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ANIMAL SCIENCE

Embryonic and post-embryonic development of the spider *Polybetes pythagoricus* (Sparassidae): A biochemical point of view

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Abstract: Analysis of energy expense during development has achieved special interest through time on account of the crucial role of the consumption of resources required for offspring survival. Spider eggs have a fixed composition as well as some initial energy that is supplied by mothers. These resources are necessary to support the metabolic expense not only through the embryonic period but also during the post-embryonic period, as well as for post emerging activities before spiderlings become self-sustaining. Depletion of these resources would be critical for spiders since it could give rise to prey competition as well as filial cannibalism. Even though spiders represent a megadiverse order, information regarding the metabolic requirements during spiders development is very scarce. In this study, we analyse the changes in protein, lipid and carbohydrate content as well as the variation in lipovitellin reserves and hemocyanin content during *Polybetes pythagoricus* development. Our results show that lipovitellins and phospholipids represent the major energy source throughout embryonic and post-embryonic development. Lipovitellin apolipoproteins are gradually consumed but are later depleted after dispersion. Phosphatidylethanolamine is mainly consumed during the post-embryonic period, while triacylglycerides are consumed after juveniles' dispersion. Finally, hemocyanin concentration starts to increase in postembryonic stages.

Key words: development, fatty acids, hemocyanin, lipids, lipovitellin, spider.

INTRODUCTION

With more than 49,000 species described (World Spider Catalog 2021), spiders represent one of the most important and megadiverse groups and are found in different habitats (Foelix 2011, Grismado et al. 2014). They are of great importance to humans both for the harmful effect that the venom present in some species generates on human health (Vetter & Isbister 2008) and for their role in the biological pest control of different agroecosystems owing to their being generalist predators (Saba et al. 2020). Although existing information on the development and reproduction of spiders is very abundant, biochemical and metabolic studies on these topics are very scarce.

The study of energetic expense during development has achieved significant interest over time, and oviparous animals have become a special model for biological studies on development. Their eggs have a fixed composition and, therefore, a certain amount of energy whose variations can be followed throughout embryo development (Garcia et al. 2008, Trabalon et al. 2018). In the case of spiders, mothers lay their eggs in silk cocoons built by themselves (Foelix 2011) thus determining the amount and quality of resources they allocate to their offspring (Salomon et al. 2011, Ruhland et

al. 2016). The presence of this cocoon influences the development of spiders due to the fact that some post-embryonic stages also take place inside this structure. Because of this, initial composition and energy resources of the eggs are needed not only to support the metabolic demand throughout the embryonic period but also to bear the metabolic cost during the post-embryonic stage and the post emergent activities before spiderlings become self-sustaining (Anderson 1978, Ruhland et al. 2016, Trabalon et al. 2018).

The different resources accumulated by mothers and transferred to the eggs may influence the offspring's traits (Salomon et al. 2011). The process of synthesis and storage of nutrients in the oocytes is known as vitellogenesis. During vitellogenesis, oocytes size increases by the progressive accumulation of yolk, lipid droplets, as well as various proteins, carbohydrates and lipids, which are required for offspring development (Byrne et al. 1989, Choi & Moon 2003, Fruttero et al. 2011, Romero et al. 2018, Thompson & Russel 1999, Trabalon et al. 1992). The depletion of yolk is a critical time for spiders, since it could give rise to competition for prey and filial cannibalism (Yip & Rayor 2014). Information regarding yolk synthesis in spiders is quite recent and very scarce (Bednarek et al. 2019, Laino et al. 2011b, 2013, Romero et al. 2019). Moreover, the use of energetic resources during development is variable and the energetic demand required for embryonic development can be supplied by lipids, proteins and carbohydrates (Campos et al. 2006, Mohamed 2000, Santana et al. 2014). However, the only contribution about the consumption of energetic resources in spiders was that by Trabalon and collaborators, who observed the key role played by trialcylglycerides, carbohydrates, and proteins during the embryonic and post-embryonic development of the spider *Pardosa saltans* (Trabalon et al. 2018).

Focusing on spiders' diversity, the main aim of this research is to make headway in the understanding of the role played by the different biomolecules during *Polybetes pythagoricus* spider development. *P. pythagoricus* is a South American spider found in Chile, Bolivia, Brazil, Paraguay, Uruguay and in north-central Argentina, where different biochemical and physiological aspects have been analysed (Cunningham & Pollero 1996, Cunningham et al. 1994, 2002, 2007, Laino et al. 2009, 2011a, b, 2015b, Romero et al. 2018, 2019). Copulation and mating, along with other relevant data about *P. pythagoricus* life cycle were previously described (Galiano 1979, Scioscia 1984), as well as post-embryonic development (Galiano 1971). However, there is no information about the different energetic resources and their use during *P. pythagoricus* development.

The aims of this study were: firstly, to determine the quantitative changes in the total content of carbohydrates and proteins. Secondly, to characterize, through immunological techniques, lipovitellins consumption and hemocyanin content, respectively. Lastly, to provide information on the lipid and fatty acids dynamics during *P. pythagoricus* development. We hypothesized that: (1) carbohydrates are gradually consumed throughout the development process; 2) lipovitellins are gradually consumed throughout development; (3) hemocyanin is synthetized in the advanced stages of development; 4) triacylglycerides are the main energy source used throughout the development process, 5) phospholipid content decreases as lipovitellins are consumed.

MATERIALS AND METHODS

Ethics statement

The species used in the present experiments (*P. pythagoricus*) is not endangered or protected. Our research conforms to the legal requirements for the treatment of animals in research facilities using invertebrate species and taking care for them using accepted ethical standards.

Spider collection and rearing

Spiders were captured in a forest of *Eucalyptus* sp. of Martin Rodriguez Park in the city of Ensenada (34°52'56" S, 57°56'07" W) and in Pereyra Iraola Park (Berazategui-La Plata) Argentina (34°50'39" S, 58°10'55" W). Capture permits were No 117/16 Protected Areas, Province of Buenos Aires. They were housed individually in cylindrical terrariums (10 cm in diameter × 5 cm in height) without any substrate, fed once a week with Turkestan cockroach (*Shelfordella tartara*) or Dubia roach (*Blaptica dubia*), and kept at 25 ± 1 °C under a 14-h light – 10-h dark photoperiodic cycle. Vitellogenic females were maintained in these conditions until oviposition. All the egg-sacs were built by the spider females. An ootheca was obtained from each female and kept in the laboratory at 25 ± 1 °C.

Experimental groups

A total of 32 egg-sacs (1.21 ± 0.48 g/egg-sac) were used and divided into eight experimental groups to analyse the different energy resources and their variation during *P. pythagoricus* development. These experimental stages were numbered from 1 to 8, the first five are stages that are develop inside the cocoon and the last three are stages after emergence (outside the cocoon). To standardize the analysis, stages inside the cocoon (stages 1 to 5) were temporarily separated by a 5-day difference between each them. Since the stages used in this study started to number from the embryonic period, and in order to allow a comparison between our study with previously reported studies (Galiano 1971, Scioscia 1984), we highlight that the post-embryonic stages 4 and 5 of this study are the non-mobile free juveniles described by Galiano (1971), while the first emergence stage analysed in the present study (stage 6) are the mobile free juveniles characterized by the same author (Galiano 1971).

The stages analysed of the embryonic period (stages after oviposition and before hatching) were: Stage 1 (egg stage, less than 12-h laying); Stage 2 (5 days after oviposition) and Stage 3 (10 days after oviposition). The stages analysed of the post-embryonic period (after hatching) that take place inside the egg-sac were: Stage 4 (15 days after oviposition) and Stage 5 (20 days after oviposition). Stages analysed outside the egg-sac were: Stage 6 (25 ± 1 day after oviposition, juveniles in gregarious stage at the moment of emergence from egg-sac), Stage 7 (35 ± 1 day after oviposition, juveniles after the first moult outside the egg-sac) and Stage 8 (42 ± 1 day after oviposition, juveniles in dispersal stage one week after the first moult outside the egg-sac). Moreover, two complementary stages were processed and analysed: an advanced juvenile stage (AJ, 1.2 ± 0.4 g) and previtellogenic females as an adult stage (A, 2.01 ± 0.6 g), which represent stages that have already been fed.

General measurements

All egg-sacs were weighed in a Mettler-Toledo New Classic MS-204S analytical balance. Before being processed, the individuals were observed using a Leica S8 APO stereomicroscope and photos were taken with a digital camera.

The individuals of each egg-sac were weighed together and frozen-killed at –20 °C, and later on, homogenized in 1.5 ml buffer potassium phosphate regardless of their weight

(50 mM, pH 7.4) with the addition of a protease inhibitor cocktail 1/1000 (v/v) (Sigma Chemical Co., St. Louis, MO, USA). For each sample, 0.75 ml was centrifuged at 10,500 g for 20 min, the pellet discarded, and supernatant stored at –70 °C until the time of proteins and carbohydrates analysis. The rest of the sample was used to extract lipids following the procedure of Folch et al. (1957). In the case of AJ and A, the entire body of each spider was homogenized in 3 ml buffer potassium phosphate (50 mM, pH 7.4) with the addition of a protease inhibitor cocktail 1/1000 (v/v) and following the same procedure as the egg-sacs.

