



Review

Egg carotenoproteins in neotropical Ampullariidae (Gastropoda: Arquitaenioglossa) ☆

H. Heras *, M.S. Dreon, S. Ituarte, R.J. Pollero

Instituto de Investigaciones Bioquímicas de La Plata, CONICET-UNLP, La Plata, Argentina, Facultad de Ciencias Médicas - La Plata, Argentina

Received 5 April 2006; received in revised form 5 September 2006; accepted 3 October 2006

Available online 24 January 2007

Abstract

Carotenoid-binding proteins are commonly found in invertebrates. Their carotenoids form non-covalent complexes with proteins giving tissues a variety of colors. In molluscs they have been described in only a few species. In particular, the egg perivitellin fluid of those Ampullariid species which deposit eggs above the waterline is provided with carotenoproteins playing several roles ranging from photoprotection, antioxidant or antitrypsin actions to nutrient provision for development. These molecules form complex glyco-lipo-carotenoproteins of high molecular weight where either free astaxanthin (3,3'-dihydroxy-β, β'-carotene- 4,4'dione) or astaxanthin esterified with fatty acids, occur more frequently. This review compiles the current knowledge on the biochemical composition and biophysical data on the chemical and thermal stability of egg carotenoproteins in ampullariid. In addition, recent data on their metabolism, their cellular site of biosynthesis during perivitellogenesis, as well as their carotenoid binding properties are reviewed, highlighting the physiological significance of carotenoproteins in the context of the reproductive biology of these molluscs.

© 2007 Published by Elsevier Inc.

Keywords: Antioxidant; Asolene; Apple snail; Carotenoid; Mollusca; Perivitellin; Pomacea; Ovourubin; Lipoprotein; Embryogenesis

Contents

1. Introduction 159
2. Nature of the complexes 160
2.1. Protein moiety 160
2.2. Lipid moiety 161
2.3. Carbohydrates 161
2.4. Carotenoids 161
3. Structural aspects 161
3.1. Binding of astaxanthin 161
3.2. Structure and stability 162
4. Synthesis of carotenoproteins in females 162
5. Functional aspects 163
5.1. Source of nutrients during embryogenesis 163

☆ This paper is part of the 4th special issue of CBP dedicated to The Face of Latin American Comparative Biochemistry and Physiology organized by Marcelo Hermes-Lima (Brazil) and co-edited by Carlos Navas (Brazil), Rene Beleboni (Brazil), Rodrigo Stabeli (Brazil), Tania Zenteno-Savín (Mexico) and the editors of CBP. This issue is dedicated to the memory of two exceptional men, Peter L. Lutz, one of the pioneers of comparative and integrative physiology, and Cicero Lima, journalist, science lover and Hermes-Lima's dad.

* Corresponding author. INIBIOLP (CONICET-UNLP), Facultad de Ciencias Médicas, 60 y 120 s/n, 1900 - La Plata, Argentina. Tel.: +54 221 4824894; fax: +54 221 4258988.

E-mail address: h-heras@atlas.med.unlp.edu.ar (H. Heras).

5.2.	Protein targeting and other suggested functions for saccharide moiety	163
5.3.	Protective functions of carotenoproteins.	163
5.3.1.	Antioxidant.	164
5.3.2.	Photoprotective action	164
5.3.3.	Carotenoid stabilization/protection	164
5.3.4.	Warning coloration	165
5.3.5.	Prevention of desiccation.	165
6.	Future directions	165
	Acknowledgements	165
	References	165

1. Introduction

The family Ampullariidae has a worldwide distribution. Commonly referred to as “apple snails”, these freshwater aquitaenioglossa are exceptionally well adapted to both temperate regions, where thermal changes during the year are large (Albrecht et al., 1999, 2005) and to subtropical and tropical regions where periods of drought may alternate with periods of excessive rainfall (Pizani et al., 2005). These adaptations are reflected in their noteworthy anatomical, physiological and ecological features. Among them is the strategy of some ampullariid genera (Fig. 1) to deposit their eggs above the waterline in a calcareous, colored clutch. This remarkable egg-laying strategy, very unusual for an aquatic invertebrate, was probably developed to protect their eggs against predation by fish and other water inhabitants (Turner, 1998), but at the same time it exposes the eggs to sunlight and desiccation. To cope with these harsh conditions ampullariids have developed some fascinating mechanisms at the biochemical level, where carotenoid pigments forming complexes with proteins play a central role.

Many of the more than 700 carotenoids found in nature are present in invertebrate species that display colored tissues. They are usually non-covalently bound to proteins forming carotenoproteins that are primarily found in two body areas: adult surfaces (exoskeleton and epidermis) and reproductive structures (gonads and eggs) (Cheesman et al., 1967). The ovaries

and egg yolk carotenoproteins usually form a water-soluble complex, and frequently, associated with sugars and lipids, forming glyco-lipo-carotenoprotein particles (Zagalsky, 1985). One particular carotenoid, astaxanthin (ASX; 3,3'-dihydroxy- β , β' -carotene-4,4'dione) is most usually isolated from invertebrate carotenoproteins, including Ampullariidae (Fig. 2). ASX is a good example of a carotenoid that can cover the complete visible absorption spectrum by forming complexes with different proteins that usually have no sequence homology among them. From an evolutionary point of view, it is remarkable that such complex coloration mechanism developed from the astaxanthin-protein interaction (Zagalsky, 1985).

Carotenoproteins have been studied in several invertebrate phyla, mostly Porifera, Cnidaria, Arthropoda and Echinodermata (Britton et al., 1982; Cheesman et al., 1967; Zagalsky, 1985; Zagalsky et al., 1990). Mollusca, however, have been given much less attention and except for ovorubin, the well studied carotenoprotein from *Pomacea canaliculata*, there are few reports on the presence of carotenoproteins in other molluscs, namely (a) Polyplacophora: the hemolymph of *Cryptochiton stelleri* (Allen, 1977), (b) Bivalvia: muscle of *Mytilus edulis* (Yang et al., 1994), extracts from whole body of *Unio pictorum* (Czeczuga, 1983), gonads of *Volsella modiolus* (Euler et al., 1934) and ovaries of *Pecten maximus* (Ceccaldi and Zagalsky, 1967; Zagalsky, 1972) and (c) Gastropoda: mantle of *Cerithidea californica* (Nakadal, 1960), ovary of *Patella vulgata* (Goodwin and Taha, 1950) and skin of *Flam-bellina iodinea* (Cheesman et al., 1967).

