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FATE OF YOLK LIPID IN AN OVIPAROUS ELASMOBRANCH FISH, *SCYLIORHINUS CANICULA* (L.)

Francine WRISEZ, Henri LECHENAULT, Claude LERAY, Bernard HAYE & Jean MELLINGER

Laboratoires de Biologie Animale et de Biochimie, Faculté des Sciences, Université de Reims Champagne-Ardenne, B.P. 347, F-51062 Reims Cedex, France. - and (C.R.), Laboratoire d'Etude des Régulations Physiologiques, Centre National de la Recherche Scientifique, B.P. 37 CR, F-67037 Strasbourg Cedex, France. - Host marine laboratory: CNRS et Université de Paris VI, Station Biologique, F-29211 Roscoff, France.

Abstract

Analyses of total lipid, total cholesterol and phospholipid were carried out on freeze-dried oocytes, ova, external yolk sacs (EYS) including yolk, mid-term embryos, and newborns of the small spotted dogfish, *Scyliorhinus canicula* (L.). In the newborn, the liver, the spiral gut, the internal yolk sac (IYS), and the rest of the body ('carcass') were separated for weighing. Individual lipid data were obtained from oocytes, ova, EYS plus yolk, and carcasses, showing a large variation. Yolk contained about 20% lipid on a dry weight basis, 30-60% of these being phospholipid and about 5% cholesterol. An average 44% of total yolk lipid disappeared during development, while at birth the IYS still contained 10% lipid and the liver 26.5 % lipid with reference to the original yolk. Liver and carcass contained 47.4% and 31.3% of the remaining lipid, respectively. This shows that storage ranks second in the developmental utilization of yolk lipid by this model elasmobranch species, next to oxidation, or biotransformation, and before membrane construction. Cholesterol loss was 40%, phospholipid loss 71%. This indicates that phospholipids stored in the yolk are not merely precursors for membrane biogenesis, but are also used as a source of energy, and probably also as a source of phosphate and a source of materials for non-lipid biosynthesis.

INTRODUCTION

Several features of elasmobranch development are highly distinctive of this fish taxon with regard to teleosts. Fertilization is always internal. Sperms are stored in the nidamental glands of the oviducts, often for months or years, and ova are fertilized as they are passing through them. Polyspermy has been described in all well-studied species, and no micropyle has ever been observed in the very thin vitelline membrane which wraps the very large, brittle ova. The diameter of mature oocytes commonly ranges from one to six centimeters, according to species.

With some exceptions occurring among viviparous species (CAPAPÉ & al. 1990), ova must be mechanically protected during development by an egg envelope comprised of an internal, glycosaminoglycan jelly, and an external, tanned protein egg-case. These fish show a unique feature among Metazoa, consisting in the anticipated digestion of the jelly and opening of the egg-case by hatching gland enzyme(s), about mid-development: this event has been termed "pre-hatching". It allows oxygenated sea water to circulate freely around the embryo, which begins growing rapidly and will fill entirely the egg-case at term, at least in oviparous species.

In the small spotted dogfish, *Scyliorhinus canicula* (L.), an oviparous species that lives along African and European coasts of the Atlantic Ocean as well as the Mediterranean coasts, pre-hatching marks the beginning of yolk transfer from the external yolk sac (EYS) into the spiral gut, where its digestion starts, and will be ended only one week after birth. Since the gut cannot accommodate all the yolk and the EYS has to be resorbed completely before birth, an internal yolk sac (IYS) develops, playing the role of a transient storage organ within the abdominal cavity. Not all elasmobranchs have an IYS, but this is very common, and *S. canicula* is appropriate as a model for the whole taxon (MELLINGER & al. 1986).

The thin EYS wall is vascularized by a dense capillary bed fed by a vitelline artery and drained by a vitelline vein, as in other meroblastic, telolecithal eggs. The main function of these blood vessels seems to be respiration, because they appear prior to the gills themselves. The wall of the EYS contains all embryonic sheaths: ectoderm, mesoderm, endoderm, and also a permanent layer of yolk syncytium. The EYS wall is an extraembryonic organ in every aspect: i) it lies outside the embryo, and ii) it finally disappears by resorption. This is in sharp contrast with the yolk sac of teleosts, which i) has no true vitelline blood system (and even no vessels at all, in marine larvae), ii) is only formed of the yolk syncytium, without any endoderm around it, iii) has no connection with the gut lumen, and iv) becomes resorbed within the abdominal cavity, whilst the surrounding parts of the skin and the mesoderm persist, giving the ventral wall of the trunk.

