J Comp Physiol B
DOI 10.1007/s00360-017-1120-7

ORIGINAL PAPER

Embryonic and post-embryonic development inside wolf spiders' egg sac with special emphasis on the vitellus

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Received: 30 May 2017 / Revised: 18 July 2017 / Accepted: 25 July 2017

Abstract The development of *Pardosa saltans* wolf spiders inside an egg sac includes two periods: an embryonic period and a post-embryonic period after hatching. We investigated spiderlings' energy expenditure to assess energetic costs during the different embryonic and post-embryonic developmental stages during which they are confined within their egg sac. We focused on the following developmental stages: egg, embryonic stages 1 and 2, and two stages, separated by a moult, during post-embryogenesis inside the egg sac: "juvenile instars 1 and 2" until emergence of 2 instar juveniles from their egg sac. We present the first biochemical characterization of the vitellus of wolf spiders' eggs, embryos and juveniles. Lipovitellins (LV) are composed of four apolipoproteins of 116, 87, 70 and 42 kDa, respectively, and LV represent 35–45% of total protein during development. The principal LV lipids are triglycerides, phospholipids, free fatty acids and sterols. Egg caloric content averaged 127 cal/g (proteins: 91 cal/g, lipids: 33 cal/g, carbohydrates: 3 cal/g). During development from undivided egg to emerged "juvenile 2", 67% of proteins, 51% of carbohydrates and 49% of triglycerides stocks were depleted. At the end of the post-embryonic period, at emergence from egg sac, body energy stock of "juveniles 2" was 38% of the initial calorie stocks in the eggs.

Keywords Lipovitellin · Biochemical status · Energetic state · Developmental period · Arachnids

Introduction

Embryogenesis in oviparous animals proceeds independently through the direct influence of maternal activity. In many animal species, a large quantity of nutritive material or yolk (vitellus) is deposited in the egg, the amount depending on the length of time before the young animal can feed itself. The yolk provides the developing embryo, be it a worm, a tadpole or a chicken, with the nutrients essential for growth within its birthplace, the egg (Byrne et al. 1989). The term yolk does not refer to any particular substance but in fact includes proteins and lipids with a relatively small amount of carbohydrates, vitamins and minerals, all of which substances occur in various proportions in the eggs of different vertebrate and invertebrate animals (Byrne et al. 1989). These compounds are usually associated leading to the formation of lipovitellins (LV) that are a major nutrient source for oviparous embryos (Kunkel and Nordin 1985; Wallace 1985). Most arthropods lay centrolecithal eggs and LV have been purified and characterized from several species: crustaceans (Chen et al. 2004; García et al. 2006; Kawazoe et al. 2000; Lubzens et al. 1997) and insects (Chino 1997; Dhadialla and Raikhel 1990; Tufail and Takeda 2008) but data concerning arachnids, except three Acari (Boctor and Kamel 1976; Chinzei et al. 1983; Tatchell 1971), and two spider species (Laino et al. 2011, 2013) are poor.

Communicated by G. Heldmaier.

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Published online: 31 July 2017

In the process of embryogenesis, utilization of reserved nutrients is auto-regulated related to the program of embryogenesis and differentiation (Masetti and Giorgi 1989; Raikhel and Dhadialla 1992). Hatching of the egg usually occurs when histogenesis is complete and newly born arthropods have diverse forms (Minelli et al. 2013). The time required to complete embryonic development varies widely among different arthropod taxa, ranging from 30 h to more than 3 months (Hinton 1981). Studies on the role of yolk during embryogenesis have been restricted mainly to changes in the yolk protein content of insect eggs (Yamashita and Indrasith 1988; Izumi et al. 1994). During insect embryonic development the carbohydrate/protein and protein/fat ratios decline with age and there is a succession of sources of energy, carbohydrate preceding protein and protein preceding fat (Dovner 2012). Little is known about embryonic development and yolk utilization in spider.

Spider lays centrolecithal eggs and most spiders enclose their eggs in some form of silken egg sac (Foelix 2011). Embryonic development of eggs occurs within this structure and is often divided into two parts by the incident of birth or hatching: the prenatal part and the postnatal part. The prenatal part (or embryonic period) encompasses development from the time eggs are fertilized until the typical shape of the spider's body is established. Descriptions of spiders' embryonic development are confusing because authors use different terms (e.g. pre-larva, larva, pullus, pre-nymph or embryo) for the different embryonic stages (Canard 1987; Canard and Stockmann 1993; Downes 1987; Mittmann and Wolff 2012; Wolff and Hilbrant 2011). After hatching, development is qualified as postnatal (or post-embryonic). Spiders hatch in the cocoon and emergence can sometimes last a few hours or days before the first instar juveniles are released (Canard 1987; Vachon 1957). Emergence from the egg sac takes place after the moult which releases a juvenile equipped with all its organs (Vachon 1958; Wurdak and Ramousse 1984). As the development of spiders inside egg sacs includes several stages, embryos have to rely on energy supplied by nutriments in the vitellus provided by mothers. Notably, the yolk of an egg cell must contain all the energy needed for embryonic development, hatching and moulting, as well as for all the activities of a young spiderling before it actually catches its first prey (Anderson 1978; Schaefer 1976). This raises questions concerning use of vitellus. Therefore, we investigated spiderlings' energy expenditure to assess their energetic costs during the embryonic and post-embryonic development periods while they were confined within their egg sac.

We selected the free-moving wolf spider, *Pardosa saltans*, as our model lycosid species. These spiders live in forests, woodlands and copses and sometimes nearby grasslands and hedgerows. The breeding season of *P. saltans* extends from April to September when adult spiders copulate and females' maternal behaviour develops. Their reproduction usually presents two peaks in France: one in late spring–early summer, and a second in autumn (Ruhland et al. 2016a, b). *P. saltans'* maternal behaviour can be divided into the following stages: construction, transport, care, perforation of the egg sac, and care of spiderlings after they emerge from the egg sac. *P. saltans* females carry their egg sacs for about 25–30 days and mothers open their egg sac to facilitate the emergence of their young, as do other spiders (Ruhland et al. 2016a, b; Whitcomb et al. 1966).

Our aim was threefold: first, to identify two embryos' late developmental stages and two juveniles' developmental stages inside the egg sac laid by female *P. saltans* and to evaluate the contribution of protein, carbohydrates and lipids to the development of spiderlings; second, to identify the LV and the lipids present during spiderlings' development; and third, to evaluate the contributions of protein, carbohydrates and triglycerides to the maintenance of juveniles' energy. We hypothesized that: (1) trophic eggs inside the egg sacs allow juveniles to reconstitute their energy reserves after hatching and before emergence; (2) triglycerides are used as a priority source of energy. The recently proposed embryonic and post-embryonic development stages for *Cupiennius salei* (Wolff and Hilbrant 2011) and *Parasteatoda tepidariorum* (Mittmann and Wolff 2012) provided us a basis for describing the developmental stages of *P. saltans*. We applied this system with the aim to establish a standardized description of spiders' developmental stages.

Materials and methods

Ethics statement

Our research conformed to legal requirements and guidelines established for the treatment of animals in research using invertebrate species and for their care using accepted ethical laboratory standards. The species used for the experiments is not endangered or protected in Europa.