During vitellogenesis the main components of the egg vitellus such as lipids, proteins and carbohydrates are synthesised. Vitellus proteins are named vitellins (Wallace et al., 1967), and have different origins. In most vertebrates, they are usually synthesised in the liver, released to circulation as precursor particles called vitellogenins (Vg), taken up by the ovary and incorporated into the developing oocyte (heterosynthetic mechanism). In most insects and some crustaceans this mechanism is similar to that in vertebrates, while in other crustaceans and some fish it has been reported that the ovary is involved in this process (autosynthetic mechanism) though some may have both vitellogenic mechanisms (Fainzilber and Zlotkin, 1992; Kanost et al., 1990; Lubzens et al., 2003). In comparison, little is known about this process in molluscs. In cephalopods (de Jong-Brink et al., 1983) and some gastropods (Barre et al., 1991; Bride et al., 1992) heterosynthesis seems to

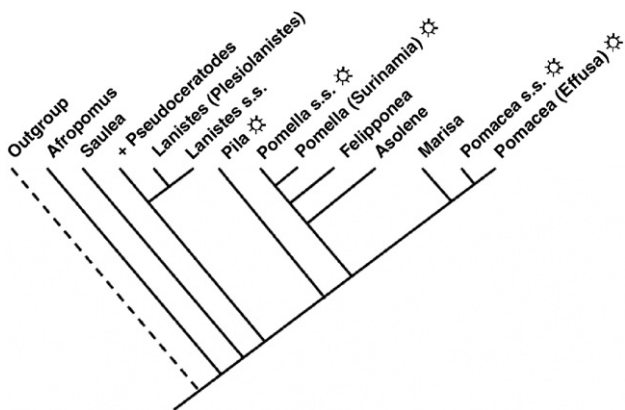
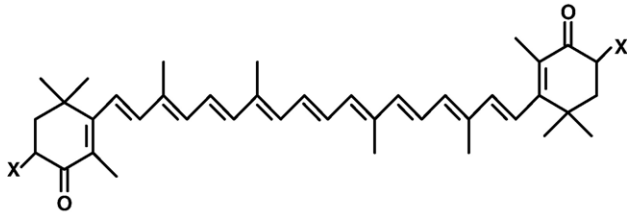


Fig. 1. Phylogenetic tree of the Ampullariidae (according to Berthold, 1989). ☆: Genus with aerial egg-laying.



Substituent (X):

-OH : Astaxanthin

-OR Astaxanthin esters (R = 16:0 and 18:1, see "Nature of the complex" for details).

Fig. 2. General formula for known carotenoids in naturally occurring carotenoproteins from *Pomacea* spp.

be the main yolk forming process. Gastropod vitellins have been described in the oocytes (Barre et al., 1991; Bottke, 1986; Miksys and Saleuddin, 1986).

Unlike this general mechanism, most gastropod eggs have a perivitellin fluid (PVF) surrounding the fertilized oocyte, that is mainly synthesised by an accessory gland of the female reproductive tract, the albumen gland. PVF represents the major supply of nutrients during embryogenesis (de Jong-Brink et al., 1983). Therefore, proteins present in PVF are termed perivitellins.

In this paper the progress made in the composition, synthesis and functions of the glycolipocarotenoproteins from the egg perivitelline fluid of Ampullariidae is reviewed. The carotenoprotein ovorubin from *P. canaliculata* is the only gastropod carotenoprotein and perivitellin for which information on structure and biosynthesis is available. Therefore it will be given special attention.

2. Nature of the complexes

The presence of a red pigment forming a carotenoid-protein complex in Ampullariidae was first reported in the eggs of *P. canaliculata* by Comfort (Comfort, 1947, 1949). This highly water soluble complex was later isolated by Cheesman (1954) and called ovorubin by analogy with ovoverdin, the green carotenoprotein of lobster eggs (Cheesman, 1956). In those early descrip-

tions proteins and glucid forming a glyco-carotenoprotein were found, but no lipids were reported (Cheesman, 1958). After these research, gastropod carotenoprotein study was virtually neglected until the late 90's when our group began to investigate the PVF biochemistry of *P. canaliculata*.

2.1. Protein moiety

Using a combination of gradient ultracentrifugation and HPLC, three lipoprotein fractions were purified from the PVF of *P. canaliculata*. They were named PV1, PV2 and PV3 (Garín et al., 1996).

PV1 is a red particle of high molecular mass (approximately 300 kDa), that shares many characteristics of ovorubin and may be considered the same perivitellin, though in his original work, Cheesman reported carotenoids as the only lipids associated. Ovorubin is actually a particle with the characteristics of a very high-density lipoprotein (VHDL). It is the major egg lipo-glyco-carotenoprotein complex isolated from *P. canaliculata* PVF, representing 57.0% of the egg total protein, while PV2 and PV3 represent 7.5 and 35.5% of the egg total protein, respectively (Garín et al., 1996). Considering its hydration density, PV2 is a lipoprotein that also falls into the VHDL range. It is a high molecular weight (MW) particle of 400 kDa. PV3 is a heterogeneous fraction, rich in lipids, composed of at least two particles with the characteristics of high-density lipoproteins (HDL) (Garín et al., 1996). This carotenolipoprotein has a strong yellowish color already mentioned for egg homogenates by Cheesman (1958).

The characteristics of these and other ampullariid perivitellins are given in Table 1. Interestingly, the N-terminal amino acid sequences of ovorubin and PV2 subunits show no homology with other related or non-related proteins (Dreon et al., 2002, and unpublished results), including those from the few reserve proteins so far sequenced in snail eggs (Miller et al., 1996; Mukai et al., 2004). The total amino acid composition of ovorubin contains a preponderance of acidic amino acids (Asp, Glu) followed by long-chain hydrocarbon amino acids (Leu, Val), Ser and Lys (Zagalsky, 1972).

A new carotenoprotein has very recently been isolated and partially purified from the eggs of *Pomacea scalaris*. Electrophoretic

Table 1
Isolated carotenoproteins of Ampullariidae

Species	Name	Description	Organ	λ_{\max} (nm)	kDa (native)	kDa (subunits)	Hydration density (g/mL)	Carbohydrate (%wt)	Carotenoid
<i>P. canaliculata</i> (1, 2, 3, 4)	Ovorubin	Glycolipoprotein	Egg/ alb. gland	(a) 480, 510, 545	300	35, 32, 28*	1.26–1.28	17.8	ASX/ASX esters
<i>P. canaliculata</i> (1, 4)	PV2	Glycolipoprotein	Egg/ alb. gland		400	67, 31	1.22–1.25	2.5	ND
<i>P. canaliculata</i> (1)	PV3	Glycolipoprotein	Egg	(a) 420	64		1.19–1.21	ND	ASX/ASX esters
<i>P. scalaris</i> (5)		Glycolipoprotein	Egg	(a) 465, 495, 532	380	35, 28, 24	1.26	ND	ASX/ASX esters
<i>P. insularum</i> (6)		ND	Egg	(b) 506	ND	ND	ND	ND	ASX

(1) Garín et al., 1996; (2) Cheesman, 1958 (3) Heras and Pollero, 2002; (4) Dreon et al., 2004a; (5) Ituarte et al., submitted for publication; (6) Clark et al., 1980.

(a) dissolved in 0.05 M Tris/HCl, pH 7.4; (b) dissolved in 0.05 M Tris/HCl, pH 6.5.

(*) Preliminary results from exhaustive chemical deglycosylation suggest that the three subunits may represent glycoforms of a single polypeptide.

