Metabolism of ovorubin, the major egg lipoprotein from the apple snail

Marcos S. Dreon, Horacio Heras and Ricardo J. Pollero

Instituto de Investigaciones Bioquimicas de La Plata (INIBIOLP), CONICET-UNLP, La Plata, Argentina

Abstract

The site of synthesis of molluscs lipoproteins is little known and was investigated for the egg lipoprotein perivitellin 1 (PV1) or ovorubin in the freshwater snail *Pomacea canaliculata*. Tissues (albumen gland, gonad–digestive gland complex and muscle) of vitellogenic females were incubated *in vitro* at 25°C for 12 h with ^{14}C Leucine. After that, soluble proteins from tissue homogenates and medium samples were analysed for *de novo* protein synthesis by electrophoresis and HPLC, and radiolabelled proteins quantified by liquid scintillation. Gonad–digestive gland complex did not synthesise ovorubin, in spite its high protein synthesis levels. Three albumen gland radiolabelled proteins (35, 32 and 28 kDa) comigrated with the subunits of ovorubin and represented 1.3% of the total labelled protein of that tissue. Western blot analysis with polyclonal antibodies confirmed that these were ovorubin subunits. *In vivo* experiments where vitellogenic females were injected with ^{3}H Leucine, revealed that ovorubin was not present in hemolymph. ELISA analysis confirmed ovorubin presence only in albumen gland and developing eggs with levels of 800 and 582 mg/g protein, which represent 30.3 and 28.4 mg ovorubin/g of tissue, respectively. Therefore, albumen gland is the single site of ovorubin synthesis as no extragland synthesis, circulation or accumulation could be detected in the apple snail. (Mol Cell Biochem **243**: 9–14, 2003)

Key words: ovorubin, lipoprotein, snail, glycolipocarotenoprotein, *in vitro* synthesis, carotenoprotein

Introduction

During vitellogenesis the main components of the egg vitellus such as lipids, proteins and carbohydrates are synthesised. Vitellus proteins are named vitellins or, if they contain lipids, lipovitellins [1], and have different origins. In vertebrates, they are usually synthesised in the liver, released to circulation as precursor particles called vitellogenins, taken up by the ovary and incorporated into the developing oocyte (heterosynthetic mechanism). In insects and some crustaceans this mechanism is similar to that in vertebrates, while in other crustaceans it has been reported that the ovary as well as other tissues are involved in this process, thus showing either autosynthetic or heterosynthetic mechanism of vitellogenesis [2, 3]. In comparison, little is known about this process in molluscs. In cephalopods [4] and some gastropods [5, 6] heterosynthesis (e.g. vitellogenesis outside the ovary) seems to be the main yolk forming process. Gastropod vitellins have been described in the oocytes of *Helix* [6], *Helisoma* [7] *Lymnaea,* and *Planorbis* [8]. Unlike this general mechanism, most gastropods have a perivitelline fluid, mainly synthesised by accessory glands of the female reproductive tract called albumen gland, that represents the major source of nutrients for the embryo. Therefore, proteinaceous yolk granules found in developing eggs do not serve the purpose of nutrient storage, but they function as primary lysosomes in charge of perivitelline fluid digestion instead [4]. The apple snail, *P. canaliculata* belongs to this latter group where yolk is a minor source of nutrients to the embryo. This freshwater snail is a plague of rice crops in Asia, and it is the vector of the human meningoencephalitis. Our laboratory has been studying the perivitelline fluid trying to understand its biochemistry during embryogenesis. Two lipoproteins perivitellin-1 (PV1) or ovorubin, and perivitellin-2 (PV2)

and one lipopoprotein fraction (PV3) were isolated [9], where ovorubin represents the major egg protein.

Ovorubin was first described by Cheesman [10] as a glyco-protein which has a carotenoid (astaxanthin) as a prosthetic group. Our laboratory further characterised it as a lipoglyco-carotenoprotein of very high density (VHDL, 1.26–1.28 g/ml), with a molecular mass of 300 kDa, and constituted by three apoprotein subunits of 28, 32 and 35 kDa. Its lipid content was only 0.3%, represented by free sterols and phospholipids [9].