Yolk can be defined as an implement of egg inclusions which are needed for growth and maintenance of embryos and early post-embryonic stages such as elasmobranch newborns, which have a direct development, or the larvae of most teleosts having an indirect development. In most anamniote vertebrates, the major part of these microscopically visible materials comprises yolk platelets, which contain crystalline assemblies of proteins and lipids (reviewed by BYRNE & al. 1989). Yolk platelets persist during development until they are digested one by one, except in many teleosts where they are fusing into a single mass of liquid yolk at the end of vitellogenesis. More or less abundant oil droplets or globules, and glycogen, are also present in egg hyaloplasm.

Besides yolk, solute biomolecules may also play an important role in embryonic growth, and this has been well demonstrated for free amino acids in marine teleost eggs (FYHN & HAHNENKAMP 1986; FYHN & al. 1987; FYHN & SERIGSTAD 1987; FYHN 1990). Other solutes are readily (electrolytes) or may be (dissolved organic material) taken up by the embryo integument from sea water, and environmental water itself is similarly absorbed during development, except perhaps in eggs from the marine telosts, which already absorb considerable amounts of water prior to ovulation (refs. in SELMAN & WALLACE 1989; McPHERSON & al. 1989).

Among nutrients, lipids have the highest energy contents, and as such they provide eggs with the more space-saving yolk constituent. Phospholipids are only stored in yolk platelets, whilst neutral lipids are partitioned between platelets and oil droplets. Compared to other biomolecules, lipids are particularly interesting to investigate because they can be used by the embryo in at least three different ways: i) energy production by oxidation, CO_2 being the main output, ii) building cell membranes, mainly by using the phospholipid moiety, and iii) storage, with regard to the physiological needs of early, non-feeding or poorly feeding post-embryonic stages.

We determined dry weights by freeze-drying and we analyzed total lipid, phospholipid, and total cholesterol in the individual yolk at the beginning of developement in *S. canicula*. In order to encompass the natural variability, this material was obtained from different, wild females. Eggs were incubated under controlled environmental conditions, and similar analyses were performed at pre-hatching and birth. Owing to its fairly large size, the newborn dogfish can be easily dissected. Two internal storage organs were studied, viz. the internal yolk sac (IYS) and the liver, which was fully developed at birth. The spiral gut has also been dissected, weighed, and analyzed for its lipid contents after pooling, while the

remainder of the body, termed 'carcass' in the present paper, was processed separately in every newborn, thus gaining another view on natural variability in this population, at birth.

Several questions have been addressed. What is the lipid composition of dogfish egg yolk ? How is yolk lipid partitioned between the IYS, the gut, the liver, and the carcass ? What part of total lipid is used as an energy source during dogfish development ? Are phospholipid and cholesterol wholly conserved, or partly consumed ? It will be shown that, even if gross estimates can be obtained, particularly for total lipid, individual variation is inexpectedly large in some cases. Fresh weight and dry weight data have already been given in another paper (LECHENAULT & al. 1993). Preliminary results on yolk lipids have already been published before (MELLINGER & al. 1988).

MATERIAL AND METHODS

Material

All the dogfish, *Scyliorhinus canicula* (L.), material used was taken from an Atlantic population of the species, which is biometrically very different from Mediterranean populations (MELLINGER & al. 1984). The Biological Station, Roscoff, France, which provided the fishes and their eggs, generally obtains dogfishes from a small, local long-line fishery in Le Diben, to the north of Morlaix, Brittany, along the southern coasts of the English Channel.