Spider collection and rearing

Our subjects were young adult females and were captured during the copulation period in a forest near the Paimpont biological station (property of the University of Rennes 1; France; 48°00′05.67″North, 2°13′46.65″West) in April–June 2015. Females were housed individually in circular terrariums (10 cm diameter × 5 cm high) without any substrate on bottom and were kept at 20 ± 1 °C, with 57 ± 1% relative humidity under a L:D, 14:10 h

photoperiodic cycle. Spiders were fed every other day ad libitum, with either juvenile cricket (*Acheta domestica* and *Nemobius sylvestris;* 5 days after egg emergence) or adult flies (*Drosophila melanogaster*). All females were checked four times a day (8–9h, 11–12h, 14–15h, 17–18h) to record oviposition.

Experimental groups

The spiders studied came from 100 egg sacs built by spiders in the laboratory. They were divided into six experimental groups related to embryonic and post-embryonic stages inside the egg sac, before emergence of young (Fig. 1). The embryonic period was divided into three groups: 1–2 h (egg stage, $n=20$), 5 days ("embryo 1" instar, $n=20$), and 10 days ("embryo 2" instar; $n=20$) after oviposition and before hatching. The post-embryonic period included two groups after hatching: juveniles after the 1st moult, i.e. 15 days after oviposition ("juvenile 1" instars; $n=20$) and after the 2nd moult, i.e. 20 days after oviposition ("juvenile 2" instars; $n=20$); and one group at the moment of emergence from egg sac, i.e. 30 days after oviposition ("juvenile 2E" instars; $n = 20$). All egg sacs were dissected on micrographs and egg, embryo, and juvenile developmental instars were investigated. Individuals from each developmental instar were placed in an Eppendorf tube (1.5 mL) then weighed using a Sartorius electronic balance (Palaiseau, France) (±0.01 g). The entire mass was divided by the number of individual inside tube.

Development period and energetic state

After weighing, individuals in the Eppendorf tubes were freeze-killed at −18 °C. Each individual was mixed in 50 µL of water, homogenized (5 min) and vortexed for 30 min and centrifuged at 10,000 rpm, for 10 min at 5 °C (Ruhland et al. 2016b). Three aliquots (10 µL/aliquot) of supernatant were taken to assay carbohydrates and proteins. Lipids from the homogenate sample were extracted with 600 µL of methanol–dichloromethane (1v/2v) (Sigma, Saint-Quentin Fallavier, France), vortexed for 30 min and centrifuged at 10,000 rpm, for 10 min at 4 °C. The pellets were discarded and the supernatant was aliquoted for further analyses. We triplicated the analysis of each sample and then calculated the ratio of lipids, proteins, and carbohydrates related to wet mass. Energetic state was assessed from 20 extracts for each developmental stage.

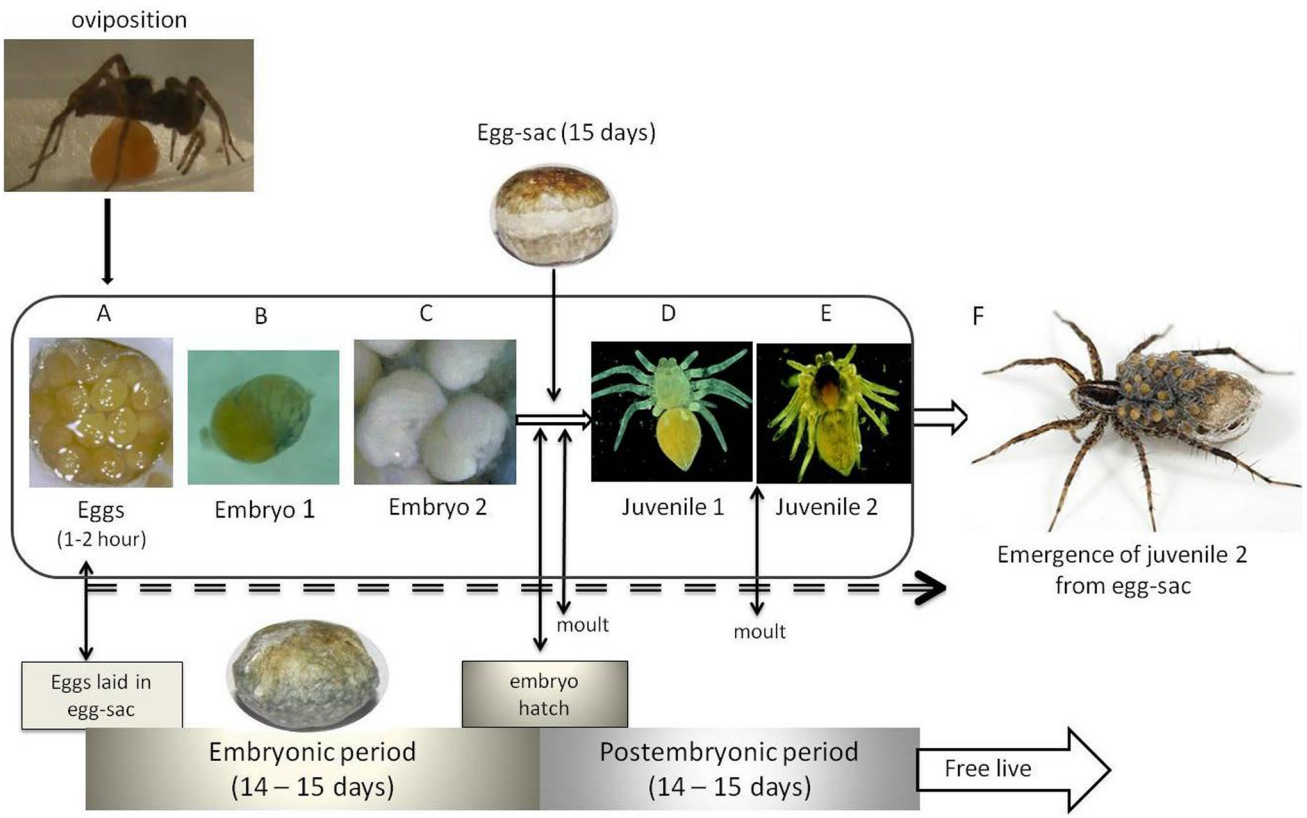

Fig. 1 Biological cycle of the embryonic and post-embryonic development of *P. saltans* (Lycosidae) in the egg sac until emergence, under laboratory conditions (20 ± 1 °C, 57 ± 1% relative humidity)

Quantification of carbohydrates and free glucose

Total carbohydrate concentration in a 10-µL homogenate sample was assessed using a colorimetric method (Total Carbohydrate Assay kit, Sigma-Aldrich, St Louis, USA) based on the phenol–sulfuric acid method in which polysaccharides are hydrolyzed and then converted to furfural or hydroxylfurfural with a yellow-orange colour as a result of the interaction between the carbohydrates and the phenol. We divided the carbohydrate level data by fresh weight to obtain concentrations in µg/mg.