Studying the synthesis, distribution and storage of *P. canaliculata* PV2 we determined that the albumen gland was the only tissue able to synthesise this lipoprotein, though it was not possible to detect any circulating form of this particle [11].

In the present report, we centred our attention in the apple snail ovorubin determining its synthesis site using *in vitro* tissue cultures, as well as its distribution, *in vivo* circulation, and levels in eggs and organs.

Materials and methods

Snails

Adults of *P. canaliculata* were collected in streams or ponds near city of La Plata, Buenos Aires province, Argentina. Eggs were collected from females either raised in our laboratory taken from the wild between November and April (reproductive season). All egg masses used had embryos developed to no more than the morula stage. Embryo development was checked in each egg mass microscopically.

Tissue extracts

Albumen gland, gut, stomach, lung, muscle and digestive gland–gonad complex were dissected from 3–4 females, repeatedly rinsed with ice-cold 20 mM Tris-HCl, pH 6.8, containing 0.8 μM aprotinin (Trasylol, Mobay Chemicals, New York, USA) and homogenised in a Potter type homogenizer (Thomas Sci., Swedesvoro, NJ, USA). The relation buffer: sample was kept at 5:1 v/w. Fertilised eggs were homogenised in the same fashion. The crude homogenates were then sonicated for 15 sec and centrifuged sequentially at 10,000 × g for 30 min and at 100,000 × g for 60 min. The pellet was discarded and the supernatant stored at –70°C until analysis. Hemolymph was collected by cardiac puncture with a 1 ml syringe with G22 needle. By this procedure, it is possible to obtain 2–8 ml from each adult. Protein content was determined by the method of Bradford *et al.* [12] using bovine serum albumin as standard.

Ovorubin isolation and purification

The egg soluble protein obtained using the above procedure was purified in a Merk–Hitachi high performance liquid chromatograph (HPLC) (Hitachi, Tokyo, Japan) with a L-6200 Intelligent Pump and a L-4200 UV detector set at 280 nm. A serial purification was done, the sample was analysed in a Mono Q HR 10/10 (Amersham-Pharmacia, Uppsala, Sweden) using a gradient of 0–1 M NaCl in a 20 mM Tris-HCl buffer. The ovorubin peak was then further purified by size exclusion chromatography (Superdex 200 HR 10/20, Amersham-Pharmacia, Uppsala, Sweden) using an isocratic gradient of sodium phosphate buffer 50 mM, 150 mM NaCl, pH 7.6. Purity of the single peak obtained was checked by native electrophoresis.

Anti-PV$_1$ rabbit serum preparation

Antibodies directed against purified PV$_1$ were prepared in rabbits. Animals were given multiple subcutaneous injections of about 1.5 mg of ovorubin emulsified in Freund's complete adjuvant (Sigma Chemicals, St. Louis, MO, USA). A booster injection with about 1.5 mg antigen mixed with Freund's incomplete adjuvant was administered after 4 weeks. Two weeks later, the rabbits were bled through cardiac puncture. The collected blood was allowed to clot overnight (4°C) and after centrifugation the serum obtained was stored at –70°C, and used in the immunochemical techniques. The specificity of the antiserum was verified by immunoblotting against all the fractions obtained from tissues. The antiserum reacted only with ovorubin fraction.

Gel electrophoresis

Proteins were measured by the method of Bradford *et al.* [12]. Protein subunits and apoproteins were analysed by sodium dodecyl sulfate (SDS)-PAGGE using a gradient of 4–15% acrylamide [13]. The gels were stained with Coomassie Brilliant Blue R-250 (Sigma Chemicals).

Immunoblotting

For Western blots, proteins were separated by SDS-PAGE electrophoresis and electroblotted for 1.5 h at 12 V (Trans-Blot SD Semi Dry Transfer Cell, Bio Rad, Hercules, CA, USA) into nitrocellulose membranes using 39 mM Tris 48 mM Gly, pH 9.2, 20% MeOH buffer [14]. After blocking overnight at 4°C with 3% (w/v) non fat dry milk in 10 mM Tris-HCl, pH 7.4, 0.15 M NaCl, the membranes were incubated for 2 h with the anti-ovorubin serum diluted (1:1,000)

in 10 mM Tris-HCl, pH 7.4, 0.15 M NaCl. Specific antigens were detected by goat anti-rabbit IgG horseradish peroxidase conjugate (Bio-Rad Laboratories) diluted (1:3,000). Immunoreactivity was visualised by ECL.

