylethanolamine (PE). A striking observation concerns the high level of diphosphatidylglycerol (DPG) in stage III oocytes, which could be indicative of a relatively larger mitochondrial population with respect to other oogenetic stages. A net increase in sphingomyelin content was found during oogenesis. This fact could be related to the role of this phospholipid in the signal transductional pathways. In PC, palmitic (16:0), linoleic (18:2) and oleic (18:1) are the major fatty acids for both types of oocytes, while in PE the main acyl groups are 18:1, 16:0, arachidonic acid (20:4n6) and 18:2. PE is more unsaturated than PC and both phospholipids are more unsaturated in stage III oocytes than in stage IV oocytes. The amount of triacylglycerols is 0.3-fold higher in stage IV oocytes than in stage III oocytes. In both stages, the main fatty acids are 18:2, 18:1 and 16:0. During oogenesis, a significant increase in 18:1 and 18:3n3, and a decrease in 18:2 of TAG were found. The unsaturation index of TAGs from stage IV oocytes is higher than that from stage III oocytes. The TAG increase during oogenesis is consistent with the putative use of these lipids as a source of energy in embryo development.

Keywords: *Bufo arenarum* oocytes, Fatty acid composition, Oogenesis, Phospholipids, Triacylglycerols

Introduction

Amphibians have been considered a model in embryological research. In Argentina, *Bufo arenarum* is one of the most important representatives in the batrachiological fauna. It has a continuous sexual cycle and, because of this, oocytes can be obtained during different stages of development at any time of the year (Veldéz Toledo & Pisanó, 1980).

In amphibians, oocytes increase in volume during oogenesis. The macromolecules produced in the liver of the female are transported through the circulatory system to the ovary where they are incorporated into the growing oocytes as storage materials which are later used as a source of energy for the developing embryo (Wallace & Jared, 1969). The time of appearance of organelles and stored substances bears no consistent relationship to the progression of oocytes through the stages of oogenesis (Webb & Smith, 1977).

Based on a series of macroscopic and histological observations during an annual cycle, five stages of oogenesis in *Bufo arenarum* have been recognised according to their most significant features (Valdéz Toledo & Pisanó, 1980). Stage III or *late vitellogenesis* oocytes (600–1200 μm) are characterised by a diffuse clear brown pigmentation having an unpolarised appearance. During stage IV or *auxocytosis* oocytes (1200–1700 μm) pigments become unequally distributed between the animal and vegetal hemispheres. During stage V or full-grown prophase-arrested oocytes (1700–1800 μm) the animal–vegetal polarity is evident with a darkly pigmented animal hemisphere also characteristic of other amphibian oocytes (Dumont, 1972). The biochemical composition of stage V *Bufo arenarum* oocytes has been analysed in previous reports (Alonso *et al*., 1987, 1992; Caldironi & Alonso, 1996). Nevertheless, no biochemical characterisation of earlier oogenetic stages has been carried out to date.

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Lipids not only provide a source of nutrient to the cell but also play a vital role in modifying the physical properties and function of biological membranes, and they produce significant effects on cell–cell interactions, cell proliferation and transport (Stubbs & Smith, 1984). In addition, the activities of some membrane proteins depend on their immediate lipid environment, and the ability of hormone receptor complexes to bind to effector molecules may be modulated by the headgroup and fatty acid composition of membrane lipids (Hirata *et al.*, 1979; Galo *et al.*, 1981). The importance of lipids in the maintenance of normal cell function is therefore unequivocal. This paper is concerned with the content of phospholipids and triacylglycerols in *Bufo arenarum* oocytes in stages III and IV of their oogenesis. The fatty acid composition of the most abundant phospholipids was also studied and, for comparative reasons, full-grown prophase-arrested oocytes were analysed.

Materials and methods

Experimental system

Mature females of the toad *Bufo arenarum* (Hensel) were obtained commercially and maintained under laboratory conditions. The ovarian tissue was surgically removed and transferred to ND 96 solution (96 mM NaCl, 1 mM $MgCl_2$, 2 mM KCl, 5 mM HEPES; pH 7.4; Sigma, St Louis, MO). According to Valdéz Toledo & Pisanó (1980), late vitellogenesis (stage III), auxocytosis (state IV) and full-grown prophase-arrested (stage V) oocytes were isolated with watchmaker's forceps under a stereotaxic microscope and the follicular cells were released.