Proteins and glycogen analysis

The total protein content of the homogenates was quantified by the method of Lowry et al. (1951) with bovine-serum albumin as standard and expressed in µg of protein/mg of wet weight of individuals. The protein-subunit analyses were performed by polyacrylamide gel electrophoresis (PAGE) under dissociating conditions adding sodium dodecyl sulphate (SDS) on a 4–23% SDS-PAGE (Laemmli 1970) and using β-mercaptoethanol as a reducing agent. Twenty µg of sample was loaded per well; and after resolution, gels were stained with Coomassie Brilliant Blue R-250 (Sigma Chemical Co.). The respective molecular weights were calculated using a lyophilized mixture of ten highly purified well-characterized proteins to use in molecular weight determination in the presence of SDS (Precision Plus Protein™ Dual Color Standards, BIO-RAD Code:1610374). The isolation of lipovitellins (LVs) of eggs and the very-high-density lipoprotein (VHDL) of *P. pythagoricus* hemolymph, the same as the obtaining of rat-serum antibodies against hemocyanin (Hc) and LV, were performed following the same steps described in Romero et al. (2019).

Western blotting analysis initially involved the separation of proteins by SDS-PAGE loaded with 15 µg of protein of each sample por well. The proteins from the resolved unstained gel were transferred to a nitrocellulose membrane following the same steps as Romero et al. (2019). Then, membranes were incubated for 2 h one with the anti-LV polyclonal antibodies (1:5000) and the other one with the anti-Hc polyclonal antibodies (1:3000), both raised in rat. Immune complexes were detected after an incubation with a Goat Anti-Rat IgG H&L (HRP) (1:2000; ABCAM Inc.) and the immunoreactivity signals emitted were detected using Chemidoc Imaging system (Bio-Rad) and analysed with ImageJ software (NIH).

The enzyme-linked immunosorbent assay (ELISA) was used to quantify Hc and LV in the homogenates of the eight development stages previously described according to the assay of Engvall & Perlmann (1972). In order to quantify LV and Hc, standards curves were produced with the isolated LV and purified Hc. The Hc quantification was carried out following the same steps taken by Romero et al. (2019). In LV case, the curve was prepared by loading with 50 µl per well of LV in the micro well plates (Nunc-Immuno™ MicroWell™ PolySorp) at different concentrations of: 0, 5, 10, 50, 100, 200, 400, 600, 800, and 1000 ng dissolved in coating buffer (35 mM $NaHCO_3$, mM Na_2CO_3, pH 9.6). The supernatants from stage 1 to 8 were incubated with anti-LV polyclonal antibodies diluted (1:2000), and then with the Goat Anti-Rat IgG H&L (HRP) diluted (1:5000) following the same steps described in Trabalon et al. (2018). The same detection and readout of data steps were carried out for both LV and Hc. After washes, 50-µL aliquots of substrate solution that contained ABTS 2,2'-Azino-bis[3-ethylbenzthiazoline-6-sulfonic acid]-diammonium salt) and H_2O_2 were added to each well, incubated during 15 min, and

finally the reaction was stopped with 2% oxalic acid (50 µL). The absorbance reading at 405 nm was performed in a Beckman Coulter DTX 880 Multimode Detector. Triplicate measurements were taken for each sample.

Glycogen of each sample was precipitated using a 66% KOH 1:1 (v/v) solution and heated for 20 minutes over a boiling water bath (100 °C). After cooled at room temperature, a solution of Na_2SO_4 10% (v/v) and ethanol 96° 0.25% (v/v) was added in each sample, and then were heated for 5 minutes over boil water. Samples were finally centrifuged at medium speed for 10 min, supernatants were discarded and the glycogen precipitates obtained were resuspended in 1 ml H_2O. The glycogen concentration of the different homogenates was determined applying a modified anthrone-based assay (Van Handel 1985) and quantified using a glycogen standard (0.1-40 µg/ml). The results were expressed as µg of glycogen/mg of wet weight of individuals.

Lipids and Fatty-acid analysis

After lipid extraction, the total lipid concentration was determined by gravimetry (Cunningham & Pollero 1996) and expressed in µg of lipids/mg of wet weight of individuals. Then, the quantitative determination of lipid classes was performed by thin layer chromatography (TLC) coupled to a flame ionization detector (FID) in an Iatroscan apparatus model TH-10 (Iatron Laboratories, Tokyo, Japan) after separation on type S-III Chromarods (Ackman et al. 1990). The different lipid classes were separated and quantified using monoacylglycerol as an internal standard and the calibration curves for each lipid presented correlation coefficients greater than 0.95. The different samples were analysed following the procedure as that used in Romero et al. (2018) using also the same kind of lipid standards and solvent systems.

Fatty-acid methyl esters from the total lipids of each sample were prepared using a BF3–MeOH solution according to the method of Morrison & Smith (1964). The fatty-acid methyl esters analysis was performed by gas–liquid chromatography (GLC) in an HP-6890 capillary chromatograph (Hewlett Packard, Palo Alto, California, USA), equipped with a flame-ionization detector and fitted with an Omegawax 250 fused silica column of dimensions 30 m × 0.25 mm and with a 0.25 µm solid phase (Supelco, Bellefonte, California, USA), and under the same conditions that were performed in Romero et al. (2018). Peaks were identified by comparison with retention times of Supelco 37 component fatty acid methyl ester mix.

Statistical analyses

Statistical comparison was performed by a one-way ANOVA after checking for normality and homogeneity of variances. The different values were expressed as the means ± standard deviations (SDs). Significant differences ($p< 0.05$, n=4) were compared by Tukey's post hoc test. Data were analysed using GraphPad InStat 3.01 (GraphPad Software, San Diego, California, USA).

RESULTS

In this study, eight developmental stages of *P. pythagoricus* were analysed (Figure 1): five took place inside the egg-sac and the other three outside the egg-sac. Females laid 209 ± 88 eggs without any substance holding them together. After egg laying, spherical green eggs with 1.91 ± 0.11 mm diameter are observed (Stage 1). Then, after 5 days of embryo development an active organogenesis could be observed where a white cumulus cells are identified in the green eggs (Stage 2), and 10 days after oviposition, the appendages of the embryo begin to be appreciated (Stage 3). The post-embryonic

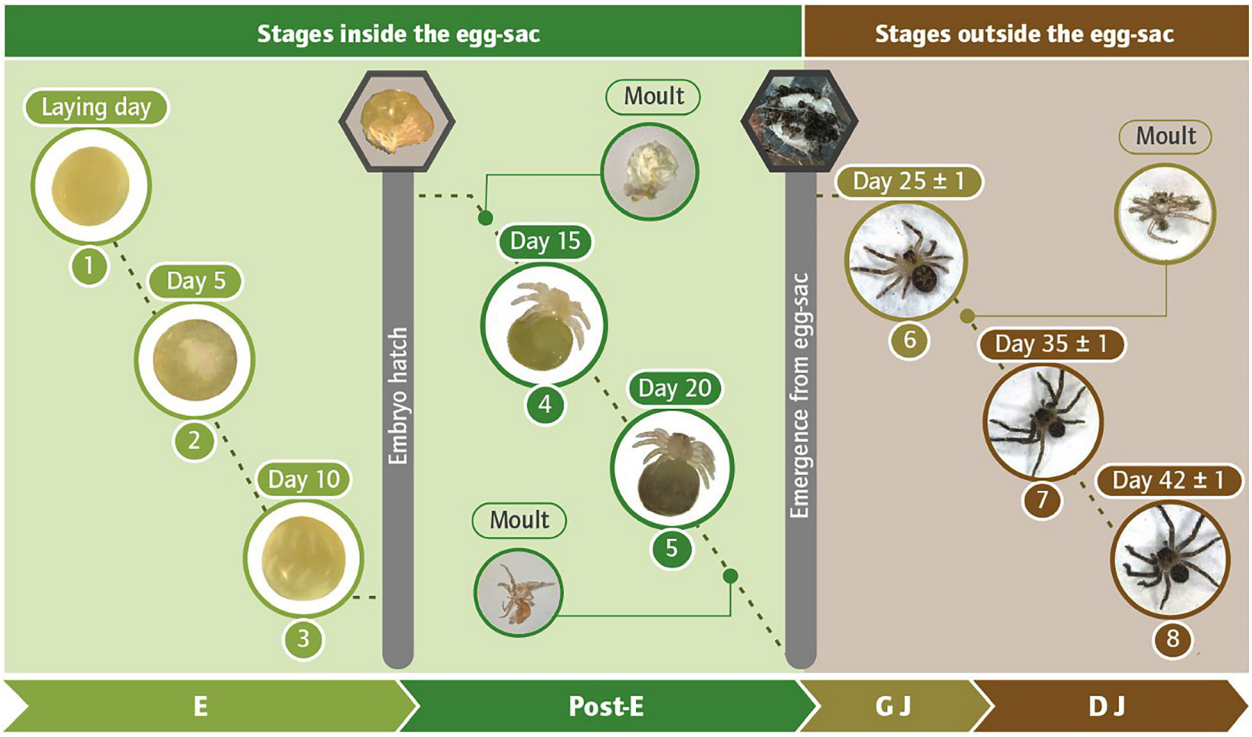

Figure 1. The analysed stages of *P. pythagoricus* development. Inside the egg-sac: the embryonic period (Stages 1 to 3) and post-embryonic period (Stages 4 and 5); Outside the egg-sac: juveniles in gregarious stage (Stage 6) and juveniles in dispersal stages (Stages 7 and 8). E: embryonic period; Post-E: post-embryonic period; GJ: juveniles in gregarious stage; DJ: juveniles in dispersal stages.