ND: not determined.

analysis showed that it is composed of a single, carotenoprotein of 380 kDa with three subunits (Table 1). It has been characterized as a VHDL complex (Ituarte et al., submitted for publication).

2.2. Lipid moiety

The three lipoprotein fractions purified from the PVF of *P. canaliculata* contain most of the lipids present in newly-laid eggs. Their lipids, which are non-covalently bound to the protein, account for, 6.7, 10.0 and 53.2% of the egg total lipids in PV1, PV2 and PV3, respectively, and were characterized by microchromatographic methods (Garín et al., 1996).

Ovorubin lipids are mainly composed of sterols and phospholipids, both typically structural lipids. Therefore this particle can be classified as a lipo-glyco-carotenoprotein, similar to other invertebrate lipovitellins (Cheesman et al., 1967; Zagalsky, 1985). Like ovorubin, the major lipid classes of PV2 are free sterols and phospholipids, but significant quantities of energy-providing triacylglycerols and free fatty acids are present. PV3 is the most lipid-rich particle (30% of the total PVF lipids), with high levels of a carotenoid pigment, hydrocarbons, free fatty acids, phospholipids, free sterols and triacylglycerols. The lipid moiety of one of PV3 subfractions (PV3-II) is mainly composed of free sterols, phospholipids and free fatty acids. They are probably two different carotenoproteins that have not been completely purified.

Details of lipid and protein compositions of the carotenoprotein fractions of *P. canaliculata* are summarized elsewhere (Heras and Pollero, 2002).

P. scalaris carotenoprotein, the other ampullariid species whose lipids have been studied, contains a high carotenoid content and less than 1% associated lipids (by wt) (Ituarte et al., submitted for publication).

2.3. Carbohydrates

In a recent work we have analyzed the carbohydrate moieties and monosaccharide compositions of ovorubin and PV2 from *P. canaliculata* (Dreon et al., 2004a) which are the only ones studied in gastropods so far. Both perivitellins contain carbohydrates as the major prosthetic group. Quantitative analysis of total carbohydrates revealed that ovorubin is highly glycosylated (17.8% w/w) in agreement with previous data by Cheesman (1958), while PV2 contains moderate carbohydrate quantities (2.5% w/w). In fact, ovorubin has the largest amount of carbohydrates reported for an animal carotenoprotein or vitellin thus far.

Monosaccharide compositions, determined by gas–liquid chromatography, showed that in both perivitellins the predominant sugar is mannose, followed by N-AcGlc in ovorubin and galactose in PV2. Liquid chromatographic analysis after chemical deglycosylation of both particles showed that the amount of O-linked oligosaccharides is higher than that of the N-linked species. We also studied the glycosylation patterns of the two perivitellins by a set of lectins, which provided information about the possible carbohydrate structure associated with the protein backbone. Lectin affinities confirmed the pre-

sence of asparagine-linked carbohydrates, probably of hybrid and high mannose type, the presence of O-linked residues derived from the T antigen, and suggested that sugars conjugated to both glycoproteins are short and structured in their biosynthetic route.

We are not aware of any study of the carbohydrate moiety in other ampullariid snail proteins, although ongoing research on *P. scalaris* carotenoprotein revealed that it is positive to the thymol-sulphuric reagent (Ituarte et al., submitted for publication).

2.4. Carotenoids

In the past few years, we have characterized the carotenoid component of ovorubin (Dreon et al., 2004b). Chromatographic analysis showed that astaxanthin is present mainly in the free form, but mono and diester forms were also found. A similar pattern was observed for *P. scalaris* carotenoprotein, also dominated by free ASX, though esterified forms were present in different proportions (unpublished results). PV3 carotenoprotein complex is the one carrying most of the carotenoids in the PVF of *P. canaliculata* (67% of the egg total).

The composition of the fatty acids that esterify ASX in ovorubin is dominated by saturated and monoenoic fatty acids (Fig. 2). A similar fatty acid pattern for ASX esters was found in other organisms (Bidigare et al., 1993). The presence of esterified forms of ASX, may represent a mechanism by which this carotenoid could be incorporated into the embryo cytoplasmic membrane in this more hydrophobic form and thus play an antioxidant role (see below).

3. Structural aspects

3.1. Binding of astaxanthin

Carotenoproteins can be classified into two groups considering an stoichiometric relationship between carotenoid and protein (true carotenoproteins), or a non-stoichiometric association (lipoproteins with lipid-associated carotenoids) where carotenoids form part of a large lipid prosthetic group (Cheesman et al., 1967). Ampullariids' carotenoproteins appear to fall into the former group as inferred by the induced fine structure absorption spectra of ovorubin and the carotenoprotein from *P. scalaris*.

The binding capacity of ASX to ovorubin was investigated by fluorescence spectroscopy (Dreon et al., in press). The apoprotein shows a single high affinity binding site with a $K_d=500$ nM. Tryptophan residues adjacent to the probe binding site exhibit resonance energy transfer (RET) to the ligand. The enhancement of the fluorescence of ANS indicates that their binding site has a non-polar character and the quenching of tryptophan fluorescence by ASX indicates that 22% of these tryptophan residues are within the carotenoid-binding site in a non-polar environment. The high affinity of ASX for apo-ovorubin indicates that ovorubin does not release this molecule during embryogenesis until it is incorporated into embryo cells (see below).

In another study, the bathochromic spectral shift of ASX and the vibrational properties of ASX-bound oворubin and a pink carotenoprotein from *Pomacea insularum* was investigated (Clark et al., 1980). The resonance Raman spectrum of the carotenoproteins, excited by irradiating within the contour of the $\pi^* \leftarrow \pi$ transition gave an estimation of the elongation of the C=C and shrinkage of the C–C bonds in the resonant excited state. A correlation was found for oворubin and the *P. insularum* carotenoprotein between the ν_1 wavenumber and $1/\lambda_{\max}$ of the $\pi^* \leftarrow \pi$ absorption band, suggesting that in both ampullariid carotenoproteins ASX bathochromic shift arises from polarization of the carotenoids which gives rise to an increased π -electron delocalization in the astaxanthin, though twisting about the double bonds of the polyene chain was not excluded in that study.

3.2. Structure and stability

It has been reported that carotenoids stabilize the native configuration of most carotenoproteins (Mantiri et al., 1996). Therefore we studied this possibility in the apple snail oворubin. The first experiments suggested that ASX was not essential for the native structure of oворubin (Dreon et al., 2004b). Further studies using circular dichroism (CD) analysis of apo-ovorubin revealed that the secondary structure ($\alpha + \beta$) was not affected by cofactor removal, confirming that, unlike other invertebrate carotenoproteins, the presence of ASX is not necessary to stabilize the native structure. Native oворubin has unusually high thermal stability up to 100 °C, while PV2 is thermolabile in the same conditions. The removal of ASX or its lipid moiety does not affect the thermal or chemical stability of the quaternary structure, as suggested by CD and fluorescence experiments (Dreon et al., in press). The carotenoprotein from *P. scalaris* also showed high thermal stability (Ituarte et al., submitted for publication), the possible role of this feature in eggs is suggested below.