Lipid analysis

Lipid extraction from oocytes was carried out following Folch *et al.* (1957). Phospholipids were separated by two-dimensional thin-layer chromatography (TLC) on silica gel H according to Rouser *et al.* (1970). Neutral lipids were separated by monodimensional TLC on silica gel G using hexane/diethylether/acetic acid (40:60:1, by volume). Phospholipids were located after exposure of the plates to iodine vapour and quantitated by phosphorus analysis after digestion with perchloric acid (Rouser *et al.*, 1970).

For the determination of fatty acid composition, the lipids were separated by TLC as described above, and spots were visualised under ultraviolet light after spraying the plates with 2´7´-dichlorofluorescein in methanol. Fatty acid methyl esters were prepared according to the method of Morrison & Smith (1964) using boron trifluoride (14% in methanol; Sigma, St Louis, MO). They were subsequently purified by TLC using hexane/diethylether (95:5, by volume) on silica gel 60 plates prewashed with methanol/diethylether (75:25, by volume). Fatty acid analysis was carried out by temperature-programmed gas–liquid chromatography using 21:0 methyl ester as internal standard. Two glass columns (2 m × 0.2 cm i.d.) packed with 10% SP2330 on 80–120 Chromosorb WAW (Supelco, Bellefonte, PA) were connected to two flame-ionisation detectors operated in the dual-differential mode. The initial and final oven temperatures were 160 and 220 °C, respectively, and the rate of increase was 5 °C/min. Injector and detector temperatures were 220 and 230 °C, respectively. The carrier gas was N_2 (30 ml/min). Chromatograms were quantitated with a CDS-111 Varian integrator (Palo Alto, CA). Peaks were identified by comparing their retention time with that of the standard. This procedure led to a tentative identification of polyunsaturated fatty acids. The unsaturation index (UI) was determined as the sum of the percentages of individual unsaturated fatty acids times the number of double bonds.

During all procedures, the lipids were kept in an N_2 atmosphere. All organic solvents were of analytical grade.

Other analytical methods

Protein concentration was determined following Lowry *et al.* (1951) after extraction with 1 N NaOH using crystalline bovine serum albumin as standard. Data on the percentage phospholipid distribution in different ovogenetic stages were subjected to ANOVA and mean values were compared using Bonferroni *t* statistics. Another statistical analysis was carried out using Student's *t*-test. In all cases, the values represent the mean and standard deviation of the total number of samples indicated in each legend.

Results and discussion

The data collected in the present study provide the first detailed lipid analysis of *Bufo arenarum* oocytes in oogenetic stages III and IV. The content of polar and neutral lipids was determined and the fatty acid composition of phosphatidylcholine (PC), phosphatidylethanolamine (PE) and triacyglycerols (TAGs) was studied. Phospholipids are present in measurable quantities in the nanomolar range in the stages analysed (Table 1). The total amount in stage IV oocytes is 0.5-fold higher than in stage III oocytes. In both cases, the main phospholipids are PC and PE. In stage III oocytes, diphosphatidylglycerol (DPG) or cardiolipin is the third phospholipid, followed by phosphatidylinositol (PI) and phosphatidylserine (PS).

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Table 1 Phospholipid composition of stage III and IV oocytes

Phospholipids	Stage III oocytes	Stage IV oocytes
Phosphatidylcholine	98.99 ± 10.80	150.83 ± 24.04
Phosphatidylethanolamine	39.16 ± 8.69	50.71 ± 9.36
Diphosphatidylglycerol	14.99 ± 2.60	3.17 ± 0.43
Phosphatidylinositol	8.49 ± 0.45	23.97 ± 3.59
Phosphatidylserine	5.27 ± 0.92	5.67 ± 0.78
Phosphatidic acid	2.95 ± 0.27	0.91 ± 0.10
Sphingomyelin	2.61 ± 0.65	16.13 ± 2.86
Total	172.46 ± 17.27	251.40 ± 33.91

Phospholipids were isolated and quantitated as described in the Materials and Methods section. Results are presented as nmol per mg protein and are mean values ± SD from three independent samples.

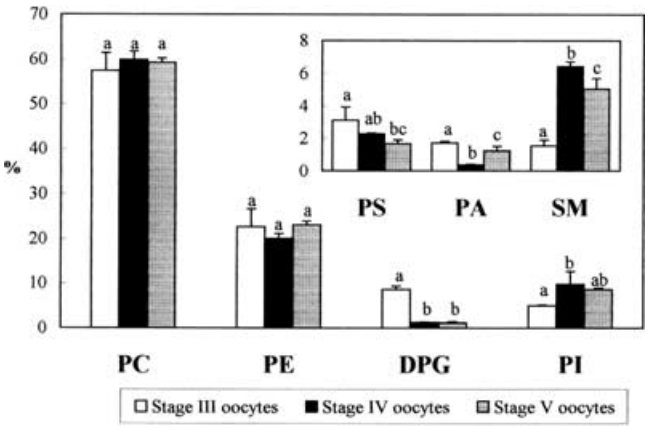


Figure 1 Phospholipid percentage in different ovogenetic stages. Results are mean values ± SD from three independent samples. Mean values are compared using Bonferroni *t* statistics. Within groups, the bars with different letters (*a–c*) are significantly different ($p<0.05$). PC, phosphatidylcholine; PE, phosphatidylethanolamine; DPG, diphosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; PA, phosphatidic acid; SM, sphingomyelin.

