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Energy sources from the eggs of the wolf spider *Schizocosa malitiosa*: Isolation and characterization of lipovitellins

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ABSTRACT

In oviparous species, proteins and lipids found in the vitellus form the lipoproteins called lipovitellins that are the major source of energy for the development, growth, and survival of the embryo. The energy resources provided by the lipovitellins have not yet been investigated in the Order Araneae. Using the wolf spider *Schizocosa malitiosa* (Lycosidae) as an experimental model, we identified and characterized the lipovitellins present in the cytosol, focusing on the energetic contribution of those lipoprotein particles in the vitellus. Two lipovitellins (LV) named SmLV1 and SmLV2 were isolated. SmLV1 is a high-density lipoprotein with 67% lipid and 3.6% carbohydrate, and SmLV2 is a very high-density lipoprotein with 9% lipid and 8.8% carbohydrate. Through electrophoresis in native conditions we observed that SmLV1 has a molecular mass of 559 kDa composed of three apolipoproteins of 116, 87, and 42 kDa, respectively. SmLV2 comprised several proteins composed of different proportions of the same subunits (135, 126, 109, and 70 kDa). The principal lipids of these lipovitellins are sphingomyelin + lysophosphatidylcholine, esterified sterols, and phosphatidylcholine. Lipovitellin-free cytosol contains abundant phospatidylcholine and triacylglyceride related to the *yolk nuclei* (the vitellus organizing center). The principal fatty acids of SmLV1 and SmLV2 are 18:2 n−6, 18:1 n−9, and 16:0. Spectrophotometry detected no pigments in either the lipovitellins or the cytosol. The egg caloric content was 92 cal/g, at proportions of 59.8% protein, 20.1% carbohydrate, and 19.9% lipid. SmLV1 and SmLV2 provided 19.5% and 17.1% of the calories, respectively. Both lipovitellins contribute mainly with proteins (15.8–18%), with the input of carbohydrates and lipids being lower than 1.3%.

1. Introduction

In oviparous species, the main content of nutrients is found in the vitellus or yolk, it being essential for embryonic development. In general the vitellus in invertebrates is composed of lipids, proteins, and carbohydrates, and in many instances, it also contains pigments. Most eggs store nutrients in the yolk in the form of a complex lipoprotein called lipovitellin (LV), or as lipid droplets scattered throughout the cytoplasm. Vitellogenesis, the process of vitellus accumulation, in most invertebrates involves a massive synthesis of vitellogenin (the lipoprotein exclusive to females in the reproductive state) along with the latter deposition in the oocyte in the form of vitellins (majority protein of eggs or LVs, which are lipoproteins present in the vitellus Wallace et al., 1967).

Although terrestrial-arthropod vitellins have been well characterized in insects (Dhadialla and Raikhel, 1990; Chino, 1997; Salerno et al., 2002; Tufail and Takeda, 2008) as well as in aquatic arthopods such as crustaceans (Lubzens et al., 1997; Kawazoe et al., 2000; Chen et al., 2004; Garcia et al., 2006; Walker et al., 2006), the relevant data for arachnids are scarce with the exception of Acari, with vitellins in that group having been reported in several publications. In that order, LVs have been characterized in several species of ticks, containing either vitellins or vitellogenins; the latter are complex hemoglycolipophosphoproteins, compounds of structures assumed to be related mainly to the hematophagia of those ticks. Their lipid and carbohydrate composition is quite similar to that of the vitellins in insects (Tatchell, 1971; Boctor and Kamel, 1976; Chinzei et al., 1983; Dhadialla, 1986; Shanbaky et al., 1990; Schriefer, 1991; Rosell and Coons, 1992; James and Oliver, 1997; Boldbaatar et al., 2010).

The vitelline body—a typical structure studied and reported only in the oocytes of many spider families (von Wittich, 1845)—was found through microscopy to be surrounded by concentric lamellae corresponding to the endoplasmic reticulum (André and Rouiller, 1957; Sotelo and Trujillo-Cenóz, 1957). With respect to function, analyzing the structure through microscopy and based on morphology, Osaki (1972) determined that the vitellin body was most likely an organizing center for the yolk.

In the Order Araneae (spiders) two LVs have been described in *Polybetes pythagoricus* (Sparassidae), named LV1 and LV2. LV1 has

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49% lipids and a density corresponding to a high-density lipoprotein (HDL) and LV2 has a much lower lipid content (9.7%) and the density of a very high density lipoprotein (VHDL) (Laino et al., 2011). The main neutral lipid found was esterified cholesterol at 16 and 24% in LV1 and LV2, respectively; though triacylglycerides (TAG) were also present at 8 and 9.5% along with diacylglycerides at 3 and 4.3%, respectively. With respect to structural lipids, phospholipids were the dominant class at 57 and 47% in LV1 and LV2, respectively. The major fatty acid was 18:1 n−9 in both LVs. Since these particles were not related to hemolymphatic lipoproteins, we assume that the two lipoproteins are reproduction specific (i.e., vitellogenin/LV). Moreover, a putative apovitellin has been described in the ovary and eggs of the spider *Tegenaria atrica* (Agelenidae) (Pourié and Trabalon, 2003).

LVs also have a significant relevance to other lipoproteins. For example, Baker (1988) determined that the vitellogenin of certain invertebrates contains domains homologous to the apoB-100 of the human LDL and human lipase. Moreover, in a comparative genomic and phylogenetic analysis in metazoa, Hayward et al. (2010) recently determined that the genes encoding the LLTPs (i.e., proteins related to vitellogenin/LV) arose significantly earlier in evolution than the arachnids and are in addition more widespread than previously proposed, with those proteins being present in numerous additional bilaterian and nonbilaterian lineages.