The data gathered thus far suggest that ASX and lipids are not essential for oворubin stability in the aqueous environment.

Except for the carotenoproteins linckiacyanin and asteriarubin from the starfish *Linckia laevigata* and *Asterias rubens* (Echinodermata: Asteroidea) (Zagalsky et al., 1990), this is the only example in invertebrate carotenoproteins where carotenoids play no part in maintaining the quaternary structure.

There is no information on structural aspects of other gastropod carotenoprotein.

4. Synthesis of carotenoproteins in females

Unlike crustaceans, little is known in molluscs about egg reserve lipoproteins. All lipoproteins of ampullariids fall into the VHDL and HDL categories. This characteristic, together with the presence of associated pigments are basically the only structural features in common between perivitellins and other invertebrate and vertebrate vitellins (Lee, 1991).

Metabolism of vitellins has been well studied in many organisms including insects, crustaceans and vertebrates (Lee, 1991; Sammar et al., 2005; Yepiz-Plascencia et al., 2000). In contrast, the metabolism of female gastropod perivitellins was

little known until recently when our group studied the site of synthesis, mobilization and storage of the two major carotenoproteins of *P. canaliculata* (Dreon et al., 2002, 2003). The use of a combination of incubations with labeled amino acids, antibodies directed against purified perivitellins and enzymatic immune assays revealed the presence of proteins with the characteristics of oворubin and PV2 only in the albumen gland. Immunochemical *in vivo* experiments using vitellogenic females, confirmed the presence of immunoreactive proteins only in the albumen gland.

Vitellogenins circulating in plasma or hemolymph of vitellogenic females have been detected in most oviparous organisms such as vertebrates, crustaceans, insects, bivalves and gastropods (Barre et al., 1991; Bride et al., 1992; Lee, 1991; Osada et al., 2004; Raikhel and Sappington, 2002; Wallace, 1985; Yepiz-Plascencia et al., 2000). For example, the pulmonate snail *Helix aspersa* synthesises its vitellogenins in the digestive gland which surrounds the ovotestis and transports them by hemolymph to vitellogenic oocytes. In other pulmonate freshwater snails (*Lymnaea stagnalis* and *Planorbarius cornutus*), a vitellogenin with characteristics of the iron reserve ferritin, has been described. This particle was also produced by the digestive gland and circulates in hemolymph (Bottke, 1986; Bottke et al., 1988). In contrast, no lipoproteins similar to oворubin or PV2 could be found in *P. canaliculata* hemolymph, although highly sensitive techniques as *in vivo* radiolabeling and immunoblot were used. In conclusion, the albumen gland is the only site for the oворubin and PV2 synthesis in the apple snail, as no extra-gland synthesis, circulation or accumulation could be detected. This is only a piece of the “big picture” of carotenoprotein metabolism that includes aspects such as precursor synthesis, hemolymph transport of carotenoid towards albumen gland, carotenoprotein assembly, etc. In contrast with the vast amount of information reported for vertebrates where different models for carotenoid transport from extraovarian tissues into eggs/oocytes have been proposed (Sammar et al., 2005) there are only indirect experiments on ampullariids studying transformations of dietary β -carotene in *P. canaliculata* (Tsushima et al., 1997). These authors have demonstrated that the digestive gland and gonad are capable of ASX synthesis and that gonad (probably albumen gland) has the highest amount of labeled ASX in the body.

To look further into the role of the albumen gland in carotenoproteins metabolism, we recently performed an ultrastructural study of the parenchymal cellular types involved in the synthesis and secretion of carotenoproteins in *P. canaliculata* (Catalán et al., 2006). The two major parenchymal cell types, albumen secretory cells (AS) and labyrinth cells (LC) undergo seasonal variations through the annual reproductive cycle. Both cellular types show maximal development and structural complexity at the reproductive period (spring and summer). AS cells have a well developed Golgi complex and rough endoplasmic reticulum. Its secretory granules show electron-dense particles of about 20 nm, probably galactogen. It was concluded that AS are the only parenchymal cells involved in oворubin and PV2 perivitellin synthesis and their secretory granules are the single storage site, as revealed by immunoelectronmicroscopy. Both carotenoproteins are contained in the same secretory granules.

The results described above led us to infer that these egg carotenoproteins do not follow an entirely heterosynthetic mechanism. Unlike other invertebrate lipovitellins, they are not transported by the hemolymph to the vitellogenic oocytes, but they are incorporated to the fertilized oocytes as a secretion of the albumen gland. Their transport from the site of origin, the albumen gland, to the eggs, can be explained on the basis of the snail reproductive tract morphology, the albumen gland being an exocrine gland attached to the oviduct (Catalán et al., 2002). As in other gastropods, perivitellins and other PVF components are secreted directly into the oviduct, coating fertilized eggs as they pass through it, later adding an outer covering membrane (de Jong-Brink et al., 1983). A similar behaviour was observed for an egg glycoprotein in the pulmonate *Helisoma duryi* by Morishita et al. (1998). Moreover, recent studies on fish and mammals report the synthesis of carotenoid binding proteins in oviduct and ovarian tissues (Eberhardt et al., 1999; Lubzens et al., 2003) which contrast with the traditional view of an heterosynthetic mechanism for yolk protein synthesis in vertebrates.

5. Functional aspects

5.1. Source of nutrients during embryogenesis

As mentioned above, the carotenoproteins ovorubin, PV2 and PV3 are the most abundant source of proteins and lipids of *P. canaliculata* eggs (Garín et al., 1996). In a study by Heras et al. (1998), their fate was followed during the embryo development. Egg development was divided into five stages from morula (stage I) to hatching (stage V). Juvenile snails (first 3-days of free life), were added as stage VI.

During embryogenesis, total protein levels undergo significant changes, decreasing in the PVF while increasing in embryos. Embryos took up 90% of PVF proteins with a sharp consumption between stages IV and V used as an energy source or converted into other components. Embryos in stages II to IV absorb ovorubin from PVF and to a lesser extent also PV2. The results also suggest that the uptake systems and the metabolic pathways of embryos are more active at stages IV and V. Finally, the PVF at stage V is very poor in proteins and mostly contains some ovorubin. As already mentioned, ovorubin is a highly thermostable protein unaltered by the relatively high temperatures to which eggs are exposed (Dreon et al., in press), and its selective conservation throughout development can be explained by its other functions (see below). Moreover, after hatching, ovorubin was still one of the most abundant snail protein recovered from whole body analysis though most of it is probably located “outside” the embryo in the lumen of the digestive gland ready to be endocytosed. In contrast, PV2 and PV3 were virtually absent.

Protein conversion efficiency showed relatively low values (29%), suggesting they are not only used for embryo structure, but also as energy source.