period takes place when the embryo hatches and moult after 14 or 15 days of oviposition. Stage 4 (15 days after oviposition) are juveniles with a transparent and unpigmented cuticle. The yolk shifted to the opisthosoma gives a characteristic green colour to this body area and whose size can double the prosoma size. In stage 5 (20 days after oviposition) juveniles show a lightly pigmented brown cuticle. These juveniles have frequent leg movements but without be able to walk and their opisthosoma remains the largest body area. Between the 22 ± 1 days after oviposition, juveniles moult inside the egg-sac producing a well-defined light pink and more rigid exuviae. The resulting juveniles after this moult present longer legs which enable them to walk and be more active, and both prosoma and opisthosoma are comparable in size and present a darker pigmentation throughout the body. Between 25 ± 1 days after oviposition, these juveniles emerge from the egg-sac and remain together as juveniles in gregarious stage (Stage 6) moving around the cocoon surface. Spiderling emergence may or may not be carried out in the presence of mothers because it was observed that they can emerge and continue to grow even when their mother is not present. Ten days after emergence, these gregarious spiderlings moult and give rise to dispersed juveniles (Stage 7 and 8). The tough exuviae presents a dark pink pigmentation. In both stage 7 (35 ± 1 days after laying) and 8 (42 ± 1 days after laying), juveniles leave the gregarious life walking and running with a higher speed than juveniles in gregarious stage. The cuticle of these juveniles has a darker pigmentation throughout the body and opisthosoma is slightly smaller than prosoma.

At this time, juveniles have long legs with the characteristic lateral position of *P. pythagoricus*.

Protein concentration remained relative constant until stage 5, where it was observed an increase that reaches 203.01 ± 6.47 µg of proteins/mg wet weight (Figure 2). After emergence, values decreased up to 88.06 ± 9.84 µg of proteins/mg wet weight in stage 8. Then, a significant increase was observed in AJ and A (already fed individuals).

Taking into account the principal polypeptides observed by SDS-PAGE analysis (Figure 3a and 4a), it was observed the same protein profile during the embryonic period (Stages 1 to 3) with the polypeptides 170, 120, 75, 67, 46, and 30 kDa present. After hatching of embryos, 75 kDa polypeptide disappeared almost completely in stage 4, while 170 and 120 kDa polypeptides disappeared completely before the emergence of juveniles (Stage 6). Low-molecular-weight polypeptides were differentially consumed, 30 kDa started to diminish after embryos´ hatching (Stage 4) but 46 kDa polypeptide did not disappear until juveniles dispersed (Stages 7 and 8). Conversely, 67 kDa polypeptide was present in all stages.

Moreover, the western blot analysis using polyclonal antibody against LV revealed the presence of 120, 75, 46, and 30 kDa polypeptides of LV during the embryonic period (Stages 1 to 3) (Figure 3b). During the post-embryonic period, 75 kDa polypeptide disappeared in stage 4 and then also 120 kDa polypeptide disappeared in stage 5, remaining only low-molecular-weight polypeptides (30 and 46 kDa). Finally, juveniles in gregarious stage only preserved the 46 kDa polypeptide (Stage 6) and no polypeptides sharing immunological identity with LV were detected in dispersed juveniles (Stage 7 and 8). Conversely, the presence of 67 kDa polypeptide was observed throughout the all stages studied applying a western blot analysis using polyclonal antibody against Hc (Figure 4b).

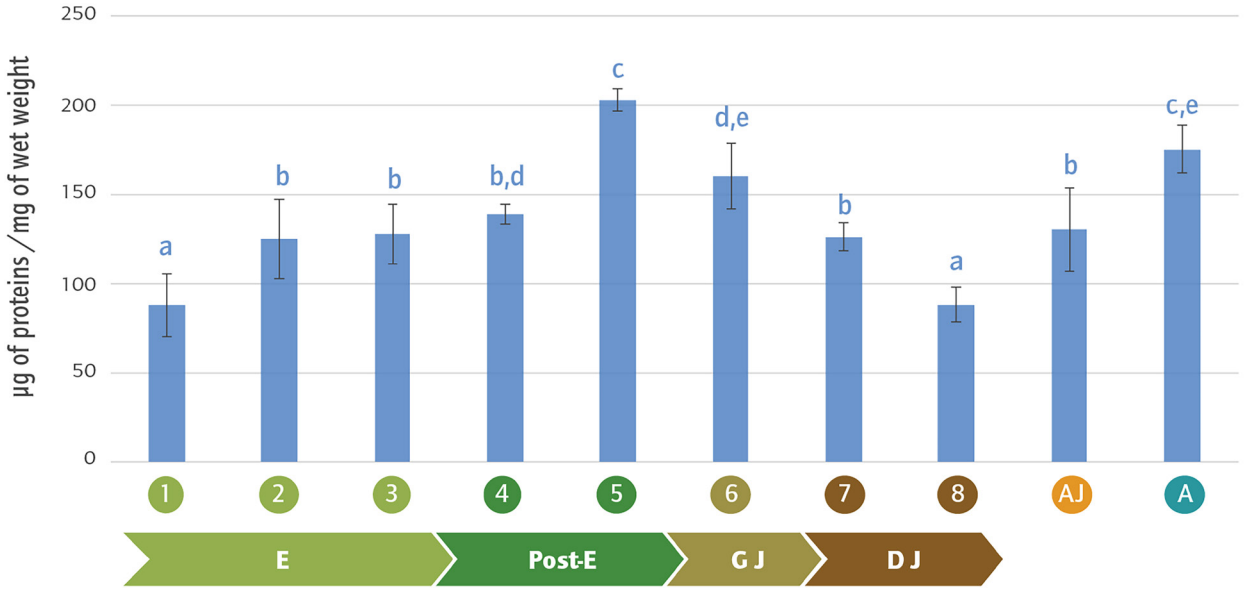

Figure 2. Analysis of protein concentration during *P. pythagoricus* development until adult's stage. Values are the means ± SDs. Different letters above the bars indicate significant differences among the different stages at $p < 0.05$, as determined by Tukey's *post hoc* test. 1 to 8: stages of *P. pythagoricus* development; AJ: advanced juvenile stage; A: adult stage; E: embryonic period; Post-E: post-embryonic period; GJ: juveniles in gregarious stage; DJ: juveniles in dispersal stages.

The content of LV and Hc in the homogenates of the eight stages was quantified using the method of ELISA with polyclonal antibody against LV and Hc (Figure 5). It was observed a gradual LV consumption beginning from protein value of 410 ± 77.7 µg LV/mg of proteins in stage 1 and reaching 296.8 ± 55.8 µg LV/mg of proteins stage 4 (after embryo hatch). Then, the LV amount decreased significantly to 102.89 ± 27.6 µg LV/mg of proteins in stage 6 (juveniles in gregarious stage) and 18.04 ± 5.53 µg LV/mg of proteins in stage 8 (juveniles in dispersal stages). LV consumption between the first stage and the last stage of juveniles in dispersal phase was linear and gradual (R^2= 0.96). By contrast, the amount of Hc presented an exponential increase (R^2= 0.81). It began with an average of 159.22 ± 27.93 µg Hc/mg of proteins, and then it reached values of 274.67 ± 1.86 µg Hc/mg of proteins in stage 5. Finally, it reached 641.58 ± 16.47 µg Hc/mg of proteins in the last stage of dispersed juveniles analysed.

During the embryonic period, it was observed an increase in the glycogen concentration in the stage 3 (725 ± 0.18 µg glycogen/mg wet weight) (Figure 6). After embryo hatch, glycogen concentration decreased gradually until values of 0.18 ± 0.02 µg glycogen/mg wet weight in stage 8. The glycogen concentration was also quantified in AJ and A, where values were strikingly lower in AJ whereas in A were comparable with the first dispersal stage.