In the present work, using the wolf spider *Schizocosa malitiosa* as an experimental model mainly due to its known embryo development lasting about 30 days (Costa and Capocasale, 1985), we identified and characterized the LVs present in the egg cytosol at an early stage of development, with an emphasis on the energetic contribution of those lipoprotein particles to the egg vitellin. The present study constitutes the first investigation of this nature performed in the widespread Order Araneae.

2. Materials and methods

2.1. Animal and lipovitellin isolation

A total of 30 subadult females of wolf spider were caught in Marinda, Canelones (Uruguay) and kept with food (mealworm larvae) in glass jars until they reach adulthood. Afterward, they mated with adult males in open arenas and were raised until spawning. Eggs from 20 egg-sacs (0.33 g/egg-sac) were collected for 3–7 days after laying (due to the great content of vitellus typical of early stages), homogenized in two pools in 3 mL of 50 mM potassium phosphate buffer, pH 7.4 (Buffer A) supplemented with 0.1% (v/v) protease-inhibitor cocktail (Sigma-Aldrich Chemicals, St. Louis, MO, USA), and sequentially centrifuged at 10,000 *g* for 20 min, and then at 100,000 *g* for 60 min to obtain the cytosol. This final cytosolic fraction, upon thawing, was used for the isolation of LVs by density-gradient ultracentrifugation as follows. All assays were performed in triplicate and with independent treatments.

Aliquots of cytosol were overlayered onto a NaBr solution (density 1.28 g/mL) containing 0.01% (w/v) sodium azide and centrifuged at 178,000 *g* and 10 °C for 24 h in a Beckman L8 70 M centrifuge, with a SW 60 Ti rotor. Saline solution of the same density as that of samples was centrifuged in parallel to check the densities of each fraction and thus verify the correctness of the gradient formation. The total volume of each tube was sampled from top to bottom in 0.2 mL aliquots and the protein content of each fraction monitored spectrophotometrically at 280 nm. The zone in the gradient containing the LVs was separated as a whole fraction. The LV-containing samples were dialyzed in Buffer A. The total protein concentration of LV was measured colorimetrically by the method of Lowry et al. (1951). Measurements obtained in the cytosol without LVs were obtained deducting the values of LVs to those of cytosol.

2.2. Electron microscopy

Samples of LV were dialyzed in Buffer A and negatively stained with 2% (w/v) phosphotungstate for examination by electron microscopy (Forte and Nordhausen, 1986). Preparations were made at least in triplicate, viewed on a JEOL 100S electron microscope (JEOL Co., Japan) at 100 kV, and photographed at a final magnification of 120,000×. Approximately 100 free standing lipoprotein particles were measured in multiple photographs taken from different areas of the grid. An additional photograph with the buffer alone was taken as a negative control to confirm the absence of artifacts.

2.3. Gel electrophoresis

Apolipoproteins corresponding to LVs and to the other cytosolic proteins were analyzed by polyacrylamide gel electrophoresis. Separations were performed in 4–23% native and sodium dodecylsulfate (SDS) gels (Laemmli, 1970). The bands obtained under native conditions for one of the LVs (SmLV2) were purified from the gels by electroelution and then run on a 4–23% SDS gel. Molecular masses were calculated as previously described (Garín et al., 1996). Gels were either stained with Coomassie Brilliant Blue R-250 (Sigma-Aldrich Chemical Co.) or silver-stained.

2.4. Lipids and fatty acid characterization

Lipids were extracted following the method of Folch et al. (1957). Quantitative determination of lipid classes was performed by thin layer chromatography coupled to a flame ionization detector in an Iatroscan apparatus model TH-10 (Iatron Laboratories, Tokyo, Japan), after separation on Chromarods type S-III (Ackman et al., 1990; Lavarías et al., 2005). Lipid classes were quantified with monoacylglycerol as an internal standard. The total lipids were determined by gravimetry (Cunningham and Pollero, 1996).

Fatty acid methyl esters from LV total lipids were prepared with BF_3-MeHO according to the method of Morrison and Smith (1992). The analysis was performed by gas–liquid chromatography in a HP-6890 capillary chromatograph (Hewlett Packard, Palo Alto, CA, USA) on an Omegawax 250 30 m × 0.25 mm fused silica column with a 0.25 μm phase (Supelco, Bellefonte, CA, USA). The column temperature was programmed for a linear increase of 3 °C per min from 175 to 230 °C. Peaks were identified by comparing their retention times with those of a mixture of standard methyl esters.

2.5. Carbohydrate determination

Total hexose content of LVs was determined by the anthrone/sulfuric method with D-glucose as the standard (Sigma-Aldrich). The samples were treated with an anthrone solution in concentrated H_2SO_4. After 15 min at 90 °C the absorbance was read at 620 nm (Dubois et al., 1956).

2.6. Absorbance spectrum of LVs and cytosol

The absorbance spectum of LVs and cytosol was measured from 200 to 550 nm in an Agilent 8453 ultraviolet spectrophotometer. For the purpose of comparison it was also measured the absorbance spectrum of the VHDL of the spider *P. pythagoricus* (a hemocyanin-containing lipoprotein) along with ovorubin, a protein from the snail *Pomacea canaliculata* with carotenoid pigment.

2.7. Energetic equivalent of proteins, lipids, and carbohydrates in LVs and cytosol

In the present work, to estimate calories provided by different molecules we used the conversion factor described by Beningher

(1984) and applied in the studies of arthropods (Heras et al., 2000; García-Guerrero et al., 2003) and other invertebrates (Heras et al., 1998). The conversion factors used here are: 4.3 kcal/g for protein, 7.9 kcal/g for lipid, and 4.1 kcal/g for carbohydrate.

3. Results

3.1. Isolation of LVs and cytosol of S. malitiosa eggs

Fig. 1A and B shows, respectively, an egg-sac of a spider five days after laying and the eggs that were used for isolating the cytosol and LVs investigated in this work.