Phosphatidic acid (PA) and sphingomyelin (SM) are minor components. In stage IV oocytes, PI and SM are important constituents and represent 9.5% and 6.4% of the total phospholipids, respectively.

Lipidic characterisation of full-grown prophase-arrested *Bufo arenarum* oocytes (stage V) was carried out in a previous report (Caldironi & Alonso, 1996). Fig. 1 shows the percentage distribution of phospholipids in oocytes in stages III, IV and V. PC and PE comprise about 80% of the total amount in all analysed cases. In *Xenopus laevis* full-grown prophase-arrested oocytes, these two phospholipids also represent about 89% of the total phospholipid content (Alonso *et al.*, 1987).

The most striking observation in the present research is the high level of DPG in stage III *Bufo arenarum* oocytes (8.6%) with respect to stage IV and V oocytes (1.3% and 1.1%, respectively). The total phospholipids/DPG ratio registers significant increases during oogenesis. DPG is an exclusive inner mitochondrial membrane lipid. The high amount of DPG found in stage III oocytes might suggest a relatively larger mitochondrial population. This coincides with the mitochondrial proliferation reaching a peak in stage III *Xenopus laevis* oocytes, which remains constant until stage 30 of embryonic development (Dumont, 1972). It is important to note that according to their morphological features, the stage III oocytes in both amphibian species are comparable.

A net increase in SM content is registered in stage IV and V oocytes with respect to stage III oocytes. Several reports suggest an important role of SM in the signal transduction pathways (Igarashi, 1997; Hla *et al.*, 1999). In *Bufo arenarum* full-grown prophase-arrested oocytes, the maturation process induced by progesterone triggered a decrease in the amount of SM (Alonso, 2000). Also, in *Xenopus laevis* a brief treatment of defolliculated oocytes with sphingomyelinase was sufficient to induce maturation, and the microinjection of ceramide or sphingosine, potential products of SM hydrolysis, was capable of inducing germinal vesicle breakdown in the absence of progesterone (Strum *et al.*, 1995). The generation of ceramide from SM is probably part of the signal transduction pathway activated in response to progesterone, and the increase in ceramide is likely to be functionally important in the resumption of the meiotic cell cycle. The larger content of SM in stage IV and V oocytes with respect to stage III oocytes could be related to the above-mentioned role of this phospholipid in the maturation process that stage V oocytes may undergo.

The sum of choline lipids, SM and PC, comprises 59% of the total content in stage III oocytes while in stage IV and V oocytes they comprise about 65%. The PC/SM and total phospholipids/SM ratios in stage III oocytes are higher than the ratios found in stage IV and V oocytes. In many systems, both phospholipids constitute about half the total level, although the ratio of the amounts of these two components varies greatly (White, 1973). Among acidic phospholipids, the amount of PI increases during oogenesis changing the PI/PA ratio. PS shows a tendency to decrease during oogenesis. It is evident that the phospholipid profiles in stage IV and V oocytes are similar and that stage III oocytes have a different one. The fatty acid

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composition of PC and PE In stage III and IV oocytes is shown in Figs. 2 and 3. In PC, palmitic (16:0), linoleic (18:2) and oleic (18:1) are the major fatty acids, representing 68 mol% of the total fatty acid amount in stage III oocytes while they represent 85 mol% in stage IV oocytes (Fig. 2). It was found that during the ovarian oocyte growth, the levels of 16:0 and 18:2 increase significantly whereas a decrease in palmitoleic (16:1) and stearic (18:0) acids was observed. In full-grown *B. arenarum* and *X. laevis* oocytes, PC has the same abundant acyl groups (Alonso *et al*., 1987; Caldironi & Alonso, 1996). Homa *et al*. (1986) found high levels of 16:0 and 18:1 in PC from immature pig oocytes. In PE, the main fatty acids are 18:1, 16:0, arachidonic acid (20:4n6) and 18:2 in both oogenetic stages (Fig. 3). They comprise 56 and 65 mol% of the total fatty acids in stage III and IV oocytes, respectively. The amount of 18:0 and 18:1 undergoes a significant increase during oogenesis while the level of linolenic acid (18:3n3) decreases. Only in stage III oocytes is an important amount of 22:5n3 found. As regards 22:5n3 and 22:6n3, it is necessary to point out that the identification of polyunsaturated fatty acids (PUFA) based on chromatographic behavior compared with known standards is tentative. The role of these fatty acids in higher animals and plants has not been fully elucidated.