The concentration of total lipids in the embryonic period showed a quite variable values

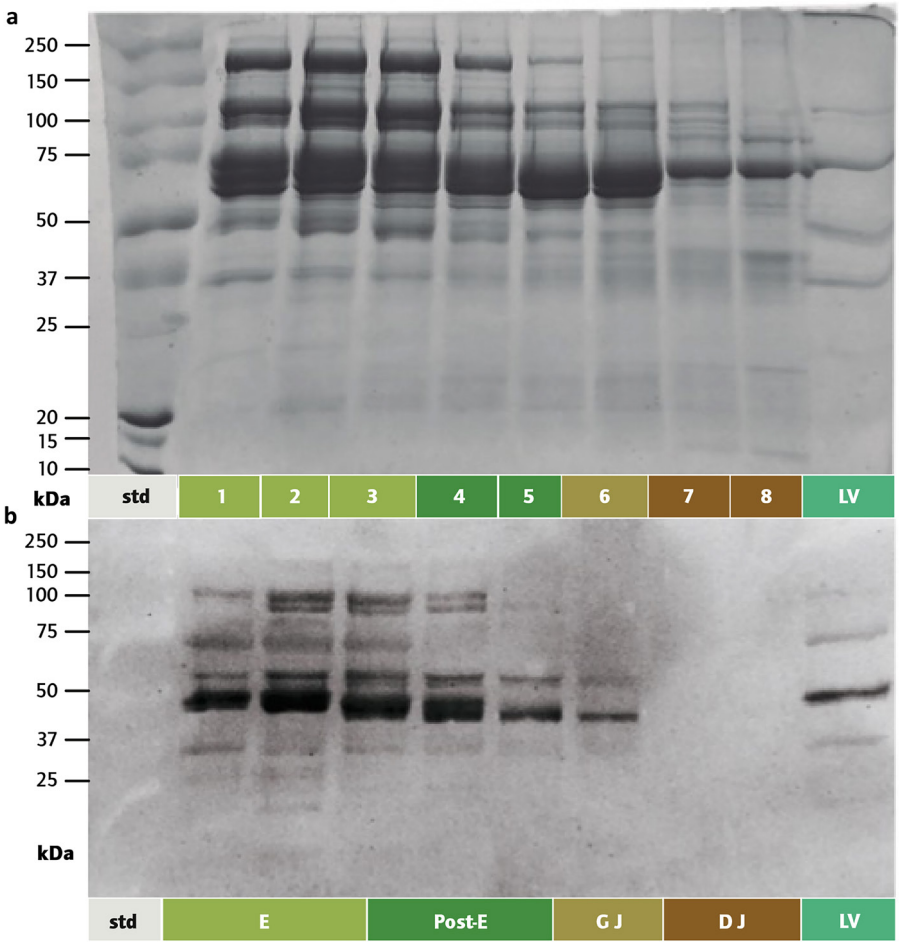

Figure 3. Analysis of soluble proteins during *P. pythagoricus* development. Electrophoresis (SDS-PAGE): 20 µg of soluble proteins per well (a) and immunoblotting with anti-LV polyclonal antibodies: 15 µg of soluble proteins per well (b). std: molecular weight standard (kDa); LV: lipovitellin isolated from egg cytosol; 1 to 8: stages of *P. pythagoricus* development. Numbers with the same colour belong to the same period of development that are delimited below the figure as: E: embryonic period; Post-E: post-embryonic period; GJ: juveniles in gregarious stage; DJ: juveniles in dispersal stages.

with an average of 60.47 ± 20.2 µg of lipids/mg wet weight, and then no significant decreases were observed with the post-embryonic stages (32.42 ± 8.2 µg of lipids/mg wet weight) (Figure 7). The amount of lipids decreased significantly to 12.3 ± 1.7 µg of lipids/mg of wet weight in the last stage of dispersed juveniles (Stage 8). Comparable values were observed in AJ and A.

Analysing the different lipid classes, the principal concentration of structural lipids in the embryonic stages was represented by phosphatidylethanolamine (PE) with values of 30.25 ± 8.05 µg PE/mg of wet weight (Figure 8). After hatching, PE content decreased until spiderlings emergence from the egg-sac (Stage 6, 2.83 ± 1.68 µg PE/mg of wet weight), values that remained relative constant in advanced stages. Phosphatidylcholine (PC) presented a similar pattern as that of PE, beginning with a concentration of 12.65 ± 1.46 µg PC/mg of wet weight in the embryonic stages and decreased until spiderlings emergence from the egg-sac (Stage 6). Sphingomyelin (SM) maintained a small proportion of less than 1.2 µg SM/mg of wet weight throughout the developmental stages occurring inside and outside the egg-sac (Figure 8). However, in A this value decreased to average concentration of 0.28 ± 0.02 µg SM/mg of wet weight.

Triacylglycerides (TAG) concentration values were relatively constant until the stage 6 (juveniles in gregarious stage), between 12.55 ± 1.8 and 17.8 ± 3.7 µg TAG/mg of wet weight (Figure 9). After dispersion, values decreased reaching

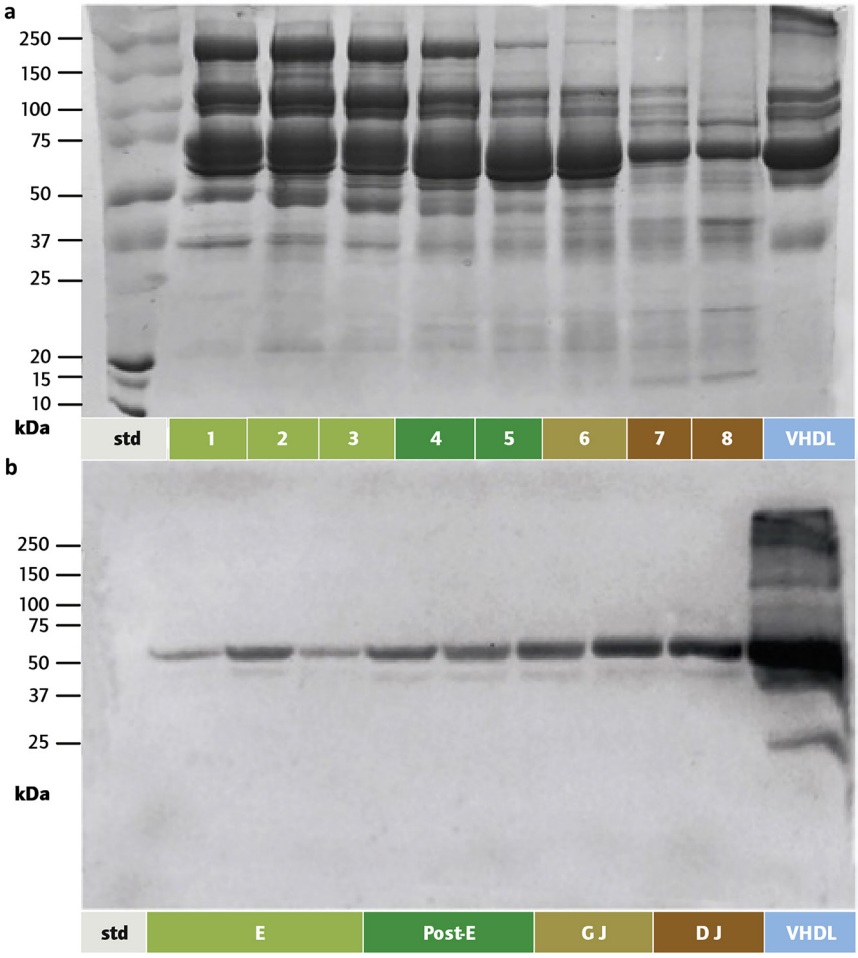

Figure 4. Analysis of soluble proteins during *P. pythagoricus* development. Electrophoresis (SDS-PAGE): 20 µg of soluble proteins per well (a) and immunoblotting with anti-Hc polyclonal antibodies: 15 µg of soluble proteins per well (b). std: molecular weight standard (kDa); VHDL: very-high-density lipoprotein of *P. pythagoricus*; 1 to 8: stages of *P. pythagoricus* development. Numbers with the same colour belong to the same period of development and are delimited below the figure as: E: embryonic period; Post-E: post-embryonic period; GJ: juveniles in gregarious stage; DJ: juveniles in dispersal stages.

0.77 ± 0.2 µg TAG/mg of wet weight in the stage 8. Then, the concentration rose to 2.6 ± 0.2 µg TAG/mg of wet weight in advanced stages. Free fatty acids (FFA) concentration had minimum values in the embryonic and post-embryonic periods (less than 2 µg of FFA/mg of wet weight), but once juveniles emerged the concentration increased to an average of 5.2 ± 0.63 µg of FFA/mg of wet weight (Figure 9). Later on, concentration values reduced to 1.2 ± 0.8 µg of FFA/mg of wet weight in AJ and A. Diacylglycerides (DAG) only appeared in small amounts during the post-embryonic development (Figure 9).

Hydrocarbons (HC) content increased during the embryonic development and remained relatively constant during post-embryonic development with averages of 1.03 ± 0.55µg of HC/mg of wet weight (Figure 10). Outside the egg-sac, dispersed juveniles increased their HC content to 4.63 ± 1.19 µg of HC/mg of wet weight and 3 ± 0.66 µg of HC/mg of wet weight in stage 7 and 8, respectively. Cholesterol (C) throughout the embryonic development fluctuated between 1.21 ± 0.12 to 1.51 ± 0.25 µg of C/mg of wet weight (Figure 10), but then decreased to 0.89 ± 0.28 µg of C/mg of wet weight when juveniles emerged from the egg-sac (Stage 6). In AJ and A, C concentration average was of 0.65 ± 0.05 µg of C/mg of wet weight. Sterol esters (SE) values were only detected in the post-embryonic stages with small amounts between 0.1 ± 0.06 and 0.96 ± 0.28 µg of SE/mg of wet weight (Figure 10).