Free glucose concentrations in a 10-µL homogenate sample were determined using a colorimetric method after enzymatic oxidation in the presence of glucose oxidase (Glucose-test, Randox, Crumlin, Co, Antrim, UK). The hydrogen peroxide formed reacted in the presence of peroxidase with phenol and 4-aminophenazone to form a red-violet quinoneimine dye as the indicator. We divided the free glucose level data by fresh weight to obtain concentrations in µg/mg.

Identification and quantification of proteins and lipovitellin

Quantification of proteins

Total protein content in a 10-µL biological sample was determined according to Bradford's method (1976) with a Coomassie protein assay kit (Thermo Scientific, Cergy Pontoise, France) using bovine serum albumin as the standard. We divided the protein level data by fresh weight to obtain concentrations in µg/mg.

Anti-lipovitellin rabbit serum preparation

Antibodies directed against purified lipovitellin of *Schizocosa malitiosa* (SmLV) were prepared in rats (Laino et al. 2013). Rats were given subcutaneous injections of about 1 mg of SmLV emulsified in Freund's complete adjuvant (Sigma Chemical Co., St. Louis, MO). A booster injection containing about 1 mg of antigen with Freund's incomplete adjuvant was administered after 4 weeks, and then 1 mg of antigen without adjuvant was administered 2 weeks later. One week later, rats were bled through cardiac puncture. The blood collected was allowed to clot for 30 min at room temperature and then overnight at 4 °C. After centrifugation, the serum obtained was stored at −70 °C until use. The specificity of the antiserum was verified by immunoblotting against different proteins. The antiserum reacted only with the proteins of the LV fraction.

Gel electrophoresis and western blot analysis

Eggs (freshly laid) were analysed by dissociating electrophoresis (SDS-PAGE) using a 12% acrylamide (Laemmli 1970) and stained with Coomasie Brilliant Blue R-250 (Sigma Chemical Co., St. Louis, MO). Molecular weights (MW) were calculated as previously described (Laino et al. 2011). Proteins were separated by SDS-PAGE electrophoresis and electroblotted for 1 h at 100 V (Trans-Blot SD Semi Dry Transfer Cell, Bio-Rad Laboratories, Hercules, CA, USA) from the unstained gel to nitrocellulose membranes using 48 mM Gly, 39 mM Tris, pH 9.2, and 20% MeOH buffer. After being blocked overnight at 4 °C with 3% (w/v) non-fat dry milk in 0.15 M NaCl, 10 mM Tris–HCl, pH 7.4, the membranes were incubated with the anti-LV serum (1:5000) for 2 h. Specific antigens were detected by goat anti-rat. IgG horseradish peroxidase conjugate (1:1000) immunoreactivity was visualized by enhanced electrochemiluminescence (ECL).

Enzyme-linked immunosorbent assay (ELISA) development

A standardized enzyme-linked immunosorbent assay (ELISA) was developed to quantify LV at different embryonic and post-embryonic instars. The procedure was based on Engwall and Perlmann's (1972) assay. The standard curve was obtained using purified SmLV. Nunc-Immunoplate Polisorp microtiter plates were loaded with 50 µL/well of the LV standard (0–400 ng) dissolved in a buffer containing 35 mM sodium bicarbonate and 15 mM sodium carbonate at pH 9.6 (coating buffer). Samples of eggs, embryos or juveniles were diluted with the coating buffer. Aliquots of 50 µL were incubated at 37 °C for 90 min. The antigen was then taken out, and each well was filled with 300 µL of PBS, pH 7.4, containing 3% (w/v) non-fat dry milk. The plates were incubated at room temperature for 2 h and subsequently washed three times with 0.05% (v/v) Tween in PBS. The anti-LV rat serum diluted in PBS-Tween (1:1000) containing 3% non-fat dry milk was poured into each well. Plates were incubated overnight at 4 °C and washed three times. Goat anti-rabbit IgG horseradish peroxidase conjugate (Thermo Scientific) diluted 1:1000 in PBS–0.05% Tween-3% non-fat dry milk was added (50 µL) and incubated 2 h at 37 °C. After four washes as before, 50-µL aliquots of substrate solution, ABTS (2, 2′-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]–diammonium salt), and H_2O_2 (Bio-Rad) were added to each well and were incubated at room temperature for 15 min. The reaction was stopped with 2% oxalic acid (50 µL). The absorbance was read at 405 nm in a Beckman Coulter, Inc. Instruments (DTX 880). Non-fat dry milk in PBS was used in all the assays as blank and negative control. All analyses of samples were triplicate and the data were averaged.

Identification and quantification of lipids

Characterization of lipids and fatty acids

Lipids were extracted from freshly laid eggs following Folch et al.'s (1957) procedure. Lipids were evaluated quantitatively by thin-layer chromatography (TLC) 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; Laino et al. 2011). The general procedure for separation and identification of lipids has been described previously by Cunningham and Pollero (1996). Fatty acid methyl esters (FAME) from total lipids of eggs were prepared with BF3–MeOH according to Morrison and Smith's (1992) method. Analyses were performed by gas–liquid chromatography (GLC-FID) using an HP-6890 capillary GLC (Hewlett-Packard, Palo Alto, CA), fitted with an Omegawax 250 fused silica column, 30 m × 0.25 mm with 0.25 µm phase (Supelco, Bellefonte, CA). The column temperature was programmed for a linear increase of 3 °C/min from 175 to 230 °C. Peaks were identified by comparison with retention times of Supelco 37 component fatty acid methyl ester mix (Supelco).

Quantification of triglycerides and cholesterol

Total triglycerides in a 10-µL biological sample were assessed using a colorimetric method (Triglycerides test, Randox, Crumlin, Co. Antrim, UK). The triglycerides were determined after enzymatic hydrolysis with lipases. The indicator was a quinoneimine formed from hydrogen peroxide, 4-aminophenazone and 4-chlorophenol under the catalytic influence of peroxidase. We divided triglyceride level data by fresh weight to obtain concentrations in µg/mg.

Cholesterol in a 10-µL biological sample was determined using cholesterol/cholesteryl ester detection kit (Abcam, Cambridge, UK) using a colorimetric method. In this assay, free cholesterol was oxidized by cholesterol dehydrogenase to generate NADH that reacts with a sensitive probe resulting in strong absorbance at 450 nm. We divided the cholesterol level data by fresh weight to obtain concentrations in µg/mg.

Energetic equivalent of proteins, carbohydrates and lipids

We estimated the total calories provided by different energy substrates (carbohydrates, proteins and lipids) using Benigher and Lucas's (1984) conversion factors applied to arthropods (Garcia-Guerrero et al. 2003; Heras et al. 2000; Laino et al. 2013). The conversion factors were: 4.3 kcal/g for protein, 4.1 kcal/g for carbohydrate and 7.9 kcal/g for lipid.

Statistical analyses

Statistical analyses were performed using STATISTICA 6.0 for WINDOWS (Statsoft Inc.). As all our data satisfied the requirements for parametric statistics, we analysed ours using one-way ANOVAs. When differences between means were significant at the $p < 0.05$ level, post hoc Tukey's (HSD) tests were applied. Linear regressions assessed variations of biochemical variables over time during embryonic and post-embryonic development. Results are expressed as means ± SE.