From the eggs laid, the cytosol was separated by homogenization and ultracentrifugation, and two lipoproteins were isolated from the cytosol and characterized by density gradient ultracentrifugation. One of these lipoproteins, SmLV1, of hydration density 1.13 g/mL, was an HDL; whereas the other, SmLV2, of density 1.24 g/mL, was a VHDL (Fig. 2).

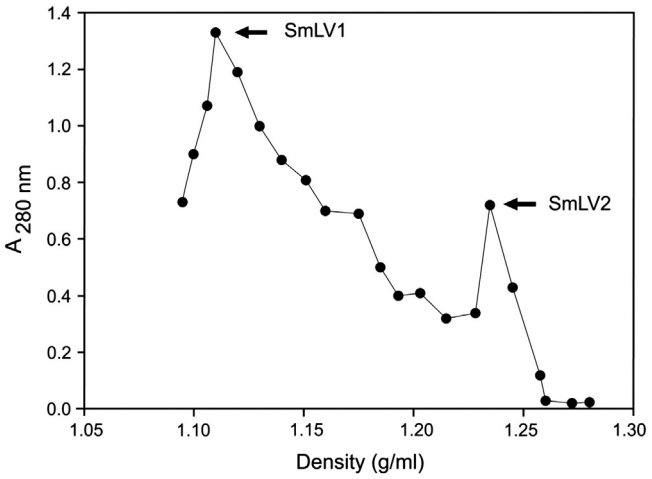

Fig. 2. Protein distribution in the egg cytosol of *Schizocosa malitiosa* after separation by density-gradient ultracentrifugation in 1.28 g/mL NaBr. The protein content of each fraction was monitored spectrophotometrically at 280 nm and density for refractometry.

3.2. Electron microscopy of LVs

Electron micrographs of LVs (Fig. 3A and B) indicated particles of homogeneity in size and a mean diameter of 21 ± 2.6 nm. Both SmLV1 and SmLV2 had a quasispherical shape without the rouleaux structure typical of lipoproteins of discoidal morphology (Forte et al., 1974), while the absence of an artifact in the measurement produced by certain buffers (Forte and Nordhausen, 1986) was verified (Fig. 3C).

3.3. Protein characterization of LVs and cytosol

Fig. 4 shows the results of the analysis of SmLV1, SmLV2, and cytosol by electrophoresis under native conditions. Electrophoresis of cytosol shows at least 7 proteins, most of them have molecular masses similar to those found for the LVs, suggesting that they are the same proteins. This coincides with the content of proteins related to lipovitellin that is 56% of total proteins (cf. below). Only certain proteins and those being found in lower quantities are exclusive to the cytosol. The pattern for SmLV1 consisted in a predominant protein of 559 kDa, whereas that of SmLV2 contained five principal bands (at mobilities corresponding to 492, 405, 293, 213, and 48 kDa).

In order to determine the nature of the interactions that bind the protein subunits together, lipovitellin was subjected to SDS polyacrylamide gel electrophoresis under oxidizing and reducing conditions. These runs indicated that SmLV1 is composed of subunits of apparent molecular masses 116, 87, and 42 kDa, and showed no differences between the electrophoretic profiles obtained in the presence or absence of β-mercaptoethanol, indicating the absence of intermolecular disulfide bridges. In contrast, the electrophoresis of SmLV2 on SDS gels under reducing conditions indicated the presence of 4 subunits of molecular masses 135, 126, 109, and 70 kDa as opposed to the six subunits of molecular masses 213, 167, 135, 126, 109, and 70 kDa observed with the same electrophoresis in the absence of β-mercaptoethanol (Fig. 5). The five bands of SmLV2 were isolated from the native gel by electroelution and analyzed by SDS polyacrylamide gel electrophoresis.

Under these dissociating conditions, lipoprotein SmLV2 fraction showed the same pattern of apolipoproteins though with different proportions, their molecular masses being 135, 126, 109 and 70 kDa (Fig. 6). The behavior of apolipoproteins coincides with that described for spider *P. pythagoricus*, in which lipovitellin LV2 was studied (Laino et al., 2011).

Table 1 indicates the quantities in mg of proteins, lipids and carbohydrates by g of egg, present in lipoproteins (SmLV1 and SmLV2), in egg cytosol without LVs, and the percentages present in each lipoprotein particle. SmLV1 and SmLV2 provide similar quantities of proteins to the egg, 3.9 and 3.3 mg/g of eggs respectively.

Of the two egg cytosol LVs (in terms of the major components) the HDL SmLV1 contains 67% protein, 28% lipid, and 3.6% carbohydrate;

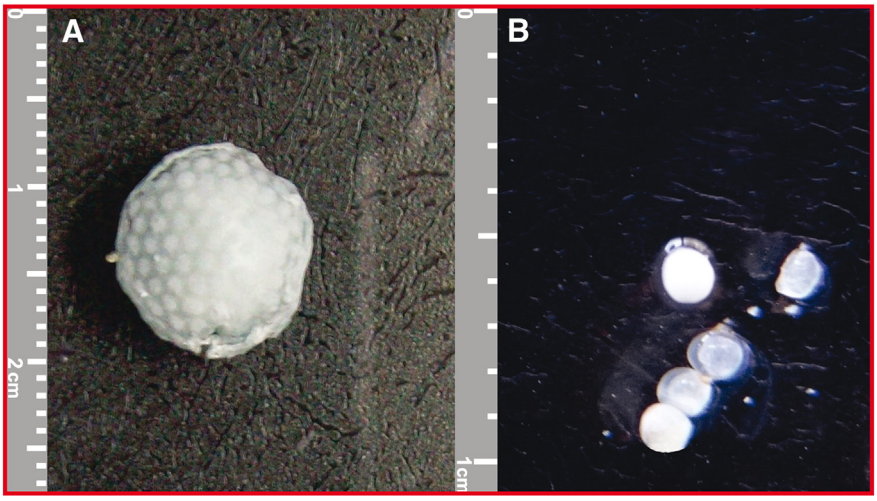

Fig. 1. A photograph of the egg-sac; B: eggs of *Schizocosa malitiosa*.