Enzyme-linked immunosorbent assay (ELISA)

This procedure is based on the assay of Engwall and Perlmann [15]. The standard curve was prepared using purified ovorubin. Nunc-Immunoplate Maxisorp microtiter plates were loaded with 50 µl/well of the PV_1 standard (0–15 ng) dissolved in a buffer containing 15 mM sodium carbonate, 35 mM sodium bicarbonate, pH 9.6 (coating buffer). Samples of hemolymph, gut, stomach, albumen gland, digestive gland, eggs and muscle were diluted with the coating buffer to load wells with 100 ng of total protein. Aliquots of 50 µl were pipeted into the wells and incubated at 37°C for 90 min. The antigen solutions were then shaken out, and each well was filled with 300 µl of PBS, pH 7.4, containing 1% (w/v) non-fat dry milk. The plates were incubated at room temperature for 2 h and subsequently washed 3 times with 0.05% (v/v) Tween in PBS. The anti-ovorubin rabbit serum diluted in PBS-Tween (1/500) containing 1% non-fat dry milk, was loaded into each well, and plates were incubated overnight at 4°C and washed 3 times as above. Goat anti-rabbit IgG horseradish peroxidase conjugate (Bio-Rad Laboratories) diluted (1/500) in PBS 0.05% Tween 1% non-fat dry milk was added to each well (50 µl) and incubated at room temperature for 2 h. After 4 washes as before, 50 µl aliquots of substrate solution, ABTS and H_2O_2 (Bio-Rad Laboratories) were added to each well and the plates were incubated at room temperature for 15 min. After colour development, the reaction was stopped by the addition of 2% oxalic acid (50 µl), and the absorbance was read at 415 nm on an EL-307C microplate reader (Bio-Tek Instruments, Winooski, VT, USA). One percent non-fat dry milk in PBS was used in all assays as a negative control and blank, and all samples were performed in triplicate and the values were averaged.

In vitro *tissue incubations*

To investigate the tissue site of ovorubin synthesis, gonad–digestive gland complex, muscle, and albumen gland were dissected from females at the end of their vitellogenic period, rinsed with sterile incubation medium, weighed and cut into 3 pieces. Each piece (approx. 50 mg, 0.2–0.4 cm^3) was weighed and incubated separately in 1 ml modified *Helisoma* medium [16] containing Hepes 5 mM, pH 7.3, NaCl 40 mM, KCl 1.7 mM, $CaCl_2$, $2H_2O$ and ^{14}C-Leu 315 µCi/nmol, (New England Nuclear, USA), final specific activity 1 µCi/ml. Incubation vials were placed in a water bath at 24°C and gently agitated for 12 h. Control incubations were carried out with media

containing labelled leucine but without tissue. After the incubation period, tissues were washed repeatedly with medium without the tracer, and homogenised as described above. Aliquots were analysed for (1) total protein [12], (2) radioactivity incorporated to protein, quantified in a Pharmacia LKB Wallac 1219 Rackbeta by liquid scintillation (Uppsala, Sweden), and (3) ovorubin was isolated by HPLC or electrophoresis as described above. Radioactivity incorporated into protein fractions separated by HPLC was either measured by Radio-HPLC (Radiomatic Flo-one beta, Packard, Meriden, CT, USA) or by collecting the peaks and counting radioactivity by liquid scintillation. Gel slabs were sliced every 0.5 mm, digested with H_2O_2 and radioactivity quantified by liquid scintillation counting.

In vivo *incubations*

Vitellogenic females were injected in the foot with 100 µl of Helisoma salts [17] containing 10 µCi of ^3H-Leu (New England Nuclear) and left in aquaria at 26°C for 18 h. After that period, hemolymph was collected from each animal and stored at −70°C for analysis.