Results

Development and instars

Females lay 35 ± 10 eggs and these eggs are initially coated with a sticky substance that holds them together (Table 1). Eggs are spherical and contain a large quantity of homogenous yolk (Fig. 1a). *P. saltans'* eggs are always enveloped in two layers, an inner vitelline membrane and an outer chorion. Egg incubation, or embryonic development, lasts 14–15 days and post-embryonic development lasts 14–15 days.

Embryonic development

After 5–10 days of incubation, "embryo 1" stage is characterized by the closure of the opisthosomal region (Fig. 1b). A frontal view shows that the chelicerae partly covered the labrum. The walking legs are segmented into seven podomeres. Yolk is shifted from the prosoma to the opisthosomal parts of the embryo. 10 days after oviposition, "embryo 2" stage is characterized by the fact that the labium starts to protrude and together with the labrum forms a beak-like structure (Fig. 1c). The eyes can be detected in their position underneath the prosomal cuticle. The walking legs show prominent endites and legs still bend ventrally. Stage 2 embryos hatch from the eggs after 14–15 days of embryonic development.

Post-embryonic development

The stage after hatching is called the juvenile instar. This instar is divided into two. Juveniles remain in the cocoon's outer chamber where they undergo one moult after hatching ("juvenile 1" instar, Fig. 1d), then a second moult 5–6 days later ("juvenile 2" instar, Fig. 1e). First instar juveniles' walking legs are arranged laterally around the prosoma. The opisthosoma is oval and can be up to twice the size of the prosoma and it still contains yolk reserves. The first instar juveniles do not move and their cuticle is

Table 1 Development of *P. saltans'* eggs and spiderlings in the egg sacs

Developmental stage	Days after oviposition	Number/egg sac	Weight (mg/egg or mg/instar)
Freshly laid eggs	0 (1–2 H)	35.1 ± 1.7	0.481 ± 0.028*
Embryonic period			
Embryo 1/undeveloped egg	5–6	31.1 ± 2.5/4.2 ± 0.5	0.589 ± 0.063
Embryo 2/undeveloped egg	10–11	28.2 ± 3.3/5.0 ± 0.8	0.602 ± 0.026
Post-embryonic period			
Juvenile 1/undeveloped egg	15–16	26.6 ± 2.3/4.8 ± 0.4	0.465 ± 0.025*
Juvenile 2/undeveloped egg	20–21	26.1 ± 2.7/4.2 ± 0.5	0.597 ± 0.032
Juvenile 2 at emergence			
Living/undeveloped egg	30	24.7 ± 1.7/5.2 ± 0.6	0.623 ± 0.036

Number and weights in relation to embryonic and post-embryonic developmental periods under laboratory conditions (20 ± 1 °C, 57 ± 1% relative humidity; $n = 100$ egg sacs). Mean levels were compared between stages using ANOVA and post hoc Tukey (HSD) tests

*Significant difference among other instars at $p < 0.05$

transparent and unpigmented. No sensory hairs are visible on the cuticle. After the second moult, the opisthosoma of instar "juvenile 2" increases in volume; their walking legs become slightly longer (Fig. 1e). The cuticle is pigmented showing brown dots and patches. Second instar juveniles are relatively active (frequent leg movements). The mother opens the cocoon 25–30 days after oviposition, and "Juveniles 2E" emerge from the egg sac and climb onto their mother's back (Fig. 1f).

Developing embryos were visible in the majority of the eggs (91%) after 5 days of incubation and only 9% of the eggs were sterile (Table 1). During the post-embryonic period, 18% of the juveniles died inside the egg sac. Weights of embryos and juveniles varied significantly during development ($F_{(5,108)} = 11.83$, $p < 0.0001$; Table 1): after hatching and their first moult, instar "juveniles 1" were significantly less heavy than instar "embryos 2" (Tukey test: $p < 0.01$); after the second moult and at emergence instar "juveniles 2" were significantly heavier than instar "juveniles 1" (Tukey test: $p < 0.02$).

Carbohydrates and free glucose levels

Levels of carbohydrates in the eggs averaged 0.83 ± 0.19 µg/mg wet mass after oviposition. Their levels varied significantly during embryonic and post-embryonic development ($F_{(5,108)} = 4.06$, $p = 0.002$; Fig. 2a). They decreased significantly from egg to "embryo 2": 0.63 ± 0.08 µg/mg (Tukey test: "embryo 2" vs "egg": $p = 0.05$) then again during post-embryonic development until emergence: 0.41 ± 0.08 µg/mg (Tukey test: "embryo 2" vs "juvenile 2E": $p = 0.01$). A linear regression indicated a significant correlation between the values of calories provided by carbohydrates and the variations of total calories during the embryonic and the post-embryonic periods ($F_{(5,94)} = 6.19$, $p = 0.0003$; $Y = -0.0813x + 0.8863$, $R^2 = 0.99$).

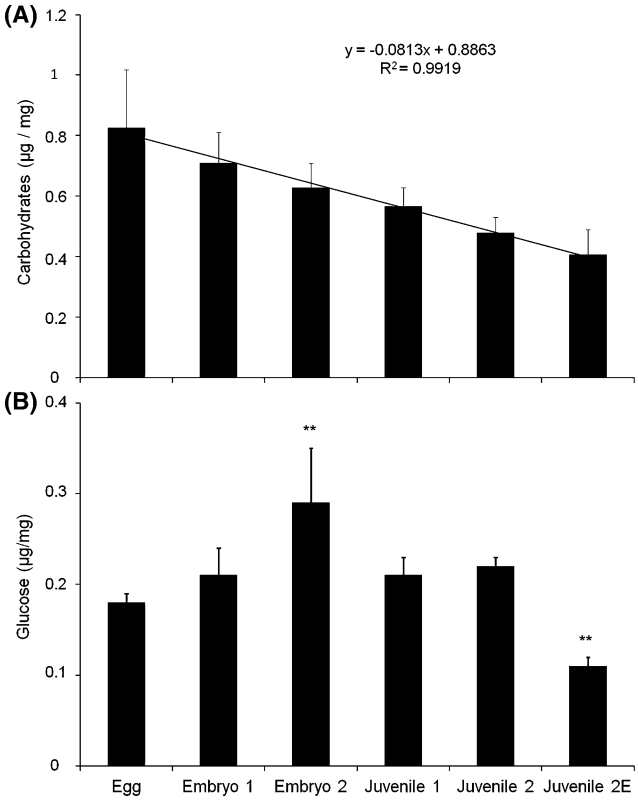

Fig. 2 Carbohydrates (**a**) and glucose (**b**) concentrations in *P. saltans'* freshly laid eggs, embryos and juveniles in relation to embryonic and post-embryonic developmental stages ($n = 20$/instar). The average levels for each instar were compared using ANOVA and post hoc Tukey (HSD) tests. **Significant difference among other instars at $p < 0.01$

Free glucose levels in the eggs averaged 0.18 ± 0.01 µg/mg wet mass after oviposition. Free glucose levels varied significantly during embryonic and post-embryonic development ($F_{(5,108)} = 4.63$, $p = 0.01$; Fig. 2b). They increased

significantly from egg to "embryo 2": 0.29 ± 0.04 µg/mg (Tukey test: "embryo 2" vs "egg": $p = 0.01$; "embryo 2" vs "embryo 1": $p = 0.01$) then decreased significantly after the first post-embryonic moult (Tukey test: "embryo 2" vs "juvenile 1": $p = 0.01$). At emergence (stage "juvenile 2E"), free glucose levels had decreased significantly: 0.11 ± 0.01 µg/mg (Tukey test: "juvenile 2" vs "juvenile 2E": $p = 0.01$). A linear regression did not indicate any significant correlation between values of calories provided by free glucose and variations of total calories during the embryonic and the post-embryonic periods ($F_{(5,94)} = 1.81$, $p = 0.147$; $Y = -0.014x + 0.2429$, $R^2 = 0.11$).