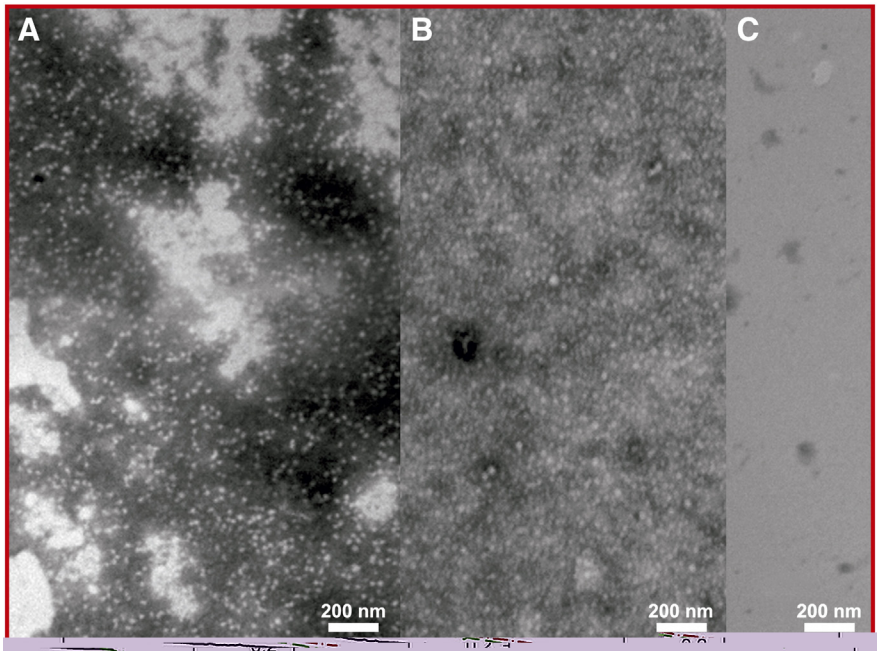

Fig. 3. Electron micrographs of negatively stained lipovitellins isolated from *Schizocosa malitiosa* eggs. Final magnification × 120,000. A: SmLV1; B: SmLV2; C: buffer without LVs used as negative control.

while the VHDL SmLV2 is richer in protein, at 81%, and poorer in lipid, at only 9%, with the remaining 8.8% constituting the carbohydrate.

3.4. Carbohydrate content of lipovitellins and cytosol

The anthrone/sulfuric method indicated the contribution of hexoses to the cytosol to be 0.11 and 0.29 mg/g for SmLV1 and SmLV2, respectively, to give a total of 0.41 mg/g. The great content of hexoses in the cytosol without LVs (4.11 mg/g of eggs) (Table 1) must be noted.

3.5. Absorbance of LVs and cytosol

The absorbance of the two LVs and the cytosol was measured between 240 and 550 nm in an Agilent 8453 spectrophotometer along with the comparable absorbances of the VHDL from the spider *P. pythagoricus* and of the protein ovorubin (with high content of carotenes) from the snail *P. canaliculata* for the purpose of comparison (Fig. 7).

Within that range of wavelength, SmLV1 and SmLV2 absorbed at 280 nm while exhibiting no further absorbance either in the ultraviolet, such as at 340 nm corresponding to oxyhemocyanin, or in the visible spectrum, such as at 450 nm, for ovorubin.

3.6. Characterization of lipids and fatty acids of LVs and cytosol

Of the total lipids in eggs, 24.3% is provided by the LVs, 19.8% from SmLV1 (at 1.6 mg/g of eggs) and 4.5% from SmLV2 (at 0.37 mg/g of eggs); (Table 1).

Apart from the LVs, the principal lipids of the cytosol are phosphatidylcholine (PC), TAG, and phosphatidylethanolamine (PE) at 2630, 2100, and 973 μg/g of egg, respectively. SmLV1 contains sphingomyelin

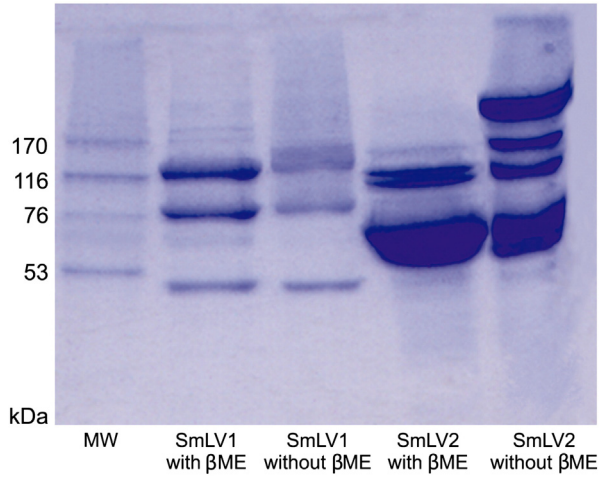

Fig. 4. Native polyacrylamide-gel electrophoresis (4–23% acrylamide gradient slab) of SmLV1, SmLV2, and cytosol. MW: molecular weight standard. Proteins were visualized by Coomassie Brillant Blue R-250.

Fig. 5. Sodium dodecylsulfate polyacrylamide-gel electrophoresis (4–23% acrylamide gradient slab) of SmLV1, SmLV2 with and without β-mercaptoethanol. MW: molecular-weight standard. Proteins were visualized by Coomassie Brillant Blue R-250.