Results

Distribution of ovorubin in different tissues

The presence of ovorubin was evaluated in homogenates of most tissues of the apple snail: albumen gland, gut, stomach, lung, digestive gland–gonad complex, hemolymph, muscle and egg cytosol using SDS-PAGGE. Figure 1A shows three bands with MW 35, 32 and 28 kDa in eggs and albumen gland that correspond to ovorubin subunits. These bands were immunoreactive to anti-ovorubin antiserum as shown by Western blots (Fig. 1 B, see below). Regarding this, only two bands were resolved in the Western blot, the upper one corresponded to the 32 and 35 kDa subunits together, and the lower one to the 28 kDa subunit.

Incorporation of ^{14}C-Leu into ovorubin

A pilot experiment was performed to determine the time course of incorporation, adjust pH, tracer concentration, and select incubation time and medium. Microscopic inspection of tissues after 12 h incubation was performed to check for necrosis (data not shown). The results indicate that tissues were viable under the conditions employed. A number of different media composition were tried and the best composition was found to be the modified *Helisoma* salts, buffered with HEPES, as mentioned in Materials and methods. Based on
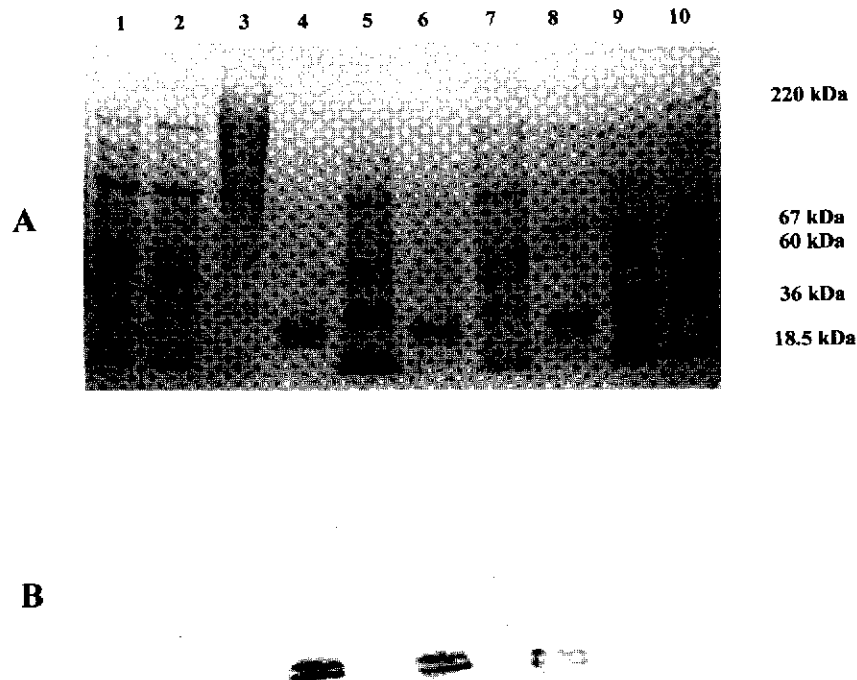
Fig. 1. Analysis of *P. Canaliculata* tissues by dissociating PAGE (A) and Western blot (B). Gels A and B prepared using an acrylamide gradient of 4–15% w/v. Lanes 1: muscle; 2: stomach; 3: hemolymph; 4: ovorubin; 5: gut, 6; albumen gland; 7: lung; 8: egg; 9:gonad–digestive gland complex; 10: STD. STD – ferritin half unit (MW 220,000), albumin (MW 67,000), catalase (MW 60,000), lactate dehydrogenase (MW 36,000) and ferritin (MW 18,500).

the time-course of incorporation, a 12-h incubation period was selected for further analysis of ovorubin synthesis.