Protein levels and lipovitellin

Protein levels in the eggs averaged 22.21 ± 0.78 µg/mg wet mass (Fig. 3) and decreased significantly during the two periods of development in the egg sac (embryo and juvenile instars) until emergence from egg sac, i.e. 6.97 ± 0.70 µg/mg in instar "juvenile 2E" ($F_{(5,108)} = 43.76$, $p < 0.0001$). A linear regression revealed a significant correlation between the values of calories provided by proteins and variations of total calories during the embryonic and the post-embryonic periods ($F_{(5,95)} = 12.61$, $p < 0.001$; $Y = -2.699x + 24.259$, $R^2 = 0.965$).

Analyses of image of dissociating electrophoresis and immunoblot analyses of proteins from eggs, embryos, juveniles and SmLV revealed the presence of different bands in 1-D gels visualized by blue staining (Fig. 4a). No significant differences could be evidenced concerning the values of bands between duplicate gels showing the reproducibility of the experiments. Four protein bands were identified during the different developmental instars: protein bands 116, 87, 70, and 42 kDa (Fig. 4a) and corresponded to LV (Fig. 3b). A standardized enzyme-linked immunosorbent assay (ELISA) was developed to quantify LV at different embryonic and post-embryonic stages (Fig. 4c, d). LV represented 35% of total proteins in the eggs. Lipovitellin levels did not vary significantly during development (35–45%) in relation to total protein.

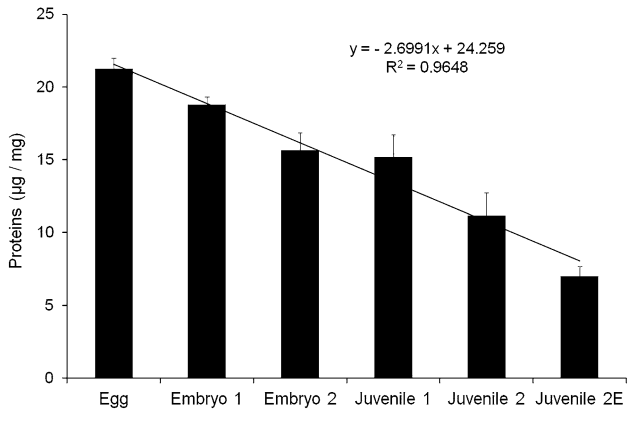

Fig. 3 Protein concentrations in *P. saltans'* freshly laid eggs, embryos and juveniles in relation to embryonic and post-embryonic developmental stages ($n = 20$/instar)

Lipids and fatty acids levels

TLC and GLC-FID from the egg extracts were composed of a mixture of lipids (Table 2). Triglycerides, phosphatidylcholine, lysophosphatidylcholine and free fatty acids accounted for the main lipid content; the levels of other lipids were lower. Oleic (C 18:1) and linoleic (C 18:2) acids were the dominant unsaturated fatty acids; palmitic (C 16:0) and stearic (C 18:0) acids were the dominant saturated fatty acids (Table 3).

Triglycerides and cholesterol levels

Triglyceride levels in eggs averaged 4.15 ± 0.38 µg/mg wet mass (Fig. 5a) and did not vary significantly during embryonic development and post-embryonic periods but at emergence, triglyceride levels stage "juvenile 2E" (2.06 ± 0.19 µg/mg) were significantly lower than those of the other instars ($F_{(5,108)} = 6.02$, $p = 0.0005$). A linear regression showed a significant correlation between the values of calories provided by triglycerides and variations of total calories during the embryonic and post-embryonic periods ($F_{(5,96)} = 3.961$, $p = 0.008$; $Y = -0.133x + 4.225*X$, $R^2 = 0.904$).

Cholesterol levels in eggs averaged 0.63 ± 0.06 µg/mg wet mass (Fig. 5b). During the "embryo 1" stage cholesterol levels were significantly lower than in the eggs 0.26 µg/mg (Tukey test: "egg" vs "embryo 1": $p = 0.0007$), then increased significantly during the juvenile post-embryonic period and at the emergence (stage "juvenile 2E") cholesterol levels were significantly higher: 0.74 ± 0.05 µg/mg. ($F_{(5,108)} = 43.76$, $p < 0.0001$).

Energetic equivalence

Just after oviposition (Fig. 6), the total caloric content of eggs was 127 ± 5 cal/g wet mass, the energy equivalent of which was represented mainly by proteins (71%) supplemented by lipids (26%), and carbohydrates (3%). During the embryonic and post-embryonic periods, total caloric contents decreased significantly between each developmental instar (ANOVA: $F_{(5,108)} = 22.62$, $p < 0.0001$; $Y = -14.715*X + 146.05$; $R^2 = 0.939$; Fig. 6a). At emergence from the egg sac, the energetic state of "juvenile 2E" (47.93 ± 5.34 cal/g wet mass) was significantly lower than those of the other developmental instars (Tukey test: "egg"