Oocytes were extracted from the ovaries of two freshly killed females at the Biological Station. Ova, i.e. freshly ovulated and fertilized oocytes with still incomplete egg-cases, were extracted from the anterior sections of both oviducts in similarly killed females. Every oviduct contained only one ovum. Encapsulated egg pairs were extracted from the caudal sections of the oviducts, either by dissection, or by pulling egg-case tendrils which appear through the cloaca of dead females at the fishery. They were shipped to Reims and incubated at 14-16°C in artificial sea water (S = 35‰; details in MELLINGER & al. 1986). Temperature in the natural environment was 10.5-12.5°C. Mortality was seldom during incubation, and no abnormalities occurred.

The embryos and newborns were immobilized with tricaine methane sulfonate (MS 222, Sandoz) before processing them by freezing (-30°C) for chemical analysis. Detailed procedures for opening the egg-case and for separating the different parts of the egg and organs of newborns have already been described elswhere (MELLINGER & al. 1984, 1986).

Chemical analyses

Weighing and freeze-drying of the biological material was performed in polypropylene tubes (n°2059, Falcon, with caps), which are keeping constant weights to the nearest 0.1 mg in varying environments and during extended time periods. Dry weight was obtained by repeated freeze-drying until the weight remained constant. The carcasses of newborns were trimmed with scissors after the first drying session, in order to speed up drying. Membranes enclosing large yolk masses were broken for the same purpose.

Total lipid was extracted and purified according to the method of FOLCH & al. (1957) and weighed at the nearest 0.01 mg. Results are expressed as total lipid percent of the dry weight of the various materials.

Phospholipid weight, expressed as a percent of total lipid weight, was estimated by measuring phosphorus contents spectrophotometrically, according to the procedure described by ROUSER & al. (1970).

Total cholesterol weight, expressed like phospholipid, was measured by a modified method, as follows: weigh about 10 mg lipid in a tight tube; add 1 ml 5% KOH solution in methanol; heat during 2 h at 90°C; cool; add 1 ml H2O, and 2 ml

petroleum ether; extract cholesterol, sample the 2 ml petroleum ether; dry; dissolve in 1 ml chloroform; take a 100 µl-aliquot, and mix in a vortex with 1 ml of 1%. (1 g l^{-1}) FeCl3 solution in ethyl acetate; add 0.8 ml concentrated H2SO4; vortex; allow cooling during 30 minutes; measure optical density at 550 nm, comparing with a standard curve from 0 to 30 µg.

The reproductibility of phospholipid and cholesterol concentration measurements was controlled with duplicate aliquots. Seventy-eight oocytes, ova, and EYS with yolk were analyzed, and 43 newborns.

RESULTS

Chronology of development and biometrical data

Pre-hatching occurred at 85-115 days, at a total length of about 40 mm, and hatching occurred at 170-220 days after egg extraction, the length of newborns being around 107 mm. At 100-106 days, the dry weights of 19 embryos taken just before pre-hatching were about 0.020 g. At 107-111 days, the dry weights of 10 pre-hatched embryo varied from 0.030 to 0.035 g. This was negligible, compared to 0.8-1.5 g of dry yolk still available. The dry weight of EYS wall tissues was less than 0.01 g in these embryos. Thus, it can be supposed that the amount of yolk used in respiration, whence CO_2 loss, was negligible during the first half of development.

At birth, a variable amount of yolk remained within the IYS, and a further, much lesser amount, within the spiral gut lumen. The dry liver averaged 0.12 g, the dry spiral gut 0.022 g, yolk contents being estimated as the half of the latter.

Newborn fresh weights were generally higher than those of ova because they contained more water (74.0-76.5% in carcasses), but dry weights were lower. Yolk utilization, as described through dry weight changes, can be summarized as follows: an average 1.2 g-ovum gave an average 0.95 g-newborn, so there was a loss of 20% from yolk dry matter.

Total lipid

There was about 20% total lipid in yolk (oocytes, ova, EYS plus its yolk at start or at pre-hatching), on a dry weight basis (range: 15.2-26%; Fig. 1). There were no differences between egg batches, nor among the successive stages of oogenesis and embryonic development. The IYS were grouped into five pools according to their weights. Total lipid weighed in extracts from IYS pools (range: 18.5-22.2%) and five pools of guts (range: 19.6-30.0%) fell into the range indicated above for yolk, taking into account the presence of both yolk and tissue lipid in the gut.