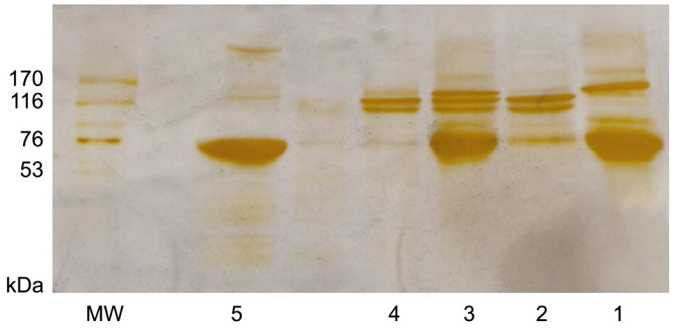

Fig. 6. Sodium dodecylsulfate polyacrylamide gel electrophoresis (4–23% acrylamide gradient slab). Bands electroluted from the native gel of SmLV2: lane 1, 492 kDa; lane 2, 405 kDa; lane 3, 293 kDa; lane 4, 213 kDa; lane 5, 48 kDa. MW: molecular weight standard. Proteins were visualized by silver staining.

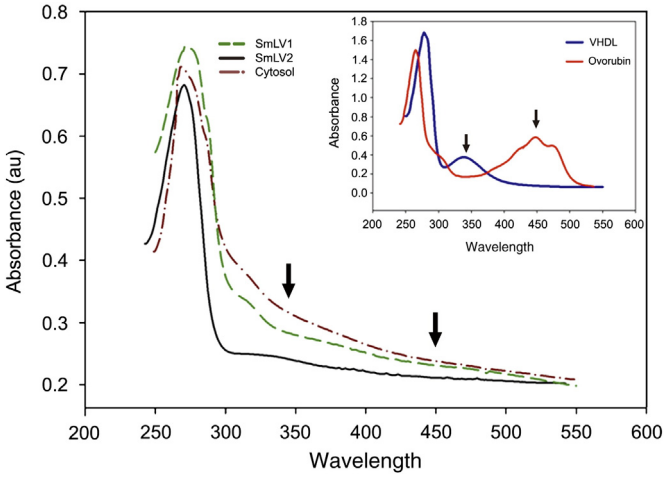

Fig. 7. UV–VIS absorption spectrum of LVs and cytosol. The absorption was recorded within the 220–550 nm range. Inset: VHDL of *P. pythagoricus* used as positive control of hemocyanin absorbance, ovorubin of *P. canaliculata* used as positive control of pigment.

(SM) plus lysoPC, PE, PC, and TAG at 1190, 278, 88, and 51 μg/g of egg, respectively. By contrast, the principal lipids of SmLV2 are SM plus lysoPC at 364 μg/g of egg with the other lipids having values lower than 5 μg/g. Nevertheless, since out of the total SM plus lysoPC of the egg, 98% is provided by the two LVs (75% by SmLV1 and 23% by SmLV2) that input constitutes a significant contribution to the egg overall lipids. Furthermore, although that total lipid content of eggs is low, 53% of the esterified sterols are provided by the LVs. For the case of PE, LVs provided 22.3% (Table 2).

With respect to fatty acids, the percentages are similar for both the LVs with 18:2, 18:1, 16:0 and 18:0 being the principal ones (each at between 20 and 25%) and the fatty acid content of the cytosol which is not significantly different, showing that lipids not related to proteins have similar composition of fatty acids (Table 3). Our analyses further indicated that the fatty acids of both the LVs and the cytosol are 43–47% saturated, 24–25% monounsaturated, and 27–31% polyunsaturated (Table 3).

3.7. Energetic equivalent of the proteins, lipids, and carbohydrates in the LVs and the cytosol

The total caloric content of the eggs was 92 cal/g, which energy equivalent is represented almost completely by protein at 59.8%, carbohydrate at 20.1%, and lipid at 19.9%.

SmLV1 and SmLV2 provide 19.5% and 17.1% of those total calories, respectively. The principal contribution of both LVs is in the form of protein at 15–18%, with the input from carbohydrate and lipid at 0–1.32%, being virtually negligible (Table 4). The caloric content of the lipids was estimated on the basis of only the most energetic species such as TAG, diacylglycerides, and free fatty acids.

4. Discussion

Two LVs were identified and isolated from the eggs of the spider *S. malitiosa*. One referred to as SmLV1, of protein concentration 3.9 mg/g of egg and containing 28% lipid, is an HDL because of its hydrated density of 1.13 g/mL. This percentage of lipids is comparable to the 49% lipid found in the LV1 of the spider *P. pythagoricus* (Laino et al., 2011).

In contrast, SmLV2 with a lipid content of 9% and a hydrated density of 1.24 g/mL is a VHDL. A similar lipid content and hydrated density were described for the LV2 belonging to *P. pythagoricus*. This scant lipid content has also been reported for tick LVs: (Boctor and Kamel, 1976; Chinzei et al., 1983). The lipid content of the vitellogenins and the egg lipoproteins of insects is also lower and ranges from 7 to 9%; (Chino et al., 1977; Chinzei et al., 1981).

With 2.02 mg of lipids/g of egg bound to the apolipoproteins out of a total vitellus lipid content of 8.02 mg/g, the SmLV1 and SmLV2 provide some 25% of the vitellus lipid. A lower value was reported for the mollusk *P. canaliculata* at 3.75 mg/g of egg (Heras et al., 1998), though a higher figure was cited for the crustacean *Macrobrachium borellii* at 98.0 mg/g of egg (Heras et al., 2000). The lipid content of invertebrate eggs would therefore appear markedly variable among the different phylogenetic groups (cf. further on).