All tissues incorporated the tracer into proteins. Tissue extracts from incubations were separated by PAGGE and HPLC (Table 1). Proteins similar to ovorubin in size were found in gonad–digestive gland complex. As specific activity was not enough to perform autoradiography analysis of gels, each gel lane was sliced, digested and quantified by liquid scintillation counting. Results showed that the only tissue incorporating ^{14}C-Leu into a protein of ovorubin MW was the albumen gland, representing 1.3% of the total labelled protein (Table 1). Radio-HPLC analysis of proteins showed a radiolabelled protein peak that eluted at ovorubin elution volume in both albumen gland and in gonad–digestive gland complex. Only the albumen gland protein, however, was immunoreactive to anti-ovorubin antiserum, confirming PAGE results. Western blot analysis using anti-ovorubin antiserum as probe, revealed that albumen gland contained immunoreactive proteins (Fig. 1B), and that immunoreactivity was not observed in homogenates of either muscle, gut, stomach, lung or gonad–digestive gland complex.

Table 1. Percentage of radioactivity incorporated into ovorubin and other protein fractions after 12 h incubation with ^{14}C-Leu

Tissue	Ovorubin		Other proteins	
Albumen gland	1.2 ± 0.9	1.3 ± 0.7	98.8 ± 1.4	98.7 ± 1.9
Gonad–digestive gland complex	Nd	Nd	Nd	Nd
Hemolymph	Nd	Nd	Nd	Nd
Muscle	Nd	Nd	Nd	Nd

Values are the mean of triplicate analysis ± S.D. of results from electrophoresis and HPLC analysis. Ovorubin identity was checked by immunoblot analysis. Nd – not detected.

Incorporation of ^{3}H-Leu into ovorubin

When vitellogenic females were injected with labelled leucine, all tissue extracts contained labelled proteins. The aim of this experiment was to search for the presence of circulating perivitellins. Then, hemolymph was particularly analysed. HPLC and electrophoretic analyses of radiolabelled hemolymph indicated that no protein with ovorubin characteris-

Table 2. Ovorubin titer in different tissues

Tissue	Soluble protein (mg/g tissue)	Ovorubin (mg/g tissue)
Egg	47.5 ± 3.5	28.4 ± 2.1
Albumen gland	37.9 ± 2.9	30.3 ± 2.0
Hemolymph	11.0 ± 0.9	Nd
Gonad–digestive gland complex	17.0 ± 1.3	Nd
Muscle	5.8 ± 0.6	Nd
Gut	7.1 ± 0.5	Nd
Stomach	14.3 ± 1.0	Nd

Values are the mean of duplicate analysis. Nd - not detected.

tics was present. Western blot analysis showed again that hemolymph did not contain any ovorubin-immunoreactive protein (Fig. 1B), though some faint cross reactivity with another hemolymph protein was present (not perceptible in Fig. 1B).

Ovorubin titer in different tissues by ELISA

This method had a linear relationship from 0–12 ng of purified ovorubin, 15 min after the addition of substrate solution. When the samples were analysed, well to well variation was similar to the variation on the standard curve.

Perivitellin was not detected in either hemolymph, muscle, gut, lung, stomach or gonad–digestive gland complex. It was only present in albumen gland and egg cytosol in large quantities (Table 2).

Discussion

The hypothesis that the albumen gland of *P. canaliculata* would be the synthesis site of ovorubin was experimentally demonstrated in two fashions. Firstly, polyclonal antibodies anti PV-1 were utilised to detect this lipoprotein in the snail. Seven tissues were assayed by Western blot, and immunoreactive proteins were found only in the albumen gland. They corresponded to the three protein subunits of the ovorubin native apoprotein [9]. The interpretation that albumen gland is the single site for the synthesis and storage of ovorubin, was also supported by the *in vitro* incubations of selected tissues (albumen gland, muscle and gonad–digestive gland complex). The analysis by electrophoresis and chromatography of the proteins revealed the synthesis of significant quantities of radiolabelled proteins in all of them, but a protein with the characteristics of ovorubin was only found in the albumen gland. Gonad–digestive gland complex was selected because there are several reports on other invertebrates supporting its importance as egg-lipoprotein synthe-

sising sites [2, 17, 18]. Both experiments showed this gland to be the only organ of *P. canaliculata* able to synthesise the protein moiety of the glycolipocarotenoprotein ovorubin.