One pool of whole embryos analyzed about pre-hatching time contained 11.2% total lipid. Individual carcasses of the newborns yielded about 6% lipid (range: 3.6-7.8%; see Fig. 1). Four pools of newborn livers contained 50.2-54.4% lipid.

Table 1 shows the partition of lipids at birth and gives a comparison of lipid amounts in newborn vs yolk. During development, about 44% of total lipid was oxidized or used in the biosynthesis of other biomolecules.

Phospholipid

The percentage of phospholipid in total lipid showed tremendous variation whenever individual measurements were made (Fig. 2). Data distributions were far from the gaussian model, so it would be meaningless to give standard deviations, and calculated means have to be used cautiously. Yolk, including that from within IYS, contained about 43% phospholipid (range: 31.7-59.0%). In extracts from the guts, lower values were obtained (range:21.7-28.6%; mean: 24.6%), which would perhaps indicate that phospholipids are more readily absorbed than neutral lipids.

Pre-hatching embryos contained 39.9% phospholipid in their lipids. Figures for newborn carcassess were 38.5% (range:29-61%). There was very scant

phospholipid in liver lipid (range:3.2-4.5%; mean: 3.9%). Using mean total lipid weights as shown in Table 1, a loss of approximately 71% can be computed for phospholipid. Such a large figure shows that phospholipids are not only used in membrane biogenesis, but also oxidized as a metabolic fuel, used a a source of phosphate, and perhaps also as a source of carbon chains for the biosynthesis of other biomolecules during development.

Total cholesterol

Cholesterol weight (Fig. 3) was about 5% of total lipid in the yolk (range: 1.8-8.8%, but only 3.0-5.5% for five IYS pools). Higher values were obtained for spiral guts (4 pools: 12-15%; mean: 14%). Pre-hatching, whole embryos contained 6.5% cholesterol, which approximates the figures obtained with newborn carcasses: 2.2-16.1% (mean: 7.8%). Livers contained about 3.3% cholesterol (range: 0.9-4.3% for the four pools analyzed).

Using again the total lipid weights of Table 1, the total weights of cholesterol can be computed from these percents, both in the original yolk and in the newborn. We found 0.012 g and 0.00719 g, respectively, so a 40% cholesterol loss, approximately, occurred during development.

DISCUSSION

We confirm a previous estimate of total lipid consumption during *S. canicula* development, which was 40-50% (MELLINGER & al. 1988). Our more accurate estimate is now 44%. Clearly, yolk lipids are used as a fuel, besides their role in membrane building. The true amount of lipid used in respiration cannot be computed from our measurements, since lipid biosynthesis has not been studied. In the chick embryo, 90% of the metabolic energy is provided by lipids (NOBLE 1987).

Yolk plus embryo probably form a closed chemical system with regard to biomolecules. A total digestibility of yolk is secured during the development of Scyliorhinus canicula by an occlusion of the rectal lumen, which persists until birth (MELLINGER & al. 1986, 1987). Carbon diffusional losses are most probably limited to CO2 and urea, and they have already been estimated by proximate analyses of C and N (MELLINGER & al. 1986; MELLINGER & WRISEZ 1989). Urine can only be expelled from the embryo when the cloacal slit opens, at pre-hatching. Urea may also be excreted through the gills, which develop earlier, but this important osmolyte appears to be largely conserved (MELLINGER & al. 1986).

Cholesterol loss was of the same order of magnitude than total lipid loss (-40%). Bile secretion begins just before pre-hatching (MELLINGER, unpublished), and bile steroids are probably recycled if one considers the presence of the rectal occlusion. We have presently no explanation for this cholesterol deficit.