In the same study, it was shown that embryos incorporate lipids together with most of the nutrients, mainly between stages IV and V. Though lipids do not represent an important energy source in this snail and function as structural components of the new tissues, it is interesting to note that about 98% of triacylglycerols are

consumed between stages V and VI (hatchlings) coincident with the fall of the triacylglycerol-rich PV2 perivitellin after hatching. Triacylglycerols may be a particularly useful energy source for hatchlings during short fasting periods, but they could also supply essential fatty acids.

It is therefore suggested that the absorption of perivitellins from the PVF in *P. canaliculata* is divided into two phases. The first one shows a mild uptake up to stage IV, followed by a second phase of very active uptake of all nutrients and a selective consumption of proteins until hatching where they would be utilized for organogenesis. After snails hatch, there would be a specific consumption of PV2 lipoprotein and its associated triacylglycerols. Regardless of the contribution of perivitellins as energy sources, the study of the dynamics of carbohydrates along development -mainly represented by the polysaccharide galactogen- indicates that this is the major energy source in *Pomacea* embryos (Heras et al., 1998).

5.2. Protein targeting and other suggested functions for saccharide moiety

As in other invertebrates, ampullariid carotenoproteins contain carbohydrates as the major prosthetic group and it is known that glycoprotein carbohydrates are involved in many physiological and biochemical processes. Among them the carbohydrate moiety may be implicated in the proper folding and tertiary and quaternary structure of the glycoprotein. To explore if this was the case with ovorubin, we performed a chemical deglycosylation of the protein, which caused a loss of quaternary and tertiary structures (unpublished results). In addition, glycosylation increases solubility in proteins and we have observed that ovorubin can be concentrated to more than 30 mg/mL without precipitating (unpublished results). Carbohydrates are important for the proper transport process and also to protect the protein from intracellular proteases (Berman and Lasky, 1985; Gibson et al., 1979). In this regard, it has been shown that ovorubin is partially auto-protected from trypsin and other proteases (Norden, 1972). Besides, the selective uptake of the ovorubin and PV2 observed during apple snail embryogenesis could also be due to their different carbohydrate composition. (Heras et al., 1998). As reported for *Xenopus laevis* oocytes (Wall and Patel, 1987) and during insect vitellogenesis (Raikhel and Dhadialla, 1992), the carbohydrate moiety could be important for receptor-mediated lipoglycoprotein endocytosis. A similar function might be attributed to carbohydrates in *P. canaliculata* perivitellins during embryo uptake of these glycoproteins from PVF where PV2 is completely consumed before hatching, while some ovorubin still remains in the digestive gland of newborn snails. Moreover, carotenoprotein glycosylation could also be a signal inside the albumen gland for fertilized-egg PVF endocytosis during perivitellogenesis. A role of carbohydrate on egg water loss is suggested below.

5.3. Protective functions of carotenoproteins

There is strong evidence pointing to carotenoproteins playing an important physiological role on ampullariid egg-laying strategy

that exposes them for about two weeks to direct sunlight, air, and high temperatures, as already mentioned in the introduction. A summary of some of the known and suggested protective functions are outlined below.

5.3.1. Antioxidant

It is generally accepted that carotenoids can act as lipid-phase antioxidants (Terao, 1989); in particular astaxanthin is a potent antioxidant in membrane models (Palozza and Krinsky, 1992). It has been reported that land snails (*Otala lactea* and *Helix aspersa*) as well as the freshwater snail *Biomphalaria tenagophila* showed an increase in their antioxidant capacity when exposed to environmental stress (Hermes-Lima and Zenteno-Savin, 2002; Ramos-Vasconcelos and Hermes-Lima, 2003). One adaptation of the aerial eggs to environmental stress would involve the provision of adequate amounts of antioxidant and photoprotective molecules to the embryo. This has been well studied in bird eggs where egg carotenoid availability has been recognized as a limiting factor for reproduction and antioxidant defense (Blount, 2004). Some studies have pointed to ovorubin as a potential candidate for these functions (Cheesman et al., 1967; Dreon et al., 2003; Heras et al., 1998). Recently, the capacity of ovorubin as a protection system against oxidative damage in eggs from *P. canaliculata* was investigated, using chemiluminescence to measure the inhibition of enzymatic and non-enzymatic membrane oxidation and so to evaluate the antioxidant properties of ovorubin (Dreon et al., 2004b). The carotenoid extract of ovorubin (a mixture of free and esterified astaxanthin) showed a strong antioxidant activity, which was dose-dependent with an IC₅₀ of 3.9 nmol/mg protein that is of the same order as the free ASX (Palozza and Krinsky, 1992). Nevertheless, native ovorubin does not have antioxidant activity *per se* (Dreon et al., 2004b), probably because of its high binding constant for ASX (Dreon et al., in press). If this is also the case with PV2 and PV3, (ovorubin, has about 23% of the total carotenoid present in the PVF) (Garín et al., 1996), then these three carotenoproteins are probably involved in the transport of membrane antioxidants to the developing embryo. The carotenoid content in eggs is approximately 72 nmol/g (calculated from Garín et al., 1996) which, interestingly, is of the same order as the amount found in several bird eggs (Blount, 2004). Assuming that 1 egg weighs 20 mg and contains 0.23 mg embryo protein (Heras et al., 1998), it therefore carries 1.5 nmol astaxanthin/egg. Hence, embryos are supplied with adequate amounts of antioxidant molecules, allowing their development under harsh conditions as above stated. The esterified forms of ASX, more hydrophobic than the free form or the astaxanthin bound to other molecules, may provide an adequate way for this pigment to pass through or being incorporated into membranes.

5.3.2. Photoprotective action

Coloration by carotenoids in *Pomacea* eggs can cover a wide range of the visible spectrum. For example orange to reddish eggs clutches are found in *P. flagellata*, *P. canaliculata*, *P. scalaris*, *P. bridgesii*, *P. paludosa* and *P. haustum*, while eggs of other species such as *P. glauca* and *P. gigas* are green or

those of *P. sordida* eggs are orange-yellow (Perera and Walls, 1996; Villela, 1956). These differences could be due to the presence of different carotenoids, but most probably they are the result of the interaction between ASX (or its esters) with different proteins. For example *P. canaliculata* and *P. scalaris* have both a similar carotenoid profile but different protein composition and their resulting absorption maxima is blue-shifted in the latter (Table 1). The ability of the protein to alter the spectral properties of ASX is even better illustrated in three carotenoproteins from the lobster *Homarus americanus* (Nephropidae, Decapoda) where ASX can confer the tissue a green color (in egg ovorubin, λ_{\max} 460 and 640 nm) a yellow color (in carapace crustochrin, λ_{\max} 409 nm) or a blue coloration (in carapace crustacyanin, λ_{\max} 632 nm) (Zagalsky et al., 1990).

The carotenoproteins solubilized in PVF of *P. canaliculata* were studied throughout embryogenesis. They absorb light throughout most of the visible range, and may exert a photoprotective effect on the embryo cells against harmful sunlight radiation at the beginning of development. In fact embryos have already taken up most of carotenoproteins from PVF incorporating them into their pigments before hatching (Heras et al., 1998). Near hatching, the need for embryos to be surrounded by a light-protecting fluid decreases, as their external melanin pigment increases. Photoprotective actions of carotenoids have also been reported for crustaceans (Hairston, 1976; Sagi et al., 1995).