In general, many of the lipoproteins related to reproduction are glycolipoproteins: in the present experimental model *S. malitiosa*, the content of hexose is slightly elevated in relation to the only LVs described in arachnids (Laino et al., 2011). In this species, SmLV1 and SmLV2 contain 3.6 and 8.8% carbohydrate, respectively; whereas in *P. pythagoricus* those same values were found to be 1.4% and 0.69% (Laino et al., 2011). In ticks the values for the lipoproteins of the hemolymph and of the vitellus have been reported to range between 3 and 12.4% (Boctor and Kamel, 1976; Chinzei et al., 1983; Maya-Monteiro et al., 2000).

The molecular mass of SmLV1 (559 kDa) is similar to that described by Laino et al. (2011) for the LV1 of *P. pythagoricus* (550 kDa). The native molecular masses of the LV2s of these two species also share similarities (cf. further on). The size of both these lipoproteins corresponds to the range described for the LVs of ticks and insects. Furthermore, Garcia et al. (2010) and Garcia-Orozco et al. (2002) have reported the same molecular-mass range (300 to 600 kDa) for the LVs of crustaceans, and in mollusks the so-called *perivitellin* was described as being of a similar molecular-size class (Kisugi et al., 1987; Garín et al., 1996). In SmLV2 the behavior seems to be similar to that described for LV2 of *P. pythagoricus* in which some proteins appear in a same density fraction after the analysis by electrophoresis in native conditions, corresponding with different aggregates of the same apolipoproteins described in dissociating conditions. Laino and collaborators in 2011 explained this situation since it is accepted in general that the vitellins of insect are

Table 1
Quantity and percentage of proteins, lipids, and carbohydrates in SmLV1, SmLV2, and cytosol without LV. Values are the means of three pools of 3 egg-sacs, with each one analyzed separately.

	SmLV1	SmLV2	Cytosol without LVs
Proteins mg/g eggs	3.9 ± 2.5	3.3 ± 0.8	5.4 ± 3.4
Lipids mg/g eggs	1.6 ± 1.0	0.37 ± 0.09	6.2 ± 3.5
Carbohydrate mg/g eggs	0.11 ± 0.03	0.29 ± 0.05	4.1 ± 0.3
Density	1.13	1.24	–
Proteins %	67	81	38.9
Lipids %	28	9.09	39.08
Carbohydrates %	3.6	8.8	21.5

Table 2
Quantities of different lipid classes in SmLV1, SmLV2, and cytosol without LV per mg of egg. Percentages are analyzed for each lipid in particular. Values are the means of three pools of 3 egg-sacs each, analyzed separately.

	SmLV1	SmLV2	Cytosol without LVs	Total
Phosphatidylcholine μg/mg of eggs	88.8 ± 8.4	3.21 ± 0.4	2630 ± 320	2722 ± 1644
%	(3.2%)	(0.11%)	(96.6%)	(100%)
Triacylglycerols μg/mg of eggs	51.4 ± 44.4	0.18 ± 0.07	2099.7 ± 449	2151.3 ± 532
%	(2.3%)	(0.008%)	(97.6%)	(100%)
Sphingomyelin + lyso phosphatidylcholine μg/mg of eggs	1190 ± 89	364.6 ± 29	21.3 ± 11.2	1577 ± 930
%	(75.5%)	(23.1%)	(1.3%)	(100%)
Phosphatidylethanolamine μg/mg of eggs	278 ± 26.3	1.34 ± 0.7	973.4 ± 141	1253.3 ± 210
%	(22.2%)	(0.10%)	(77.6%)	(100%)
Cholesterol μg/mg of eggs	17.2 ± 3.9	0.93 ± 0.6	429 ± 320	447.3 ± 210
%	(3.8%)	(0.20%)	(96%)	(100%)
Diacylglycerol μg/mg of eggs	9.0 ± 6.4	0.29 ± 0.1	146.6 ± 112	156 ± 113
%	(5.8%)	(0.19%)	(93.9%)	(100%)
Free fatty acids μg/mg of eggs	3.1 ± 1.1	0.18 ± 0.003	22.3 ± 13	25.6 ± 17
%	(5.5%)	(0.44%)	(94%)	(100%)
Hydrocarbons μg/mg of eggs	0.18 ± 0.2	0.03 ± 0.003	6.5 ± 4.8	6.7 ± 5.7
%	(2.7%)	(0.5%)	(96.8%)	(100%)
Esterified sterols μg/mg of eggs	0.59 ± 0.1	0.59 ± 0.03	1.04 ± 0.27	2.2 ± 0.6
%	(26.4%)	(26.7%)	(46.8%)	(100%)

less soluble than their corresponding vitellogenins (Laino et al., 2011). This fact is thought to facilitate storage inside oocytes, following the loss of certain lipids during the uptake from hemolymph (Kunkel and Nordin, 1985) as well as generating protein aggregates. Similarly, other investigators determined the possibility of the formation of aggregates in tick vitellogenin (Chinzei et al., 1983).

The electrophoresis under dissociating conditions indicated the absence of disulfide bridges in SmLV1, but the presence in SmLV2. The interpretation of this result is difficult since no data on the egg LVs in arachnids are available. The closest observation was on the hemolymphatic lipoproteins of the scorpion *Scorpio maurus*, where the existence of disulfide bridges was found (Maaroufi and Lamy, 1993).

In egg cytosol, a great quantity of proteins is present whose molecular masses correspond to those of the LVs, an observation that would appear reasonable since 56% of the cytosolic proteins are provided by the LVs. The non-LV proteins possibly correspond in part to embryo structures. Nevertheless, even though the egg cytosol was isolated before the seventh day of embryonic development, we cannot rule out the possibility that the presence of small fragments might be a result of the action of proteases hydrolyzing the LVs similar to what has been observed in crustacean lipovitellins (Garcia et al., 2008) and tick vitellins (Chinzei et al., 1983).