Analysing the fatty-acid methyl esters (Figure 11), fatty acids percentages remained constant until the gregarious stage (Stage 6); however, significant changes appeared after dispersion (Stages 7 and 8). Palmitic acid (16:0) decreased from 13.1 ± 2.7% (Stage 6) to 6.9 ±

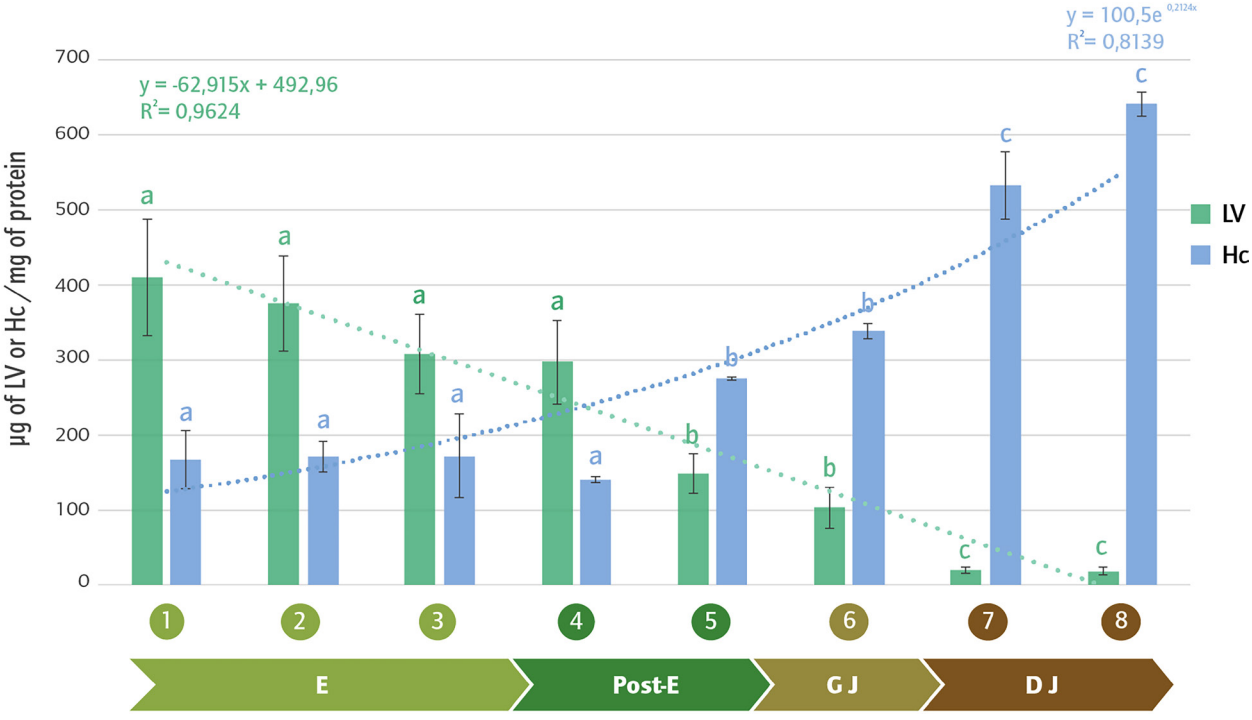

Figure 5. Analysis of lipovitellin (LV) and hemocyanin (Hc) concentration during *P. pythagoricus* development. Values are the means ± SDs. Different letters above the bars indicate significant differences among the different stages at $p < 0.05$, as determined by Tukey's *post hoc* test. 1 to 8: stages of *P. pythagoricus* development; E: embryonic period; Post-E: post-embryonic period; GJ: juveniles in gregarious stage; DJ: juveniles in dispersal stages.

0.16% (Stage 8), whereas stearic acid (18:0) increased from 7.2 ± 2.7% to 12.6 ± 0.17%. In the case of monounsaturated fatty acids, oleic acid (18:1) diminished from 45.2 ± 0.3% (Stage 6) to 24.8 ± 1.7% (Stage 8), whereas linoleic acid (18:2) began with 23.4 ± 0.7% and enriched to 38.45 ± 0.4%. Moreover, eicosatetraenoic acid (20:4) and eicosapentaenoic acid (20:5) increased their values from less than 1.5% to 6% in stages 7 and 8. Throughout the development, no changes were observed in the total amount of saturated fatty acids (SFA), while the unsaturated fatty acids underwent changes after juveniles emerged. The monounsaturated fatty acids (MUFA) content decreased from 49.45 ± 1.35% to 26.04 ± 2.02%, while the polyunsaturated fatty acids (PUFA) increased from 28.6 ± 1.93% to 52.2 ± 2.08%.

DISCUSSION

The presence or absence of maternal care, gregarious stages and competition for food and intra-species cannibalism are behaviours that may influence a spider's development. Thus, the study of resource variation during development may provide new information to explain or accompany behavioural studies. Up to the present it has been clear that the identity of the energetic resources that support embryonic and post-embryonic development in spiders and its understanding is limited in this megadiverse group.

Our results showed that *P. pythagoricus* had no variation in total protein concentration when accompanied by gradual consumption of LV during the early stages of development. These findings would suggest that there is a balance between new protein synthesis and yolk protein degradation. This has also been

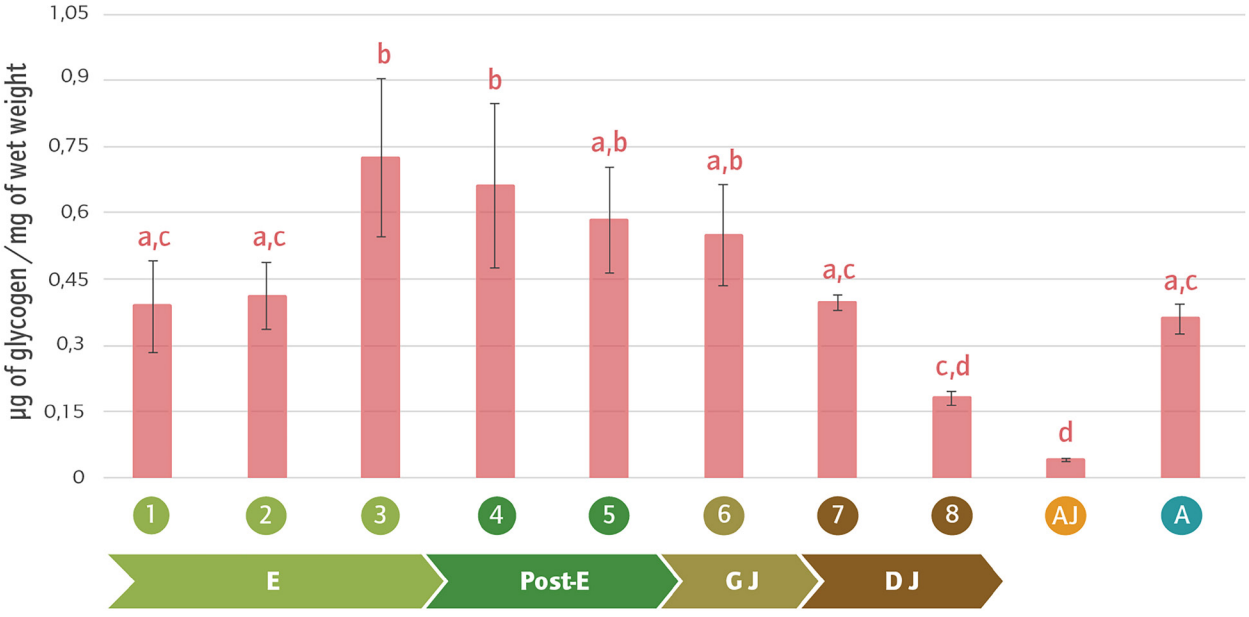

Figure 6. Analysis of glycogen concentration during *P. pythagoricus* development until adult's stage. Values are the means ± SDs. Different letters above the bars indicate significant differences among the different stages at $p < 0.05$, as determined by Tukey's *post hoc* test. 1 to 8: stages of *P. pythagoricus* development; AJ: advanced juvenile stage; A: adult stage; E: embryonic period; Post-E: post-embryonic period; GJ: juveniles in gregarious stage; DJ: juveniles in dispersal stages.

described for other arthropods (Campos et al. 2006, Kamel & Ragaa 1981). However, something different was reported in *P. saltans* spider, where gradual consumption of proteins was observed, even when LV levels did not decrease during embryonic development (Trabalon et al. 2018), which showed some differences between both species. *P. pythagoricus* showed differential consumption of LV polypeptides: higher-molecular-weight polypeptides (120 and 75 kDa) were consumed in post-embryonic instars, whereas lower-molecular-weight polypeptides (46 and 30 kDa) were consumed after juveniles emerged. Higher-molecular-weight polypeptides might be found to be more exposed to the proteolytic enzyme than lower-molecular-weight polypeptides during development as was previously described for other arthropods where LVs was observed to have a higher subunit (surrounding smaller polypeptides) that was more susceptible to enzymatic attack (Garcia et al. 2008). After juveniles of *P. pythagoricus* emerged from egg-sacs, total protein consumption and LV reserve exhaustion was probably a consequence of the intense activity of the mobile juveniles, especially during dispersion instars (Stage 7 and 8). Therefore, LV proteolysis would be linked to the energetic and structural needs of the organism (Lu & Warner 1991, Perona & Vallejo 1985, Warner et al. 1995).