The large (-71%) phospholipid loss agrees with previous observations on teleost development (TOCHER & al. 1985 a, b). These have shown that yolk phospholipids are not only brickstones for membrane biogenesis within embryos and larvae, but also a source of energy, particularly phosphatidyl choline, which is the dominant phospholipid class. However, phospholipids may also be used as additional sources for phosphate, since phosphate is a rare electrolyte in sea water and is probably needed in large amounts by huge embryos like those of elasmobranchs. This point will deserve further investigations. Another possibility is that phospholipids are also used as a additional source of materials for the biosynthesis of other biomolecules during development, even if amino acids derived from yolk proteins seem to be more appropriate candidates for that purpose. A complete biochemical picture of yolk utilization will only be produced when all yolk and newborn constituents have been fully analyzed, and the activity of the metabolic pathways been explored. Studies on the utilization of lipids for constructing the main organs, particularly myelin sheaths in the central nervous system, would be also useful and they seem within reach in this biological material.

We also showed that about 10% of original yolk lipid remained stored in the IYS at birth. The gut contained less than 2%, on the way of being digested. The liver of the newborn typically contained 26.5% lipid with reference to the original yolk, and the carcass 17.5%.

When referring to lipid still present at birth time, their partition was 47.4% in the liver and 31.3% in the carcass, and this better enlightens the outstanding storage function of the liver during the perinatal period. However, some experiments (MELLINGER, unpubl.) have shown that the fasting young dogfish reaches a "point of no return" in a few days. The IYS then disappears after one week, and liver stores also seem to be depleted during this fast. The story of IYS and liver depletion remains to be studied in normally growing newborns. These stores can only allow the wild dogfish a short training period in the hunting of its natural preys, but this is probably vital to it.

Liver stores were probably composed of triacylglycerol for the most part, as cholesterol and phospholipid percents were very low in this organ. Thus, this store probably corresponds to the adult condition, and may be considered as a stable, renewable one, whereas IYS and gut stores are only transitory. The function of liver oil is metabolically fundamental in elasmobranchs, since fatty acids cannot be oxidized outside the liver (see SINGER and BALLANTYNE 1989). It also plays a role in regulating buoyancy.

In the vertebrates, lipids and their digestion products may be conveyed from the yolk to the various organs under two forms: i) lipoproteins, ii) free fatty acids. It is now admitted that free fatty acids cannot be transported by the blood plasma of elasmobranchs, in which the usual transporting, albumin-like protein is either lacking or non functional (FELLOWS and HIRD 1981). If yolk lipid is mobilized by the EYS or the syncytial layer, it is most probably transported as lipoprotein. In other vertebrates, secretion of lipoproteins is effected by enterocytes and hepatocytes. In the trout, lipoproteins of the VLDL type are also produced by the yolk syncytium (VERNIER & SIRE 1977). These secretory activities have not yet been described in elasmobranchs.

In conclusion, yolk lipids were largely consumed during dogfish development, particularly phospholipids which did not merely participate in membrane biogenesis, but also in the maintenance of the embryo body. More than 10% yolk lipid remained untouched at birth, as a store for growth and maintenance during the first week of free living. About 25% were conserved as triacylglycerol in the liver of the newborn. Finally, only a minor part was incorporated into the remainder of the newborn body.

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FIGURE LEGENDS

Fig. 1. Statistical distributions of total lipid weight (expressed as percent of dry weight) in newborn 'carcass' (NBC) and egg yolk (YOLK). n = number of individuals. Class width is indicated by the graduations on X-axis.

Fig. 2. Statistical distributions of phospholipid weight (expressed as percent of total lipid weight) in newborn 'carcass' (NBC) and egg yolk (YOLK). n = number of individuals. Class width is indicated by the graduations on X-axis.

Fig. 3. Statistical distributions of total cholesterol weight (expressed as percent of total lipid weight) in newborn 'carcass' (NBC) and egg yolk (YOLK). n = number of individuals. Class width is indicated by the graduations on X-axis.

Table 1

Total lipid at start and end of development

(average values)

	Start	Newborn			
	Yolk	Yolk	Gut	Liver	'Carcass'
Dry weights, in g	1.2	.12	.O22	.12	.7
Lipid, in g	.24	.024	.0044	.064	.042
Lipid, %	20	20	20	53	6
Estimated % of :					
- original lipid	100	10	1.8	26.5	17.5
- remaining lipid	-	17.9	3.2	47.4	31.3
% of original lipid	100	56			
lipid, in g	.24	.134			