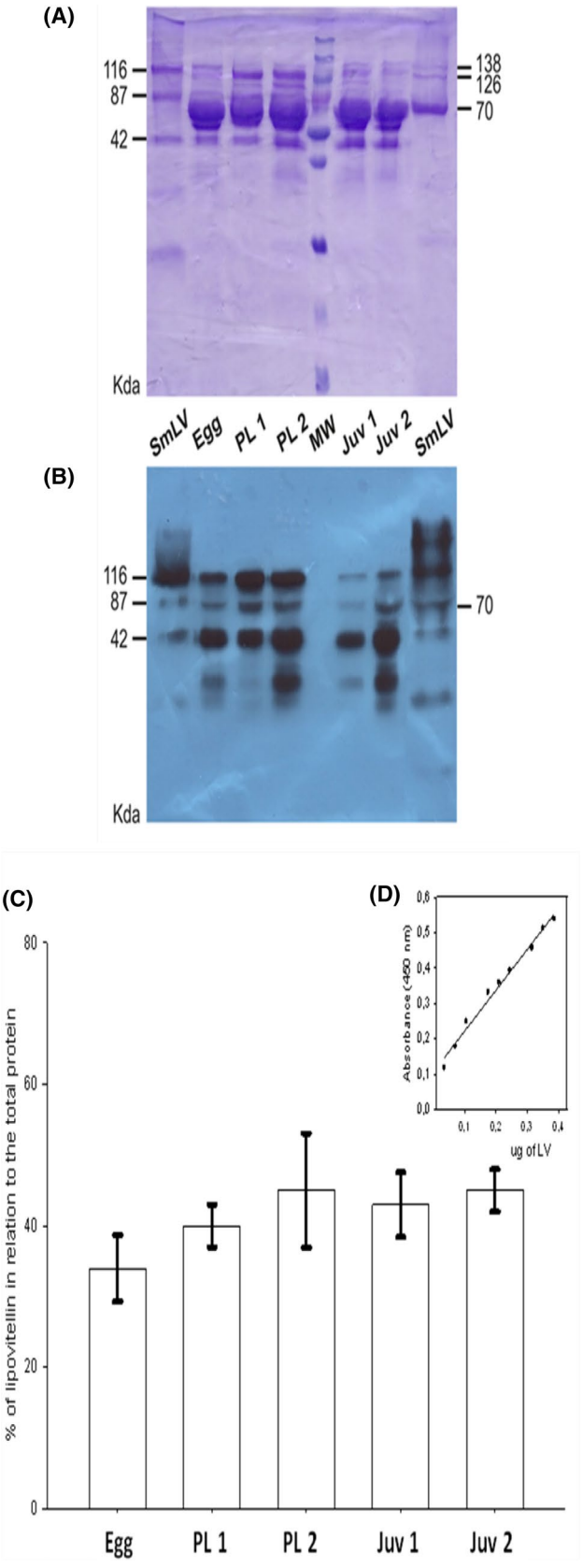

Fig. 4 Soluble proteins of *P. saltans'* freshly laid egg in relation to developmental stage, in percentage of LV in relation to the total proteins (mean ± SD) ($n = 3$). Electrophoresis (SDS-PAGE) of 20 µg of soluble protein for each well (**a**) and immunoblot of 5 µg of soluble protein for each well (**b**). *SmLV* lipovitellin of *S. malitiosa, Emb 1* embryo 1, *Emb 2* embryo 2, *MW* standard, *Juv 1* juvenile 1, *Juv 2* juvenile 2. **c** Changes of LV levels during *P. saltans'* development. LV was quantified by ELISA. Polyclonal antibody against egg LV was used to immunoblot and ELISA. **d** *Inset* dose–response titration of LV

vs "juvenile 2E", $p < 0.0001$), and its energy equivalent was mainly due to proteins (62%) and lipids (34%) (Fig. 6b).

Discussion

Our results show that the development of *P. saltans* in the egg sac can be divided into two periods: an embryonic period lasting 14–15 days and a post-embryonic period lasting for 15 more days before spiderlings emerge. The mortality rate was low, only 11% of the embryos/juveniles died during this developmental period. Some authors divided spiders' embryonic period into an early and a late development period (Canard 1987; Foelix 2011; Vachon 1957). Our study considered only the late development period that we divided into two stages: "embryo 1" and "embryo 2". These two stages are morphologically comparable to stages 13 and 14 of *Parasteatoda tepidariorum* (Mittmann and Wolff 2012) and stages 19 and 21 of *Cupiennius salei* (Wolff and Hilbrant 2011). By their morphological descriptions, our "juvenile 1" and "juvenile 2" instars are also comparable to the two "post-embryo" stages of *P. tepidariorum* (Mittmann and Wolff 2012) and *C. salei* (Wolff and Hilbrant 2011). This pattern of post-embryonic development is similar to that found in some solitary (Downes 1987) and social spiders (Viera and Ghione 2007). Moreover, our observations show that only 9% of the *P. saltans* eggs were sterile and not eaten by the juveniles inside their egg sac, in contradiction to our hypothesis and Canard's report (1987), as we found them in the egg sacs after the emergence of the juveniles. These eggs are, therefore, not trophic eggs and the only energy resource of juveniles during their development inside egg sacs is their yolk reserve.

Chemical identification of vitellus in eggs

Eggs of *P. saltans* consist largely of vitellus which is distributed in fine homogeneous granules and composed of proteins, lipids and carbohydrates. Carbohydrates are a minor constituent (3%) of *P. saltans* eggs, as for eggs of other arthropods (i.e. crustaceans: Heras et al. 2000; Holland 1978). Our results show that LV is the predominant lipoprotein in newly laid eggs as for crustaceans (Chaffoy

Table 2 Lipid composition of *P. saltans'* freshly laid eggs, in percentage (mean ± SD) of total weight and quantified by TLC-FID

Lipids	Percentage/egg
Diacylglycerols	2.1 ± 0.9
Free fatty acids	5.6 ± 1.7
Hydrocarbons	3.8 ± 0.6
Phospholipids	38.6
Phosphatidylcholine	28.4 ± 0.3
Lysophosphatidylcholine	5.5 ± 2.3
Phosphatidylethanolamine	3.0 ± 0.9
Sphingomyelin	1.7 ± 0.5
Sterols	3.5
Cholesterol	2.8 ± 0.7
Esterified sterols	0.7 ± 0.4
Triglycerides	45.9 ± 6.4

N = three independent analyses of three pools from ten egg sacs each

Table 3 Fatty acid composition of *P. saltans'* freshly laid eggs, in percentage (mean ± SD) of total weight and quantified by TLC-FID

Free fatty acids	Percentage/Egg
Saturated	45.5
Myristic a. (C 14: 0)	2.4 ± 1.1
Pentadecanoic a. (C15:0)	2.1 ± 0.1
Palmitic a. (16:0)	24.0 ± 0.6
Stearic a. (C18:0)	15.2 ± 1.9
Arachidic a. (C20:0)	1.6 ± 0.4
Unsaturated	52.8
Palmitoleic a. (C16:1)	4.2 ± 2.1
Oleic a. (C18:1)	24.1 ± 1.2
Linoleic a. (C18:2)	19.9 ± 1.1
Linolenic a. (C18:3)	2.4 ± 0.3
Arachidonic a. (C20:4)	2.2 ± 0.1

N = three independent analyses of three pools from ten egg sacs each

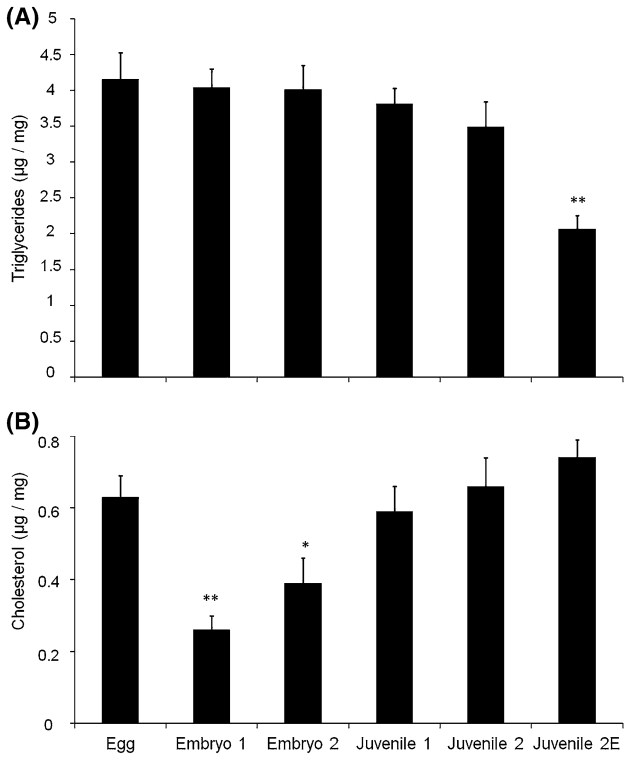

Fig. 5 Triglycerides (**a**) and cholesterol (**b**) concentrations in *P. saltans'* freshly laid eggs or bodies in relation to embryonic and post-embryonic developmental stage ($n = 20$/instar). Mean levels were compared between stages using ANOVA and post hoc Tukey (HSD) tests. *Significant difference among other instars at $p < 0.05$; **significant difference among other instars at $p < 0.01$

de Courcelles and Kondo 1980; Telfer and Kulakosky 1984) and is involved in lipid storage.