Vitellogenins circulating in plasma of vitellogenic females have been detected in most oviparous organisms such as vertebrates, crustacean, insects [19, 20] and one report in gastropods [6]. Highly sensitive techniques as *in vivo* leucine radiolabelling and immunoblot were used, though no lipoprotein similar to that of PV1 could be found in vitellogenic *P. canaliculata* hemolymph. This led us to infer that this perivitelline does not follow an entirely heterosynthetic mechanism. The transport of PV1 from the site of origin – the albumen gland – to that of storage – the egg – can be explained on the basis of the snail reproductive tract morphology, the albumen gland being an exocrine gland attached to the oviduct [21]. Consequently, ovorubin and other perivitellin fluid components would be secreted directly into the oviduct, coating fertilised eggs as they pass through the oviduct, adding later an outer covering membrane [4]. A similar behaviour was observed in our laboratory for other *P. canaliculata* perivitelline [11] and by Morishita *et al.*, for a glycoprotein from the pulmonate *Helisoma duryi* [22].

Other gastropods, such as the garden snail *Helix aspersa*, show an heterosynthetic mechanism, the vitellogenins being synthesised in the digestive gland that surrounds the ovotestis, and transported by hemolymph to vitellogenic oocytes [5, 6].

During embryogenesis perivitellus ovorubin is preferentially absorbed since the early stages [23], giving clear proof of the importance of ovorubin during the whole development as a source of structural metabolic precursors and energetic compounds; nevertheless, its principal function for the embryo is not energetic, as reserve egg lipoproteins usually are. Moreover, stabilising and photoprotective functions have been attributed to the carotenoid pigments present as an ovorubin prosthetic group [24, 25]. After hatching, ovorubin becomes the only perivitelline protein present, as previously reported by our laboratory [23]. Quantification of PV1 by ELISA in just-laid eggs showed that it was the most abundant perivitellus lipoprotein. Regarding adult snail ovorubin levels, females assayed were at the end of their vitellogenic period, which was reflected in their albumen gland by the high levels of ovorubin as determined by ELISA and the low rate of ovorubin synthesis (Tables 1 and 2). This suggests again that the albumen gland represents the storage site for the newly synthesised ovorubin, and probably the rate of synthesis assayed at an earlier vitellogenic stage would be greater.

PV1 showed similarities and differences when compared to other vitellinic lipoproteins. Its lipid moieties, mainly free cholesterol and lesser amounts of phospholipids evidenced similar characteristics to other lipoproteins found in *P. canaliculata* [9], and quite different from those lipovitellins in other invertebrates including molluscs [19,20].

14

In a study of *H. duryi* vitellogenesis, Morishita *et al.* [22] found that the albumen gland of this pulmonate snail secretes 3 perivitellins, one of them has a mass of 288 kDa, similar to ovorubin, whereas its apoprotein composition is different. Even more, the three subunits from PV1 did not show any similarity in the N-terminal regions compared to the above mentioned glycoprotein (results not shown).

Nevertheless, additional comparative work on several groups of gastropods will be required to further define the different characteristics of egg reserve proteins synthesis in this heterogeneous taxa.

Acknowledgements

References

1. Wallace RA, Walker SL, Hauschka PV: Crustacean lipovitellin. Isolation and characterization of the major high-density lipoprotein from the eggs of decapods. Biochemistry 6: 1582–1590, 1967

2. Fainzilber M, Tom M, Shafir S, Applebaum SW, Lubzens E: Is there extraovarian synthesis of vitellogenin in Penaeid shrimp? Biol Bull 183: 233–241, 1992

3. Kanost MR, Kawooya JK, Law JH, Ryan RO, Van Heusden MC, Ziegler R: Insect hemolymph proteins. In: P.D. Evans and V.B. Wigglesworth (eds). Advances in Insect Physiology, Vol 22. Academic Press, London, 1990, pp 299–396

4. De Jong-Brink M, Boer HH, Joosse J: Reproductive biology of invertebrates. In: K.G. Adiyodi and R.G. Adiyodi (eds). Mollusca, Vol 1. John Wiley and Sons, New York, 1983, pp 297–355

5. Bride M, Petraca B, Faivre D: The synthesis of vitellogenins by the digestive gland of *Helix aspersa*: Evidence from cell-free translation of mRNA. Cell Mol Biol 38: 181–187, 1992