Spheroid morphology of these LVs observed by electron microscopy with negative staining is similar to that described for the two LVs of *P. pythagoricus* (Laino et al., 2011) and for a few lipoproteins of crustaceans and insects (Gilbert and Chino, 1974; Pattnaik et al., 1979; Soulages and Wells, 1994, and Garcia et al., 2002). This spheroid morphology may result from the ability of neutral lipids to generate a hydrophobic core (Garcia et al., 2010; Laino et al., 2011). In *S. malitiosa*, however, the neutral-lipid content of the SmLV1 is little and of the SmLV2 very little. Therefore, in these lipoproteins the hydrophobic amino acids may come together within the structure of the protein to generate a portion of the core.

Although in certain phylogenetic groups such as the crustaceans the binding of pigments to vitellins is common in order to form lipocarotenoprotein (Wallace et al., 1967; Zagalski, 1985; Sagi et al., 1995), up to the present pigments associated with the LVs have not been found in *S. malitiosa*, *P. pythagoricus*, or other arachnids (Laino et al., 2011). When present, the different pigments impart a coloring to the eggs (Zagalski, 1985) that protects the ovaries and embryos from radiation of wavelengths in the visible and ultraviolet range (Hairston, 1976, 1979; Sagi et al., 1995). With spiders, that sort of protection may be conferred by the silk of the cocoon in which the eggs are laid. This silk maintains a favorable level of humidity and provides thermal insulation (Hieber, 1985). Thus, the characteristic off-white color of *S. malitiosa* eggs results from the absence of such pigments. Moreover, the arachnid LVs contain no hemocyanin (as evidenced by the absence of oxyhemocyanin-associated spectroscopic absorbance), and therefore the pigment is probably produced either at some time after birth or in the late stages of embryo development, similar to the circumstance in mollusks (Decleir and Richard, 1970).

The major lipid constituents of eggs were—apart from TAG—the structural lipids PC, PE, and SM. These same lipid classes are also the principal ones found in the crustacean *M. borellii*, in which species

Table 3
Fatty acid composition of SmLV1, SmLV2, and the cytosol isolated from eggs of *S. malitiosa*. Data on fatty acid classes are expressed as weight percentage and quantified by GLC-FID. Values are the mean ± SD of three independent analyses of two pools from ten egg-sacs each.

Fatty acid % of	SmLV1	SmLV2	Cytosol
14:0	2.4 ± 2.4	6.8 ± 0.29	1.4 ± 0.15
15:0	0.6 ± 0.06	1.06 ± 0.49	0.59 ± 0.06
16:0	20.6 ± 0.6	24.9 ± 0.2	18.4 ± 0.59
16:1	1.8 ± 0.09	1.33 ± 0.83	1.4 ± 0.07
16:2	0.7 ± 0.003	0.46 ± 0.32	1.07 ± 0.005
18:0	19.4 ± 5	14.6 ± 0.42	23.9 ± 6.2
18:1	23.9 ± 3.3	23.9 ± 1.7	22.4 ± 3.1
18:2	25.6 ± 2.3	23.7 ± 0.62	24.5 ± 2.2
18:3	2.7 ± 0.21	1.51 ± 0.66	2.9 ± 0.23
20:1	0.17 ± 0.10	0.63 ± 0.53	0.45 ± 0.28
20:4	1.04 ± 0.06	0.27 ± 0.18	1.44 ± 0.08
20:5	0.83 ± 0.05	0.61 ± 0.5	1.44 ± 0.08
Σ saturates	43 ± 5.2	47.4 ± 0.7	44.4 ± 7
Σ monounsaturates	25.8 ± 3.4	25 ± 3.03	24.2 ± 3.4
Σ polyunsaturates	30.8 ± 1.1	27.3 ± 1.6	31.1 ± 2.5

Table 4
Quantity of calories provided by proteins, lipids, and carbohydrates in SmLV1, SmLV2, and the lipovitellin-free cytosol of *S. malitiosa* eggs. Values are expressed as total calories/g (wet mass). Percentages are expressed related to the total of calories/g eggs.

	SmLV1	SmLV2	Cytosol without LVs	Total
Proteins calories/g egg	17	14.5	23.6	55.2
% Total calories/g eggs	(18.4%)	(15.8%)	(25.5%)	(59.8%)
Lipids calories/g egg	0.49	trace	17.9	18.4
% Total calories/g eggs	(0.53%)	(0%)	(19.4%)	(19.9%)
Carbohydrate calories/g egg	0.47	1.2	16.8	18.5
% Total calories/g eggs	(0.51%)	(1.32%)	(18.2%)	(20.1%)
Total calories/lipoproteins	18.02	15.7	58.3	92.2
%	(19.5%)	(17.1%)	(63.3%)	(100%)

the structural lipid content of the vitellus is 30% (Heras et al., 2000). Presence of PC and SM may be related to organogenesis since these lipids play a fundamental structural role in biomembranes. The high levels of structural lipids in the cytosol of *S. malitiosa* correlate with the substantial amounts of these species that are present in both SmLV1 and SmLV2. In the LV1 and the LV2 of *P. pythagoricus*, those same structural lipids constitute 55.9 and 46.9% of the total lipids, respectively (Laino et al., 2011). PC is furthermore the major lipid in the LVs of the ticks *Ornithodoros moubata* (at 77%) and *Boophilus micropulus* (at 43%); (Chinzei et al., 1983; Maya-Monteiro et al., 2000) as well as in *M. borellii* (at 30%); (Garcia et al., 2004). In lipoproteins related to insect reproduction, phospholipids have also been found to be the most abundant lipid class, with values ranging from 51% to 84% (Chino et al., 1977; Izumi and Tomino, 1980; Chinzei et al., 1981).