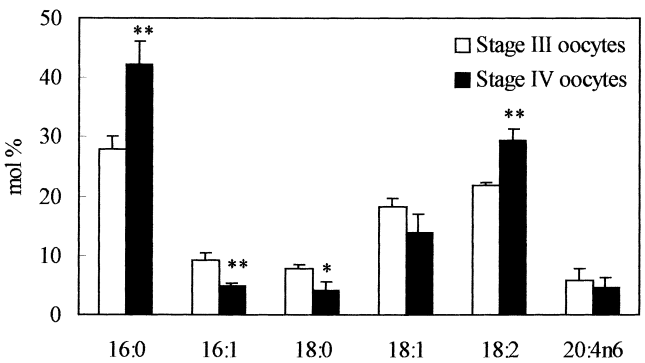


Figure 2 Percentage distribution of major fatty acids in phosphatidylcholine of stage III and IV oocytes. Results are mean values ± SD from three independent samples. Significant differences with respect to stage III oocytes: *(p<0.05), **(p<0.01).

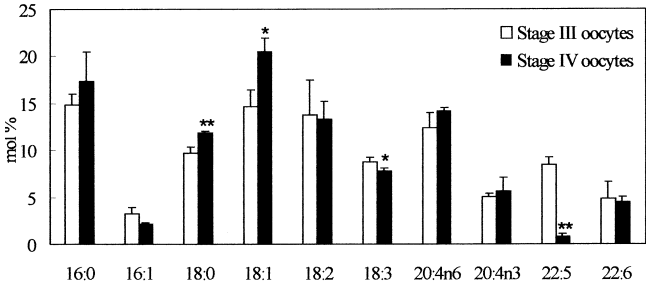


Figure 3 Percentage distribution of major fatty acids in phosphatidylethanolamine of stage III and IV oocytes. Results are mean values ± SD from three independent samples. Significant differences with respect to stage III oocytes: *(p<0.05), **(p<0.01).

The values of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), PUFA and unsaturation index (UI) in PC and PE from two types of oocytes are shown in Table 2. With respect to PC, in stage IV oocytes the amount of SFA is higher than in stage III oocytes while MUFA register a lower level. Then, the UI is minor. In PE, the PUFA content is lower in stage IV oocytes than in stage III oocytes. In conclusion, PE is more unsaturated than PC and both phospholipids are more unsaturated in stage III oocytes than in stage IV oocytes.

In *Bufo arenarum* full-grown oocytes, PE also has an important amount of 20:4n6 (Caldironi & Alonso, 1996). In unfertilised *Rana nigromaculata* eggs, PE with 20:4n6 at the 2-position was the most abundant among the molecular species (Ryuzaki, 1986). Many vital cell functions are intimately dependent on the type and extent of polyunsaturation in the membrane (Stubbs & Smith, 1984). Enrichment of arachidonic acid in sterified sites may be indicative of the capacity of oocytes to synthe-

Table 2 Fatty acids of phosphatidylcholine and phosphatidylethanolamine from stage III and IV oocytes

	Phosphatidylcholine		Phosphatidylethanolamine	
	Stage III oocytes	Stage IV oocytes	Stage III oocytes	Stage IV oocytes
SFA	37.43 ± 2.15	47.33 ± 4.92	25.42 ± 1.58	31.99 ± 3.33
MUFA	27.34 ± 1.37	18.62 ± 2.74	21.36 ± 1.09	22.69 ± 1.38
PUFA	35.23 ± 1.58	34.05 ± 3.53	53.23 ± 2.51	45.32 ± 2.32
UI	130.27 ± 7.60	100.79 ± 11.75	227.19 ± 6.60	187.73 ± 10.21

Results are shown as mean values ± SD (mol%) from three independent samples for SFA (saturated fatty acids), MUFA (monounsaturated fatty acids) and PUFA (polyunsaturated fatty acids).
UI, unsaturation index obtained as described in Materials and Methods.

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sise eicosanoids as prostaglandins, thromboxanes and leukotrienes and various hydroxyeicosatetraenoic acids and epoxyeicosatrienoic acids. Alternatively, arachidonic acid can be re-esterified into phospholipids by acyltransferases. It seems likely that arachidonic acid plays a second messenger role in the signal-transduction mechanisms (Naor, 1991; Sorbera *et al*., 2001). Recently, the idea that eicosanoids act in the biology of all animals has been proposed (Nor Aliza *et al*., 2001).