6. Barre P, Bride M, Beliard R, Petracca B: Localization of yolk proteins and their possible precursors using polyclonal and monoclonal antibodies in *Helix aspersa*. Cell Mol Biol 37: 639–650, 1991

7. Miksys S, Saleuddin ASM: Ferritin as an exogenously derived yolk protein in *Helisoma duryi* (Mollusca: Pulmonata). Can J Zool 64: 2678–2682, 1986

8. Bottke W: Immunolocalization of ferritin polypeptides in oocytes and somatic tissue of the freshwater snails *Lymnaea stagnalis* L.
and *Planorbarius corneus* L. Cell Tissue Res 243: 397–404, 1986

9. Garin CF, Heras H, Pollero RJ: Lipoproteins of the egg perivitelline fluid of the snail *Pomacea canaliculata* (Mollusca: Gastropoda). J Exp Zool 276: 307–314, 1996

10. Cheesman DF: Ovorubin, a chromoprotein from the eggs of the gastropod mollusk *Pomacea canaliculata*. Proc R Soc Lond 149: 571–587, 1958

11. Dreon MS, Lavarias S, Garin CF, Heras H, Pollero RJ: Synthesis, distribution and levels of an egg lipoprotein from the apple snail *Pomacea canaliculata* (Mollusca Gastropoda). J Exp Zool 292: 323–330, 2003

12. Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal Biochem 72: 248–254, 1976

13. Laemmli VK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685, 1970

14. Towbin H, Staehelin T, Gordon J: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc Natl Acad Sci USA 76: 4350–4354, 1977

15. Engwall E, Perlmann P: Enzyme-linked immunosorbent assay, ELISA III. Quantitation of specific antibodies by enzyme-labelled anti-immunoglobulin in antigen-coated tubes. J Immunol 109: 129–135, 1972

16. Kater SB, Mattson MP: Extrinsic and intrinsic regulators of neurite outgrowth and synaptogenesis in isolated, identified *Helisoma* neurons in culture. In: D.J. Beadle, G. Lees, S.B. Kater (eds). Cell Culture Approaches to Invertebrate Neuroscience. Associated Press, London, 1988, pp 1–32

17. Yano I, Chizei Y: Ovary is the site of vitellogenin synthesis in kuruma prawn, *Penaeus japonicus*. Comp Biochem Physiol 86B: 213–218, 1987

18. Meusy JJ: Vitellogenin, the extraovarian precursor of the protein yolk in Crustacea: A review. Reprod Nutr Develop 20: 1–21, 1980

19. Yepis-Plascencia G, Vargas-Albores F, Higuera-Ciapara I: Penaeid shrimp hemolymph lipoproteins. Aquaculture 191: 177–189, 2000

20. Lee RF: Lipoproteins from the hemolymph and ovaries of marine invertebrates. In: R. Gilles (ed). Advances in Comparative and Environmental Physiology, Vol 7. Springer-Verlag, London, 1991, pp 187–208

21. Hinsch GH, Vermeire PE: Histochemistry and ultrastructure of the capsule gland duct of the prosobranch gastropod *Pomacea paludosa*. Invertebr Reprod Dev 17: 203–201, 1990

22. Morishita F, Mukai ST, Saleuddin ASM: Release of proteins and polysaccharides from the albumen gland of the freshwater snail *Helisoma duryi*: Effect of cAMP and brain extracts. J Comp Physiol 182: 817–825, 1998

23. Heras H, Garin CF, Pollero RJ: Biochemical composition and energy sources during embryo development and in early juveniles of the snail *Pomacea canaliculata* (Mollusca: Gastropoda). J Exp Zool 280: 375–393, 1998

24. Cheesman DF, Lee WL, Zagalsky PF: Carotenoproteins in invertebrates. Biol Rev 42: 132–160, 1967

25. Zagalsky PF: Comparative studies on the aminoacid compositions of some carotenoid-containing glycolipoproteins and glycoproteins from the eggs and ovaries of certain aquatic invertebrates. Comp Biochem Physiol 41B: 385–395, 1972