Looking at the geographical distribution of the Ampullariidae it is evident that the genera that deposit the eggs above the waterline (Fig. 1) are the most widespread worldwide. In fact *P. canaliculata*, which has been introduced in Asia, is rapidly expanding and becoming a serious pest for several crops. If we assume that egg carotenoproteins are only present in those genera, their importance in the adaptation to the harsh aerial conditions becomes manifest. To test this hypothesis, we analysed the eggs of *Asolene pulchella*, a species that deposits their eggs underwater. After a thorough acetone extraction of total egg homogenates, neither carotenoids and hence nor carotenoproteins were found (unpublished results). There is no reason to believe that this may be different in other ampullariid species that lay their eggs underwater, as all of them deposit eggs, embedded in a transparent gelatinous mass which is protected from sunlight by the filtering effect of water and the particles in suspension of their turbid habitats.

Further research is still needed on the laying habits and egg biochemistry of many Ampullariidae, especially the African and Asian species, to have a complete picture. However, we would predict, on the basis of our studies in *Pomacea* and *Asolene*, that carotenoproteins would be present only in aerial eggs, protecting them from the damaging effects of light as demonstrated in *P. canaliculata* (Dreon et al., 2004b).

5.3.3. Carotenoid stabilization/protection

Ovorubin not only transports carotenoids to the eggs, but also protects or stabilizes carotenoids from damage in the PVF before they are delivered to the embryo, as shown by the shorter half-life of the free pigment as compared to the protein-associated

carotenoid. In fact, free astaxanthin is significantly more sensitive to photo-oxidation than its combined forms, such as native or *in vitro*-reconstituted ovorubin (Dreon et al., 2004b). It is known that carotenoids are altered or partially destroyed by light, especially in combination with oxygen and/or heat. This results in the discoloration and loss of activity that is usually found when working with carotenoids or carotenoproteins. *In vitro* experiments showed that under severe oxidizing conditions, most of the soluble astaxanthin is damaged, while only a small part of the ovorubin-protected astaxanthin is altered. As physiological environmental conditions are not so rigorous, the docking of carotenoids to protein ensures that virtually all carotenoids would remain undamaged and functional throughout the snail embryogenesis.

The carotenoprotein isolated from *P. scalaris* probably serves a similar function and it is very likely that this protection of the pigment may be a common feature in other Ampullariidae species laying aerial egg clutches (Fig. 1).

5.3.4. Warning coloration

Another function of coloration may involve the use of carotenoproteins as a warning coloration. In fact, field evidence of egg unpalatability is provided by the fact that most animals foraging in habitats where the apple snails live ignore these eggs. Moreover, when apple snail eggs from *P. paludosa* were offered to captive predators, though they ate them at first, refused them after repeated feeding (Snyder and Snyder, 1971).

5.3.5. Prevention of desiccation

It is interesting to note that even under the severe conditions suffered by aerial egg masses, no significant changes in the water content during development are observed in *P. canaliculata* (Heras et al., 1998). Since native proteins have a much higher binding capacity for water than denatured ones, the significant thermal stability of ovorubin and the carotenoprotein isolated from *P. scalaris* may also play a role in the conservation of egg water at high temperatures. This function would be enhanced by the elevated carbohydrate content of the protein. The combination of glycoproteins, with the high amount of galactogen present in the PVF and the egg calcareous shell should be efficient means to avoid water loss, maintaining an adequate environment for the embryos.

6. Future directions

We felt that the time had come for an assessment of the present state of knowledge on the subject in Ampullariidae. However, there are several essential questions that have not yet been addressed. The nature of the complexes in other members of the family and in molluscs as a whole is still entirely unknown, and therefore generalizations regarding the occurrence, functions and distribution of carotenoproteins in this phylum cannot be made until further work has been accomplished. There are also several questions unanswered regarding carotenoprotein folding, assembly, post translational modifications and quaternary structure. The knowledge of the primary sequence from perivitellin would allow establishing patterns and

homologies with other proteins, a subject of great evolutionary interest. More experiments are also required to ascertain the carotenoid site of synthesis, precursors, hemolymph mobilization and storage in molluscs.

Additional comparative work on different families and species of gastropods is required to further confirm the strategies for egg protein synthesis in this complex group.

Acknowledgements

M.S.D. and R.J.P. are members of CICBA, Argentina. H.H. is member of CONICET, Argentina. S.I. is a CONICET scholarship fellow.

References

- Albrecht, E.A., Carreño, N.B., Castro-Vazquez, A., 1999. A quantitative study of environmental factors influencing the seasonal onset of reproductive behaviour in the south American apple-snail *Pomacea canaliculata* (Gastropoda: Ampullariidae). *J. Molluscan. Stud.* 65, 241–250.
- Albrecht, E.A., Koch, E., Carreño, N.B., Castro-Vazquez, A., 2005. Control of the seasonal arrest of copulation and spawning in the apple snail *Pomacea canaliculata* (Prosobranchia: Ampullariidae): Differential effects of food availability, water temperature, and day length. *Veliger* 47, 169–174.
- Allen, W.V., 1977. Interorgan transport of lipids in the blood of the gumboot chiton *Cryptochiton stelleri* (Middendorff). *Comp. Biochem. Physiol.* 57, 41–46.
- Barre, P., Bride, M., Beliard, R., Petracca, B., 1991. Localization of yolk proteins and their possible precursors using polyclonal and monoclonal antibodies, in *Helix aspersa*. *Cell. Mol. Biol.* 37, 639–650.
- Berman, H., Lasky, L., 1985. Engineering glycoproteins for use as pharmaceuticals. *Trends Biotechnol.* 3, 51–53.
- Berthold, T., 1989. Comparative conchology and functional morphology of the copulatory organ of the Ampullariidae (Gastropoda, Monotocardia) and their bearing upon phylogeny and palaeontology. *Abh. Nat. Wiss. Ver. Hambg.* 28, 141–164.
- Bidigare, R., Ondrusek, M., Kennicutt, M.C., Iturriaga, R., Harvey, H.R., Hoham, R.W., Makro, S.A., 1993. Evidence for a photoprotective function for secondary carotenoids of snow algae. *J. Phycol.* 29, 427–434.
- Blount, J.D., 2004. Carotenoids and life-history evolution in animals. *Arch. Biochem. Biophys.* 430, 10–15.
- Bottko, W., 1986. Immunolocalization of ferritin polypeptides in oocytes and somatic tissue of the freshwater snails *Lymnaea stagnalis* and *Planorbis cornuus*. *Cell Tissue Res.* 243, 397–404.
- Bottko, W., Burschuk, M., Volmer, J., 1988. On the origin of the yolk protein ferritin in snails. *Roux's Arch. Dev. Biol.* 197, 377–388.
- Bride, M., Petracca, B., Faivre, D., 1992. The synthesis of vitellogenins by the digestive gland of *Helix aspersa*: evidence from cell-free translation of mRNA. *Cell. Mol. Biol.* 38, 181–187.
- Britton, G., Armitt, G.M., Lau, S.Y.M., Patel, A.K., Shone, C.C., 1982. Carotenoproteins. In: Britton, G., Goodwin, T.W. (Eds.), *Carotenoid Chemistry and Biochemistry*. Pergamon Press, Oxford, pp. 237–253.
- Catalán, N.M., Fernández, S.N., Winik, B.C., 2002. Oviductal structure and provision of egg envelopes in the apple snail *Pomacea canaliculata* (Gastropoda Prosobranchia, Ampullariidae). *Biocell* 26, 91–100.
- Catalán, M., Dreon, M.S., Heras, H., Pollero, R.J., Fernández, S.N., Winik, B., 2006. Pallial oviduct of *Pomacea canaliculata* (Gastropoda): ultrastructural studies of the parenchymal cellular types involved in the metabolism of perivitellins. *Cell Tissue Res.* 324, 523–533.
- Ceccaldi, H.J., Zagalsky, P.F., 1967. Studies on the carotenoprotein from the stomach wall of *Aristeus antennatus*. *Comp. Biochem. Physiol.* 21, 435–438.
- Cheesman, D.F., 1954. A chromoprotein from the eggs of *Pomacea canaliculata*. *Biochem. J.* 58, 38.
- Cheesman, D.F., 1956. Ovorubin. *J. Physiol. (Lond)* 131, 3P–4P.
- Cheesman, D.F., 1958. Ovorubin, a chromoprotein from the eggs of the gastropod mollusk *Pomacea canaliculata*. *Proc. R. Soc. Lond. [Biol.]* 149, 571–587.