Increase in the total amount of proteins in advanced stages of *P. pythagoricus* could be due to prey consumption. It was described that spider growth could be maximized with higher protein content diets (Jensen et al. 2011) since this may be needed to build new tissues and fulfil the basic requirements of metabolism, maintaining protein homeostasis in adults (Jensen et al. 2011, Romero et al. 2019, Wilder 2011). Salomon et al. (2011) showed that a protein-enriched diet in *Stegodyphus lineatus* resulted in higher juveniles' survival in this subsocial spider species.

The importance of Hc in O_2 transport in aqueous medium in arthropods and other invertebrates has been shown in different

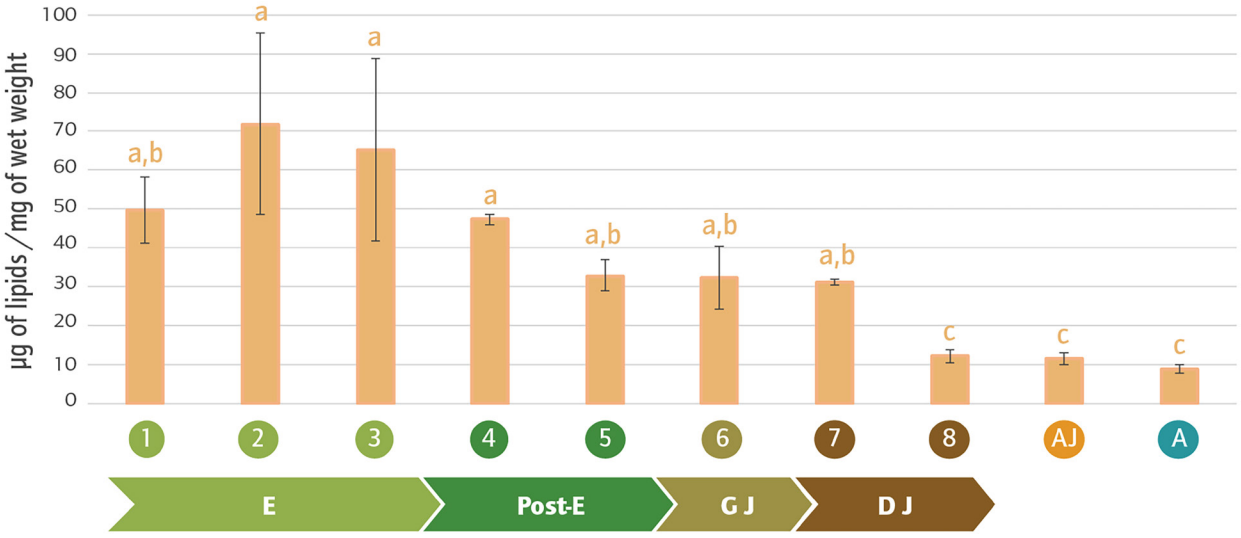

Figure 7. Analysis of total lipid concentration during *P. pythagoricus* development until adult's stage. Values are the means ± SDs. Different letters above the bars indicate significant differences among the different stages at $p < 0.05$, as determined by Tukey's *post hoc* test. 1 to 8: stages of *P. pythagoricus* development; AJ: advanced juvenile stage; A: adult stage; E: embryonic period; Post-E: post-embryonic period; GJ: juveniles in gregarious stage; DJ: juveniles in dispersal stages.

research papers (Foelix 2011, Markl & Decker 1992), this being the protein's main function. The presence of Hc in the eggs of several arthropods has been demonstrated over time (Chen et al. 2017), and it has also been recently described for the present model of study (Romero et al. 2019). This passage of Hc from the mother to the eggs could provide an initial pool of Hc to the embryo until this can be able to synthesize its own Hc; even more if we consider that Hc is not only an oxygen-carrying protein but also a protein storage of this molecule (Foelix 2011, Starrett et al. 2013). Active involvement of Hc in oxygen exchange in embryos of *Locusta migratoria* was previously described, where the authors proposed that hemocyanin plays an important role in oxygen transport during embryogenesis (Chen et al. 2017). By means of a process called inversion, during the embryonic development of Araneomorphae spiders, it has been observed that there is a great change in the organization of the body that results in the incorporation of yolk mass in the embryo (Wolff & Hilbrant 2011). Thus, the embryo would have a pool of Hc to supply the demand for oxygen which cannot be met by simple diffusion produced by the high rate of aerobic metabolism involved in embryonic development (Ingrisch 1987, Rakshpal 1962). Moreover, this could be an important fact to be highlighted if we consider that the eggs are small and isothermal in their local microclimate, and they are often challenged by oxygen shortage in fluctuating environments, even at mildly high temperatures (Woods et al. 2005, Woods & Hill 2004).

Although we cannot rule out the early synthesis of embryonic Hc, we might consider the beginning of Hc expression during development as occurring in late stages, the same as what was described for other arthropods (Chen

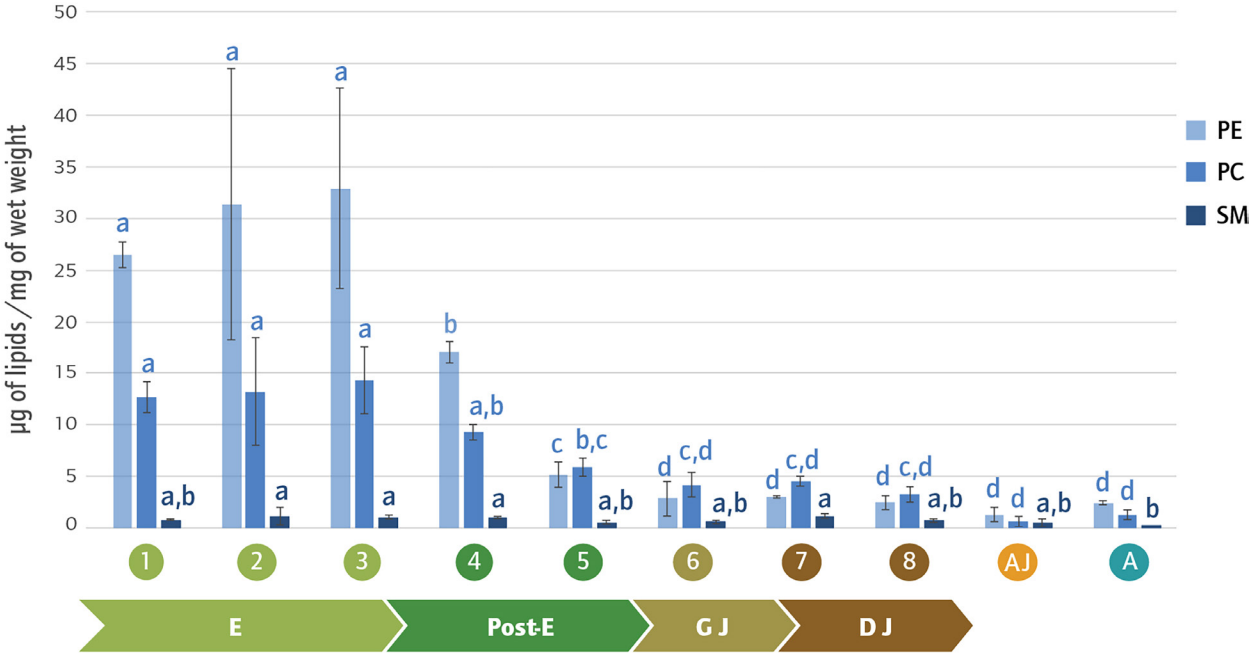

Figure 8. Concentration of the main phospholipids during *P. pythagoricus* development until adult's stage: phosphatidylethanolamine (PE), phosphatidylcholine (PC) and sphingomyelin (SM). Values are the means ± SDs. Different letters above the bars indicate significant differences among the different stages at $p < 0.05$, as determined by Tukey's *post hoc* test. 1 to 8: stages of *P. pythagoricus* development; AJ: advanced juvenile stage; A: adult stage; E: embryonic period; Post-E: post-embryonic period; GJ: juveniles in gregarious stage; DJ: juveniles in dispersal stages.

et al. 2017, Pick et al. 2010), even in members of Subphylum Chelicerata where an increase in Hc content was observed after the first embryonic moulting (Sugita & Sekiguchi 1979). The Hc increase observed during the post-embryonic stages of *P. pythagoricus* would allow juveniles to deal with the new challenges of the environment after hatching, since Hc has a great variety of functions such as the defence against pathogens (Coates & Nairn 2014, Riciluca et al. 2012), transporting hormones of the moulting (Jaenicke et al. 1999), acting as lipid transporter (Cunningham et al. 2007, Cunningham & Pollero 1996, Laino et al. 2009, 2015a), being part of the cuticle component (Paul et al. 1994) and having phenoloxidase activity (Jaenicke & Decker 2008). This last function is associated with the sclerotization process, a remarkable function if we consider that the pigmentation of the cuticle begins to show in this stage (Stage 5).