SM has numerous functions that appear to be related to embryo development—e.g., cell cycle arrest (Jayadev et al., 1995), stimulation of inositol-phosphate production (Orlati et al., 1998), cell proliferation and differentiation, cellular-membrane trafficking, among others (Yunoki et al., 2008; An et al., 2011). The presence of SM in the lipoproteins related to the reproduction of crustaceans has also been reported (Garcia et al., 2002). In *M. borellii*, for example, the presence of SM was found in both the vitellogenin and the LV, but not in the hemolymphatic lipoprotein, thus pointing to a reproduction-related function (Garcia et al., 2002, 2004). In this regard, the presence of SM may certainly be associated with any of the above-mentioned functions as well, apart from participating structurally in organogenesis.

Using electron microscopy, Sotelo and Trujillo-Cenóz (1957) studied what they referred to as the *vitellin body* of the oocytes from spiders of the families Lycosidae and Thomisidae. The authors determined that the structure contained a thick layer of multiple, concentrically arranged lamellae surrounding a central core composed of granular or vesicular elements. Their so-called "Balbiani vitelline bodie" was subsequently referred to as *Balbiani bodies*, or nowadays simply *yolk nuclei*. Jedrzejowska and Kubrakiewicz (2010), using the spider *Clubiona* sp., demonstrated that the yolk nuclei also contain endoplasmic-reticulum cisternae, mitochondria, lipid droplets, and other organelles. This thick-layered structure is possibly composed of the polar lipids cited above that would serve as structural elements for embryogenesis. These lipids may possibly contribute to the content of polar lipids found in the cytosol.

High percentage of esterified cholesterol in both the LVs is striking. This characteristic is in agreement with the findings for the lipoproteins of *P. pythagoricus*, in which species esterified cholesterol was the major neutral lipid. The large amount of cholesterol ester present in spider LV can be regarded as a sterol reservoir in the egg for providing structural components and metabolic precursors for embryonic development (Laino et al., 2011).

The TAG content of the LVs is low compared to that of the vitellus, similar to the circumstance with *M. borellii* (Heras et al., 2000). Although the percentage of that class is quite low, the TAG nevertheless represents major lipidic energy stores within the composition of SmLV1 and SmLV2, as is also true with *P. pythagoricus* (Laino et al., 2011). These lipid species are probably one of the principal energetic resources for the developing embryo. By contrast, diacylglycerides are the predominant circulating energy-rich lipids both within the hemolymphatic lipoproteins of the tarantula *Eurypelma californicum* and in insects (Chino et al., 1981; Chinzei et al., 1981; Haunerland and Bowers, 1987).

The fatty acid profile of the LVs contains 45% saturated fatty acids, consisting mainly 16:0 and 18:0. The corresponding values for the LVs of *P. pythagoricus* were found to be somewhat lower, but those figures were still within the range reported for arthropods in general. The predominant monounsaturated and polyunsaturated fatty acids of *S. malitiosa* in the present work were 18:1 n−9 and 18:2 n−6. The pattern of the principal LV fatty acids of *S. malitiosa* corresponds to that previously reported for *P. pythagoricus*, those data being the only ones on spiders available up to the present time (Laino et al., 2011). These same major fatty acids are present in the midgut diverticula of adult arachnids, their main organ of lipid synthesis (Laino et al., 2009).

At the present time no data are available on the function of fatty acids in the eggs of the Order Araneae. The most closely related information in the literature pertains to the Order Acari (*Amblyomma hebraeum*), where the fatty acids 16:1 and 18:2 of whole eggs have been reported to have an antimicrobial activity against Gram negative bacteria (Yu et al., 2012). Since 16:1 and 18:2 are the most abundant unsaturated fatty acids in the eggs of *S. malitiosa*, the possibility that these fatty acids in the spider eggs might likewise have some degree of antimicrobial activity is certainly within reason and would warrant investigation.

Oviparous animals usually undergo embryogenesis in the absence of exogenous nutrients, utilizing exclusively the components (nutrients) of the vitellus: proteins, lipids, and carbohydrates. The proportions of those three components in the egg vitellus are variable among different arthropods, and this variability may stem from the differences in their reproductive strategies. The general thinking on the role of nutrition in arthropod reproduction often implicitly assumes that reproductive biomass, measured as the product of offspring size and number, is proportional to the maternal energetic investment (Sloggett and Lorenz, 2008). Furthermore, the literature on energy metabolism within this context is usually concerned with lipid consumption as a whole (Heras et al., 1998), while few studies have discriminated among the different lipids. In the present work the analysis of energetic resources (calories) has been restricted to TAG, diacylglycerides, and free fatty acids, in order to generate a more detailed understanding of the functioning of the various energy resources.

In the insect *Adalia bipunctata* proteins are the major constituents of the eggs followed by lipids and to a lesser extent by carbohydrates (Sloggett and Lorenz, 2008). Though exceptions exist, in mollusks — and in particular in the Class Bivalvia— proteins are likewise the principal component at 40–68% by weight, with lipids comprising 10–14% and carbohydrates constituting only 1–6% (Holland et al., 1975). In the acari Ixodidae, proteins are the most abundant component of the yolk, and vitellin is the major protein. These proteins, however, are not used until a late stage of embryogenesis. Rather, during early embryonic development, lipids and carbohydrates constitute the major energy sources (Campos et al., 2008). Similar to the Ixodidae tick eggs, those of the spider *S. malitiosa* contain mainly proteins, which component provides a greater caloric input, with lesser amounts of lipids and carbohydrates in similar proportions. More specific details on the role of these different sources of energy during the various stages of embryogenesis in the spider must await the appropriate biochemical analyses.

Meanwhile, these basic studies have been necessary in order to garner an initial understanding of the energetic and biochemical mechanisms involved in the reproduction of the members of this numerous and diverse Order of the Araneae, where the data heretofore have been either scarce or totally lacking.

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