Protein extracts

The levels of total egg proteins were similar to those in the eggs of other arthropods (Kunkel and Nordin 1985; Wallace 1985; Yamashita and Indrasith 1988). The results of ELISA and western blot analyses show that 35% of the total proteins present in *P. saltans* eggs were LV as in eggs of *Schizocosa malitiosa* (Laino et al. 2013) and *Polybetes pythagoricus* (Laino et al. 2011). LV is composed of four apoproteins of 116, 87, 70 and 42 kDa, respectively, and was present during embryonic and post-embryonic development. During arthropods embryogenesis, proteins present in the vitellus and those forming LVs are degraded by hydrolytic enzymes for embryo nutrition and energy supply (González Baró et al. 2000; Subramoniam 1991).

Lipid extracts

Our results show that the composition (triglycerides, phospholipids, sterols and hydrocarbons) of the lipid extracts of *P. saltans* eggs was similar to that reported for other arthropods (i.e. Chen et al. 2004; Garcia et al. 2006; Salerno et al. 2002; Tufail and Takeda 2008; Walker et al. 2006). Triglycerides and phospholipids are the most abundant constituents of egg extracts as for *S. malitiosa* (Laino et al. 2013), but proportions differed between these two species. This difference could be caused by different food habits (*Pardosa* ate flies in the laboratory during tests whereas *S. malitiosa* were fed mealworm larvae), similar to that observed for the hemolymph of *Eurypelma californicum* (Schartau and Leidescher 1983) and *Brachypelma albopilosum* (Trabalon 2011) spiders. Triglycerides and phospholipids are lipids necessary for organogenesis, as for instance

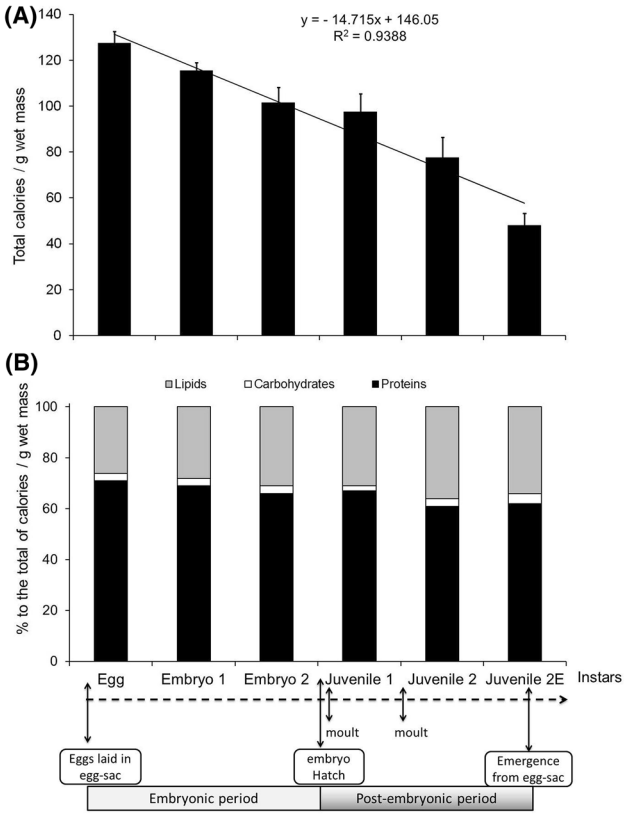

Fig. 6 Calories provided by proteins, triglycerides and carbohydrates in *P. saltans*' freshly laid eggs, embryos and juveniles during the embryonic and post-embryonic periods ($n = 20$/instars). **a** Total calories/mg wet mass. **b** Total calories/mg wet mass in percent

biomembrane formation, and are main energetic resources (Heras et al. 2000).

The phospholipids present in egg extracts are phosphatidylcholine, lysophosphatidylcholine, phosphatidylethanolamine and sphingomyelin. Phosphatidylcholine, accounts for about 95% of the total choline in tissues (Ueland 2011) and is a major plasma lipid constituent of vertebrates (Li and Vance 2008) and invertebrates (Laino et al. 2013), and is a major component of biological membranes, especially cellular membranes. Fatty acids and cholesterol are transported in the plasma by lipoproteins. Phosphatidylcholine synthesis is required for the transport of fat and cholesterol (Noga et al. 2002; Noga and Vance 2003). This phospholipid can be synthesized from choline *via* the cytidine diphosphocholine pathway or through methylation of another phospholipid, phosphatidylethanolamine (Gibellini and Smith 2010) present in extracts of *P. saltans* eggs.

Sphingomyelin (or ceramide phosphorylcholine), another phospholipid present in extract of *P. saltans* eggs, is a type of sphingosine containing phospholipid that is synthesized by the transfer of a phosphocholine residue from a phosphatidylcholine to a ceramide. Sphingomyelins constitute an important class of phospholipids present in the membranes of most eukarotic cells and lipoproteins (Slotte 2013; Slotte and Ramstedt 2007). This compound plays an important role in the expression of specific cellular functions, such as transmission of intracellular information transmission and maintenance of membrane structure. Sphingomyelin interacts favourably with cholesterol and other sterols and sphingomyelins and cholesterol are co-located on the surface of lipoprotein particles in *P. saltans* as in other species (Slotte and Ramstedt 2007). Sphingomyelin seems to regulate the distribution of cholesterol within membranes. Concerning yolk-derived sphingomyelin, palmitic acid accounts for about 80% of the fatty acid chains bound by amide bonds. Phosphatidylcholine and sphingomyelin are precursors for intracellular messenger molecules such as diacylglycerol that are also present in the extracts of *P. saltans* eggs. Diacylglycerol is released by degradation of phosphatidylcholine by phospholipases. Sterols (cholesterol and esterified sterols) in eggs represented 3.5% of the lipids in eggs. Cholesterol plays an important role in arthropod physiology as a cuticular surface wax, as a constituent of membranes in the lipid bilayer, and as a precursor for the synthesis of steroid hormones involved in moulting (Andersen 1979; Martin-Creuzburg et al. 2007; Merzendorfer and Zimoch 2003).