In various arthropods, carbohydrate mobilization has been described as being responsible for obtaining the energy which will finally be used for the growing embryo (Campos et al. 2006, Ludwig & Ramazzotto 1965, Mohamed 2000, Santana et al. 2014, Santos et al. 2008, Trabalon et al. 2018). Although glycogen is stored in eggs and is used in the early stages of development, its content has been observed to increase in late embryonic stages in *Drosophila melanogaster*, when organogenesis takes place (Waltero et al. 2020, Yamazaki & Nusse 2002). This is similar to what was observed in *P. pythagoricus* whose glycogen level increased at the end of the embryonic development (Stage 3). In advanced stages of development, different sources of carbohydrates, such as glycogen, may be important for chitin biosynthesis, which requires great amounts of glucose (Chippendale 1978). Our results showed a gradual and constant consumption of carbohydrates after

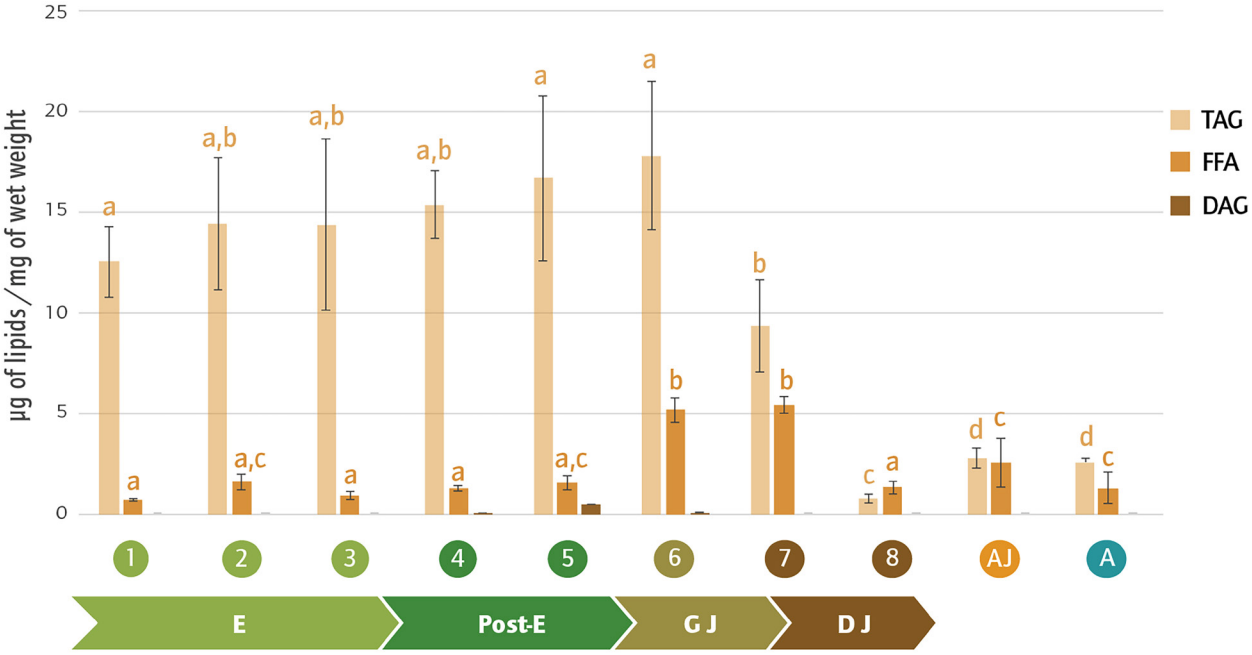

Figure 9. Concentration of energetic lipids during *P. pythagoricus* development until adult's stage: triacylglycerides (TAG), free fatty acids (FFA), and diacylglycerides (DAG). Values are the means ± SDs. Different letters above the bars indicate significant differences among the different stages at $p < 0.05$, as determined by Tukey's *post hoc* test. 1 to 8: stages of *P. pythagoricus* development; AJ: advanced juvenile stage; A: adult stage; E: embryonic period; Post-E: post-embryonic period; GJ: juveniles in gregarious stage; DJ: juveniles in dispersal stages.

hatching, similar to that observed in *P. saltans* spider (Trabalon et al. 2018). However, these carbohydrates represented less than 1% of the total reserves in *P. pythagoricus* eggs.

In *P. pythagoricus*, lipid content during development was greater in the embryonic and non-dispersed juvenile stages than in dispersed juveniles and adults. The same was observed in other arachnids and in various holometabolous insects, where this energy reserve would offset the partial or total energy required for adult life stages (Lease & Wolf 2011). Throughout the time, it has been highlighted that energetic lipids represent an important resource for the embryonic development of many insects, mites and crustaceans (Campos et al. 2006, Geister et al. 2009, Santos et al. 2011, Van Handel 1993). However, in most spiders this does not seem to be true. *P. pythagoricus* eggs initially have 72.2% of structural lipids, and only 24.3% of energetic lipids. This agrees with the analysis performed in *Schizocosa malitiosa* (Lycosidae) where it was observed that eggs had 67 ± 27% of structural lipids (PE + PC + SM), only 26.6 ± 18.5% of energetic lipids (TAG + FFA) and 5.5 ± 3.7% of C + SE + HC (Laino et al. 2013). Nevertheless, this does not coincide with the values observed in *P. saltans* (Lycosidae), where the reported figures were 33.1 ± 1.2% for structural lipids, 51.5 ± 8.1% for energetic lipids, and 12.8 ± 4% for C + SE + HC + LPL (Lysophosphatidylcholine) (Trabalon et al. 2018). According to the authors, the difference found in the lipid composition of eggs of these Lycosidae spiders could be due to the difference

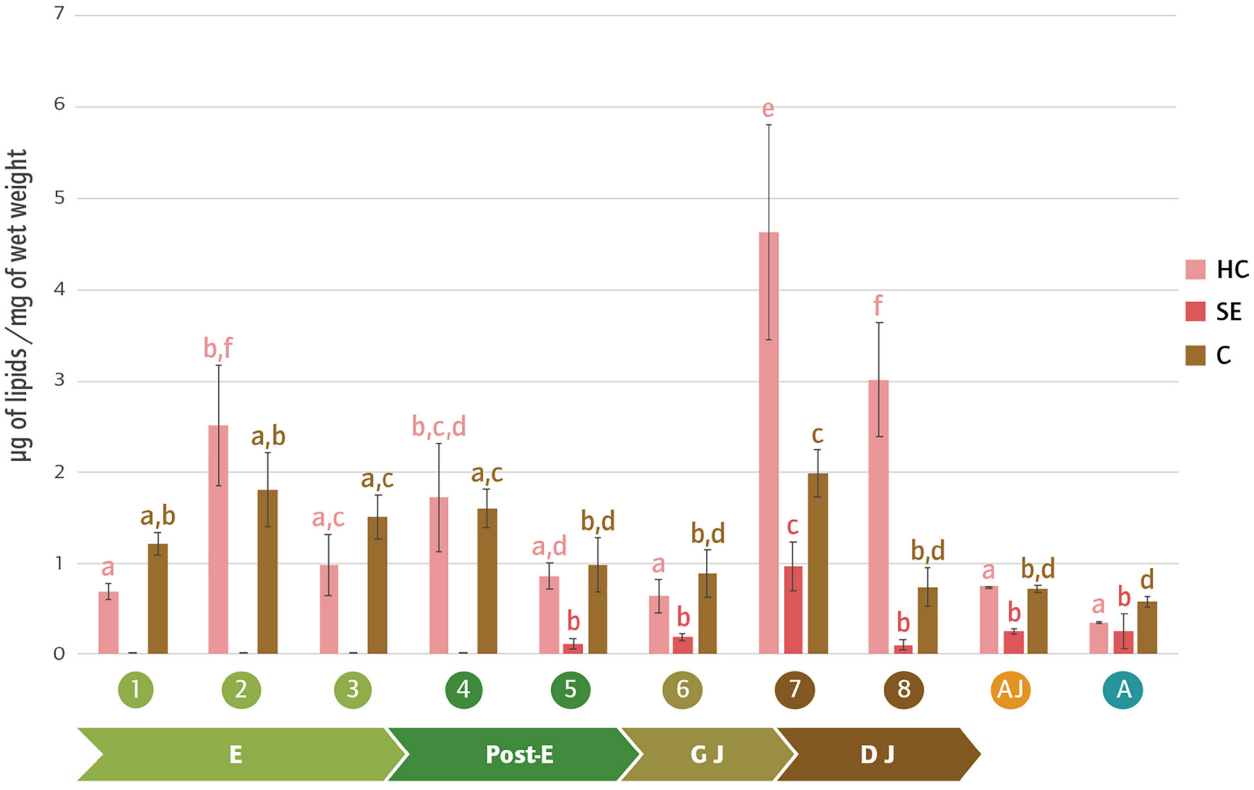

Figure 10. Concentration of modulating or signaling lipids during *P. pythagoricus* development until adult's stage: hydrocarbons (HC), cholesterol (C) and sterol esters (SE). Values are the means ± SDs. Different letters above the bars indicate significant differences among the different stages at $p < 0.05$, as determined by Tukey's *post hoc* test. 1 to 8: stages of *P. pythagoricus* development; AJ: advanced juvenile stage; A: adult stage; E: embryonic period; Post-E: post-embryonic period; GJ: juveniles in gregarious stage; DJ: juveniles in dispersal stages.

in the type of food mothers received during the test, as it was observed in the hemolymph of *Eurypelma californicum* and *Brachypelma albopilosum* that were fed with different animals (Schartau & Leidescher 1983, Trabalon 2011, Trabalon et al. 2018).