- Cheesman, D.F., Lee, W.L., Zagalsky, P.F., 1967. Carotenoproteins in invertebrates. *Biol. Rev.* 42, 132–160.
- Clark, R.J.H., D'Urso, N.R., Zagalsky, P.F., 1980. Excitation profiles, absorption and resonance raman spectra of the carotenoprotein ovorubin, and a resonance raman study of some other astaxanthin proteins. *J. Am. Chem. Soc.* 102, 6693–6698.
- Comfort, A., 1947. Lipochromes in the ova of *Pila*. *Nature* 160, 333.
- Comfort, A., 1949. Lipochromes in the ova of *Pila canaliculata* (Lamarck). *Proc. Malacol. Soc. Lond.* 27, 262.
- Czczuga, B., 1983. Investigations of carotenoprotein complexes in animals. 8. *Unio pictorum* (L.) as a representative of freshwater molluscs. *Comp. Biochem. Physiol. B* 75, 541–543.
- de Jong-Brink, M., Boer, H.H., Joosse, J., 1983. Mollusca. In: Adiyodi, K.G., Adiyodi, R.G. (Eds.), *Reproductive Biology of invertebrates. Oogenesis oviposition and oosorption*, vol. 1. John Wiley & Sons Ltd., New York, pp. 297–355.
- Dreon, M.S., Lavarias, S., Garin, C.F., Heras, H., Pollero, R.J., 2002. Synthesis, distribution, and levels of an egg lipoprotein from the apple snail *Pomacea canaliculata* (Mollusca: Gastropoda). *J. Exp. Zool.* 292, 323–330.
- Dreon, M.S., Heras, H., Pollero, R.J., 2003. Metabolism of ovorubin, the major egg lipoprotein from the apple snail. *Mol. Cell. Biochem.* 243, 9–14.
- Dreon, M.S., Heras, H., Pollero, R.J., 2004a. Characterization of the major egg glycolipoproteins from the perivitellin fluid of the apple snail *Pomacea canaliculata*. *Mol. Reprod. Dev.* 68, 359–364.
- Dreon, M.S., Schinella, G., Heras, H., Pollero, R.J., 2004b. Antioxidant defense system in the apple snail eggs, the role of ovorubin. *Arch. Biochem. Biophys.* 422, 1–8.
- Dreon, M.S., Ceolin, M., Heras, H., in press. Astaxanthin binding and structural stability of the apple snail carotenoprotein ovorubin. *Arch. Biochem. Biophys.* doi:10.1016/j.abb.2006.12.033.
- Eberhardt, D.M., Jacobs, W.G., Godkin, J.D., 1999. Steroid regulation of retinol-binding protein in the ovine oviduct. *Biol. Reprod.* 60, 714–720.
- Euler, H.v., Hellström, H., Klussman, E., 1934. Über den Carotinoidgehalt einiger Evertibraten. *Hoppe-Seyler's Z. Hoppe-Seyler Z. Physiol. Chem.* 228, 77–89.
- Fainzilber, M., Zlotkin, E., 1992. A new bioassay reveals mollusc-specific toxicity in molluscivorous *Conus* venoms. *Toxicon* 30, 465–469.
- Garin, C.F., Heras, H., Pollero, R.J., 1996. Lipoprotein of the egg perivitellin fluid of *Pomacea canaliculata* snails (Mollusca: Gastropoda). *J. Exp. Zool.* 276, 307–314.
- Gibson, R., Schlesinger, S., Kornfeld, S., 1979. The non glycosylated glycoprotein of vesicular stomatitis virus is temperature sensitive and undergoes intracellular degradation at elevated temperatures. *J. Biol. Chem.* 254, 3600–3607.
- Goodwin, T.W., Taha, M.M., 1950. The carotenoids of the gonads of the limpets *Patella vulgata* and *Patella depressa*. *Biochem. J.* 47, 249–251.
- Hairston Jr., N.G., 1976. Photoprotection by carotenoid pigments in the copepod *Diaprotomus nevadensis*. *Proc. Natl. Acad. Sci. U. S. A.* 73, 971–974.
- Heras, H., Pollero, R.J., 2002. Lipoproteins from plasma and perivitellin fluid of the apple snail *Pomacea canaliculata*. *Biocell* 26, 111–118.
- Heras, H., Garin, C.F., Pollero, R.J., 1998. Biochemical composition and energy sources during embryo development and in early juveniles of the snail *Pomacea canaliculata* (Mollusca: Gastropoda). *J. Exp. Zool.* 280, 375–383.
- Hermes-Lima, M., Zenteno-Savin, T., 2002. Animal response to drastic changes in oxygen availability and physiological oxidative stress. *Comp. Biochem. Physiol. C* 133, 537–556.
- Iuarte, S., Dreon, M.S., Pollero, R.J., Heras, H., submitted for publication. Isolation and partial characterization of a new lipo-glyco-carotenoprotein from *Pomacea scalaris* (Gastropoda: Ampullariidae). *Mol. Cell. Biochem.*
- Kanost, M.R., Kawooya, J.K., Law, J.H., Ryan, M., Van Heusden, M.C., Ziegler, R., 1990. Insect haemolymph proteins. In: Evans, P.D., Wigglesworth, V.B. (Eds.), *Advances Insect Physiology*. Academic Press, London, pp. 299–396.
- Lee, R.F., 1991. Chp. 3. Lipoproteins from the Hemolymph and Ovaries of Marine Invertebrates. In: Gilles, R. (Ed.), *Advances in Comparative and Environmental Physiology*. Springer-Verlag, London, pp. 187–208.
- Lubzens, E., Lissauer, L., Levavi-Sivan, B., Avarre, J.C., Sammar, M., 2003. Carotenoid and retinoid transport to fish oocytes and eggs: what is the role of retinol binding protein? *Mol. Aspects Med.* 24, 441–457.
- Mantiri, D.M.H., Negresadargues, G., Charmantier, G., Trilles, J.P., Milicua, J.C.G., Castillo, R., 1996. Nature and metabolism of carotenoid pigments during the embryogenesis of the European lobster *Homarus gammarus* (Linné, 1758). *Comp. Biochem. Physiol. A* 115, 237–241.