Furthermore, the fatty acid composition, dominated by palmitic, oleic and linoleic acids, of *P. saltans* eggs is similar to that of another wolf spider, *S. malitiosa* (Laino et al. 2013) and its pattern is also comparable to that of *P. pythagoricus* LV (Laino et al. 2011). A major characteristic of fatty acids in general is that they function as a structural component of membranes to maintain proper fluidity and permeability (Trabalon 2011).

We found that the energy content of *P. saltans'* eggs was equivalent to 127 cal/g, a value similar to that for *Schizocosa malitiosa* eggs (Laino et al. 2013), but lower than those for other spiders (Anderson 1978; Laino et al. 2013), crustaceans (Amsler and George 1985; Petersen and Anger 1997; Clarke et al. 1990; Heras et al. 2000), or beetles (Sloggett and Lorenz 2008) (Table 4). For example, values for 3 species of spiders (Anderson 1978) ranged from 26 to 29 J/mg ash-free dry mass (7 cal/mg). These differences could be related to differences between indirect methods (respirometer and size egg) used by Anderson to measure the level of energy in eggs and probable values were overestimated by indirect methods. It thus remains difficult to make clear generalizations about the ecological and evolutionary implications of egg compositional differences in spiders as well as other arthropods. To facilitate cross-species comparisons, clear and comparable methods are required.

Table 4 Comparison of relative lipid, protein and calories in arthropod species with different taxonomic position habitat (carbohydrates were considered constant during embryogenesis by authors)

Arthropod species	Egg (mg)	Lipid/protein in freshly laid eggs (%)	Calories/mg of egg	Lipid/protein in larvae or juvenile freshly hatched (%)
Aquatic crustacean				
Euphausia superba (Amsler and George 1985)	30	31/57	–	51/49
Hyas araneus (Petersen & Anger 1997)	69	31/34	0.38	66/34
Macrobrachium rosenbergii (Clarke et al. 1990)	51	28/61	–	100/0
Macrobrachium borellii (Heras et al. 2000)	–	29/29	1.31–0.6	–
Terrestrial spiders				
Pardosa saltans (present study)	0.481	26/71	0.13	34/62
Schizocosa malitiosa (Laino et al. 2013)	–	20/60	0.10	–
Filistata hibernalis (Anderson 1978)	1.28–1.56	–	6.1–6.3	–
Nuctenea cornuta (Anderson 1978)	0.44–0.55	–	6.1–6.4	–
Peucetia viridans (Anderson 1978)	1.67–1.85	–	6.6–6.8	–
Terrestrial insects				
Adalia bipunctata (Sloggett and Lorenz 2008)	0.13–0.14	42/50	1.43	27/72
Adalia decempunctata (Sloggett and Lorenz 2008)	0.11	40/53	1.67	–
Anissosticta novemdecimpunctata (Sloggett and Lorenz 2008)	0.10	44/49	1.79	–

Chemical variations during embryonic and post-embryonic development

As embryonic development proceeds, maternal reserves of vitellus are sequentially degraded. Our results show that carbohydrates (total sugars and free glucose), protein and lipid (triglycerides and cholesterol) levels varied during embryonic and post-embryonic development.

During development inside egg sacs, only 51% of the initial carbohydrate stock was used. During embryonic development, carbohydrate levels decreased gradually and free glucose levels increased in instar "embryo 2" just before they hatched, probably due to glyconeogenesis or hydrolysis of glycogen. We hypothesized that this increase was related to the embryos' need to tear the chorion apart, an activity that requires rapid mobilization of energetic components. Thus, during this period, 24% of the initial carbohydrate stock was used by embryos. Total carbohydrate and free glucose levels decreased after hatching, maybe as a consequence of chitin synthesis during cuticle formation, as in *Zaluska vincula* (Sloggett and Lorenz 2008). During post-embryonic development, 27% of the initial carbohydrate stock was used by juveniles and at the emergence from egg sacs, 49% of the initial carbohydrate stock was still present in instar "juvenile 2E" but the levels of free glucose level were lower.

Levels of total proteins decreased gradually during embryonic and post-embryonic development. This decrease was not linked to the decrease of LV. Indeed, our results showed that *P. saltans* consumed LV proportionally to the other proteins as no significant variations of the LV/protein ratio could be evidenced at any stage. This may be because spiderlings are born with an important reserve of LV that should satisfy their energy needs until they are able to feed themselves. Acari *Boophilus microplus* present a similar strategy as their larvae consumed only 35% of the LV deposited in oocytes (Campos et al. 2008). The variations observed of protein levels could be linked to glyconeogenesis that enables embryos and juveniles to develop. Thus, during these periods 67% of their total protein stock was used.

Triglyceride levels, contrary to proteins, did not vary significantly during development until the "juvenile 2" instar. Degradation of these triglycerides was lower (16%) during embryonic and post-embryonic development than in many other invertebrate embryos that normally used 40–60% of the initial stock (e.g. Amsler and George 1985; Needham 1950; Petersen and Anger 1997). Contrary to our hypothesis, triglycerides appear not representing the major fuel for embryonic development for *P. saltans* as for other invertebrates (Clarke et al. 1990; Sasaki et al. 1986). The greatest degradation of triglycerides was caused by instar "juvenile 2" after their second moult and before emergence: 33% of the triglyceride stock was used during this last period and when instar "juvenile 2E" emerged they still had 49% of the initial stock. This conservation strategy of carbohydrate and triglyceride stocks enables young *P. saltans* to survive without eating for 6–7 days after emergence and to moult. A previous study showed that the spiderlings start foraging

7–8 days after emergence, after their first post-emergence moult (Ruhland et al. 2016b).

Our results show that cholesterol levels increased gradually during development. We hypothesize that these variations correspond to embryos' and juveniles' needs for moulting, as arthropods moulting is controlled by steroid hormones (Cheong et al. 2015; De Almeida et al. 2003; Trabalon and Blais 2012). Indeed, our results show that cholesterol levels were high just before juveniles moulted. These compounds would be appropriate to satisfy embryos' sterol requirements but they have not yet been investigated.

In conclusion, contrary to our hypothesis, *P. saltans* do not lay trophic eggs and juveniles only develop from the vitellus. Variations of carbohydrate, lipid (particularly triglycerides) and protein levels induce energetic modifications. Intriguingly only 62% of the total initial energetic stock was used to form the embryos and the juveniles. The remaining vitellus was used by embryos. This suggests that part of the vitellus content is used for embryonic differentiation and another part for juvenile differentiation, in and out of the egg. Vitellus is used during post-embryonic life during juvenile differentiation. Decrease of energetic stocks is linked mainly to the use of proteins (67% of the initial stock), carbohydrates (51%) and triglycerides (49%) stocks. Contrary to our hypothesis, triglycerides are not the main energy source for embryonic development. The energetic stock provided by *P. saltans* mothers to their eggs is important and enables young to develop and to emerge with sufficient energetic reserves (38% of the initial energy stock) to last until they can forage by themselves, i.e. 7–8 days after emergence. It will be interesting to study at how the vitellus changes after emergence until vitellus stock is depleted.

Acknowledgements The authors would like to thank Dr Ann Cloarec and Pr Simon N. Thornton for reading and correcting the English. The work was partly supported by funding from the project ECOS-MINCyt (France-Argentine) No. A16B03.

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