As regards neutral lipids, the content and composition of triacylglycerols were analysed in this study (Table 3). The total amount of TAG is 0.3-fold higher in stage IV oocytes than in stage III oocytes. In both types of oocytes the main fatty acids are 18:2, 18:1 and 16:0 followed by 18:3n3 and 16:1. They comprise 91% and 89% of the total fatty acids in stage III and IV oocytes, respectively. A significant increase in 18:1 and 18:3n3, and a decrease in 18:2, were found when stage IV oocytes were compared with stage III oocytes. In addition, a decrease at the level of 14:0 and 15:0 and an increase in 16:1, 20:4n6 and 22:5n3 were observed. Only in stage IV were significant levels of 14:1, 17:0 and 22:6n3 acids found. The UI of TAGs in stage IV oocytes is significantly higher than that of stage III oocytes. Caldironi & Alonso (1996) reported that TAG from full-grown oocytes has the same main acyl groups. Homa *et al*. (1986) have found that 16:0 and 18:1 are the principal fatty acids in immature pig oocytes.

Table 3 Fatty acid composition of triacylglycerols of stage III and IV oocytes

	Triacylglycerols (nmol FA/mg protein)		
Fatty acid	Stage III oocytes	Stage IV oocytes	% change
14:0	8.10 ± 0.03	3.08 ± 0.67**	–62
14:1	–	5.68 ± 0.95	–
15:0	5.31 ± 0.34	1.22 ± 0.21**	–77
16:0	93.28 ± 10.72	114.17 ± 12.82	n.s.
16:1	33.86 ± 9.89	58.16 ± 6.67*	+72
17:0	–	8.86 ± 0.62	–
17:1	5.20 ± 1.54	6.50 ± 0.50	n.s.
18:0	10.07 ± 6.06	10.84 ± 0.98	n.s.
18:1	94.61 ± 6.61	120.99 ± 9.24**	+28
18:2	149.24 ± 11.59	127.35 ± 6.75*	–15
18:3n3	32.00 ± 8.13	84.84 ± 6.86**	+165
20:4n6	4.66 ± 1.49	7.28 ± 0.59*	+56
20:4n3	4.69 ± 1.54	4.91 ± 0.98	n.s.
22:5n3	3.63 ± 0.98	7.21 ± 1.43*	+99
22:6n3	–	4.37 ± 1.05	–
Total FA	444.65 ± 13.06	565.48 ± 41.03**	+27
Total TAG[a]	148.22 ± 4.35	188.49 ± 13.68**	+27
SFA	116.77 ± 7.98	138.18 ± 14.21	n.s.
MUFA	133.66 ± 13.38	191.33 ± 16.53**	+40
PUFA	194.22 ± 2.42	235.97 ± 13.03**	+22
UI	131.32 ± 4.12	143.62 ± 3.81**	+9

Results are shown as mean values ± SD from three independent samples.
[a]Total amount of triacylglycerols fatty acids divided by three.
Significant differences with respect to stage III oocytes: *($p<0.05$), **($p<0.01$); n.s., not significant.
FA, fatty acids. Other details as in Table 2.

In general, there are no data on unusual fatty acids such as 14:0, 14:1, 15:0, 17:0 and 17:1 in different biological systems. The presence of these fatty acids may involve an oxidation mechanism different from β-oxidation. Furthermore, it may also be possible that some of these unusual fatty acids are synthesised from propyonylCoA.

The significant levels of 16:0, 18:1 and 18:2n6 found in neutral and polar lipids (Table 3, Figs 2 and 3) indicate that they may serve as a storage pool for metabolic precursors for fatty acid elongation and desaturation or that they may constitute a form of fuel via β-oxidation. In vertebrates, linoleic acid is an essential fatty acid that is important as a precursor to physiological C20 PUFAs. The latter are incorporated into phospholipids to serve as integral components of biomembranes and are also metabolised by two major oxygenation systems into biologically active products (Nor Aliza *et al*., 2001). TAG increase during oogenesis is consistent with the putative use of these lipids in supplying the energy required after fertilisation, when a dramatic decrease takes place (Alonso *et al*., 1986). This decrease is not accompanied by a parallel increase in diacylglycerols or free fatty acids, indicating that the hydrolysis of triacylglycerols is followed rapidly by the utilisation of triacylglycerol acyl groups in other metabolic reactions such as β-oxidation.

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