- Miksys, S., Saleuddin, A.S., 1986. Ferritin as an exogenously derived yolk protein in *Helisoma duryi* (Mollusca: Pulmonata). *Can. J. Zool.* 64, 2678–2682.
- Miller, A.N., Ofori, K.L.F., Knight, M., 1996. *Schistosoma mansoni*: use of subtractive cloning to search for RFLPs in parasite-resistant *Biomphalaria glabrata*. *Exp. Parasitol.* 84, 420–428.
- Morishita, F., Mukai, A.S., Saleuddin, A.S., 1998. Release of proteins and polysaccharides from the albumen gland of the freshwater snail *Helisoma duryi*: effect of cAMP and brain extract. *J. Comp. Physiol., A* 182, 817–825.
- Mukai, S.T., Hoque, T., Morishita, F., Saleuddin, A.S., 2004. Cloning and characterization of a candidate nutritive glycoprotein from the albumen gland of the freshwater snail, *Helisoma duryi* (Mollusca: Pulmonata). *Invertebr. Biol.* 123, 83–92.
- Nakadal, A.M., 1960. Carotenoids and chlorophyll pigments in the marine snail *Cerithidea californica* Haldeman, intermediate host for several avian trematodes. *Biol. Bull.* 119, 98–108.
- Norden, D.A., 1972. The inhibition of trypsin and some other proteases by ovorubin, a protein from the eggs of *Pomacea canaliculata*. *Comp. Biochem. Physiol. B* 42, 569–576.
- Osada, M., Harata, M., Kishida, M., Kijima, A., 2004. Molecular cloning and expression analysis of vitellogenin in scallop, *Patinopecten yessoensis* (Bivalvia, Mollusca). *Mol. Reprod. Dev.* 67, 273–281.
- Palozza, P., Krinsky, N.I., 1992. Astaxanthin and cantaxanthin are potent antioxidants in a membrane model. *Arch. Biochem. Biophys.* 297, 291–295.
- Perera, G., Walls, G.H., 1996. *Apple Snails in the Aquarium*. T.F.H. Publications Inc., Neptune. 121 pp.
- Pizani, N.V., Estebenet, A.L., Martín, P., 2005. Effects of submersion and aerial exposure on clutches and hatchlings of *Pomacea canaliculata* (Gastropoda: Ampullariidae). *Am. Malacol. Bull.* 20, 55–63.
- Raikhel, A.S., Dhadialla, T.S., 1992. Accumulation of yolk proteins in insect oocytes. *Annu. Rev. Entomol.* 37, 217–251.
- Raikhel, A.S., Sappington, T.W., 2002. *Reproductive Biology of invertebrates. A. Progress in Vitellogenesis*, vol. XII. Science Publishers Inc., Endfield, NH, USA.
- Ramos-Vasconcelos, G.R., Hermes-Lima, M., 2003. Hypometabolism, antioxidant defenses and free radical metabolism in the pulmonate land snail *Helix aspersa*. *J. Exp. Biol.* 206, 675–685.
- Sagi, A., Rise, M., Isam, K., Arad, S., 1995. Carotenoids and their derivatives in organs of the maturing female crayfish *Cherax quadricarinatus*. *Comp. Biochem. Physiol. B* 112, 309–313.
- Sammar, M., Levi, L., Hurvitz, A., Lubzens, E., 2005. Studies on retinol-binding protein during vitellogenesis in the Rainbow Trout (*Oncorhynchus mykiss*). *Gen. Comp. Endocrinol.* 141, 141–151.
- Snyder, N.F.R., Snyder, H.A., 1971. Defenses of the Florida apple snail *Pomacea paludosa*. *Behaviour* 40, 175–215.
- Terao, J., 1989. Antioxidant activity of carotene-related carotenoids in solution. *Lipids* 24, 659–661.
- Tsushima, M., Katsuyama, M., Matsuno, T., 1997. Metabolism of carotenoids in the apple snail, *Pomacea canaliculata*. *Comp. Biochem. Physiol. B* 118, 431–436.
- Turner, R.L., 1998. Effects of submergence on embryonic survival and developmental rate of the Florida applesnail, *Pomacea paludosa*: implications for egg predation and marsh management. *Florida Sci.* 61, 118–129.
- Villela, G.G., 1956. Carotenoids of some Brazilian freshwater gastropods of the genus *Pomacea*. *Nature* 178, 93.
- Wall, D., Patel, S., 1987. The intracellular fate of vitellogenin in *Xenopus oocytes* is determined by its extracellular concentration during endocytosis. *J. Biol. Chem.* 262, 14779–14789.
- Wallace, R., 1985. Vitellogenesis and oocytes growth in nonmammalian vertebrates. In: Browder, L.W. (Ed.), *Developmental Biology: a comprehensive synthesis*, vol. 1, pp. 127–178.
- Wallace, R.A., Walker, S.L., Hauschka, P.V., 1967. Crustacean lipovitellin: isolation and characterization of the major high-density lipoprotein from eggs of decapods. *Biochemistry* 6, 1582–1590.

- Yang, H.P., Lee, A.J., Kim, Y.T., Kim, S.K., 1994. Purification and characterization of the red carotenoprotein from the muscle of blue mussel, *Mytilus edulis*. Bull. Korean Fish. Soc. 482–494.
- Yepiz-Plascencia, G., Vargas-Albores, F., Higuera-Ciapara, I., 2000. Penaeid shrimp hemolymph lipoproteins. Aquaculture 191, 177–189.
- Zagalsky, P.F., 1972. Comparative studies on the amino acid compositions of some carotenoid-containing lipoglycoproteins and a glycoprotein from the eggs and ovaries of certain aquatic invertebrates. Comp. Biochem. Physiol. B 41, 385–395.
- Zagalsky, P.F., 1985. Invertebrate carotenoproteins. In: Law, J.H., Rilling, H.C. (Eds.), Methods in Enzymology. Steroids and Isoprenoids, vol. 111 part B. Academic Press, New York, pp. 216–247.
- Zagalsky, P.F., Eliopoulos, E.E., Findlay, J.B., 1990. The architecture of invertebrate carotenoproteins. Comp. Biochem. Physiol. B 97, 1–18.