Oocytes of different spider families show the presence of aggregates of organelles and inclusions usually arranged into orderly concentric zones called Balbiani bodies or yolk nucleus, with concentric lamellae corresponding to the endoplasmic reticulum (Andre & Rouiller 1957, Jędrzejowska & Kubrakiewicz 2010, Sotelo & Trujillo-Cenoz 1957), which might show a high content of structural lipids. Moreover, the major presence of these structural lipids in yolk lipoproteins was previously described in *P. pythagoricus* (Laino et al. 2011b), where their percentages were 49 ± 1% for LV1 and 37.6 ± 1% for LV2, while energetic lipids represented only 9.5 ± 0.4%. During vitellogenesis, the presence of structural lipids was highlighted as a key component of the ovary in *P. pythagoricus* females; this organ became charged with 63% of these lipids (especially with PE) and only with 23% of energetic lipids (Romero et al. 2018).

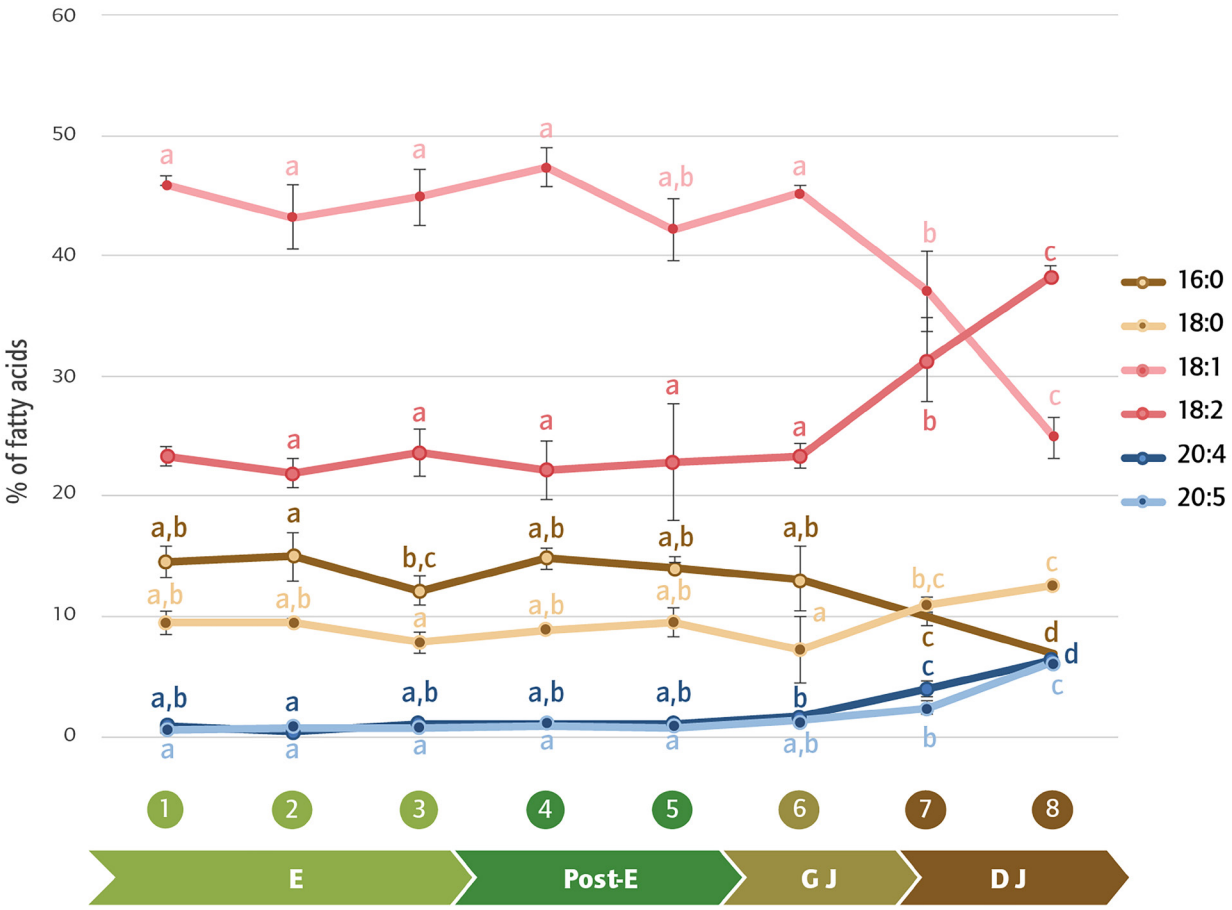

Figure 11. Composition of the major fatty acids during *P. pythagoricus* development: palmitate (16:0), stearate (18:0), oleate (18:1), linoleate (18:2), eicosatetraenoic acid (20:4) and eicosapentaenoic acid (20:5). The values are the percentage means ± SDs. Different letters indicate significant differences among the different stages at a $p < 0.05$, as determined by Tukey's *post hoc* test. 1 to 8: stages of *P. pythagoricus* development; E: embryonic period; Post-E: post-embryonic period; GJ: juveniles in gregarious stage; DJ: juveniles in dispersal stages.

Likewise, the eggs of this species have a great percentage of PE, which coincides with the composition reported for their own LVs (Laino et al. 2011b) and with that reported for some embryos of crustaceans (Wang et al. 2015). Up to the present, it has been proposed that PE needs a unique structure to form the inner mitochondrial membranes, thus improving the functioning of the electron transport chain (Ikon & Ryan 2017, Romero et al. 2018, Tasseva et al. 2012, Teague et al. 2013). However, its partial consumption in the post-embryonic period (over 50%) and its great consumption in the emerged juveniles (over 90%), represent considerable evidence that PE could also have an energetic function as some authors suggest for structural lipids (Hagen et al. 1996, Mayzaud et al. 2003). Furthermore, it has been described that phospholipids can act as energetic resource during the early development of some crustaceans (Sibert et al. 2004). This hypothesis could be reinforced by the scarce content of this phospholipid in dispersed juveniles and adults (0.4% and 0.21% of the initial content of PE). PE would be hydrolysed mainly in the post-embryonic stages, thus providing an ethanolamine moiety that could covalently modify several proteins (Vance & Tasseva 2013). Great consumption of phospholipids during the post-embryonic stages would reveal that phospholipase activity may probably be greater than lipase activity.

TAG are found in yolk forming lipid droplets (Schie et al. 2013, Walther & Farese 2012), and they are the most important energetic molecules because of their great caloric capacity and the way they get stored (Laino et al. 2011b, 2013). TAG were consumed in the more active stages analysed in *P. pythagoricus*: 50% in stage 7 and 96% in stage 8 (juveniles in dispersion instar). This kind of late hydrolysis of TAG was also observed in *P. saltans* spider (Trabalon et al. 2018). Although the *de-novo* synthesis of FFA cannot be discarded, in the present work it can be inferred that the increase of fatty acids in juveniles in the gregarious stage (Stages 6) is probably due to phospholipid hydrolysis (mainly PE), whereas in the dispersed juveniles (Stage 7 and 8) it could be due to TAG hydrolysis.

HC in spiders could have several functions such as that of being the first chemical barrier to prevent the entry of pathogens, serving as kairomones for entomopathogenic fungi and bacteria (Lecuona et al. 1991), participating as controllers of hydric homeostasis (Gibbs 1998, Hadley 1981) and having an active role in the chemical communication among congeners (Blomquist & Bagnères 2010). HC content varies to a great extent in *P. pythagoricus*, mainly in embryonic stages and in juveniles in dispersion This may be due to the de *novo* synthesis that was previously described for arthropods (Fan et al. 2008). On the other hand, both C and SE are membrane components and moulting hormone precursors (Andersen 1979, Martin-Creuzburg et al. 2007). Therefore, it is clear that, although HC, C, and SE are minor in adults, they play an important biological role such as the formation of ecdysteroids (Andersen 1979, Martin-Creuzburg et al. 2007). However, the lack of information with relation to the subject hinders the adequate interpretation of the role played by these lipids in spiders' development.

In *P. pythagoricus,* egg reserve mostly contains unsaturated fatty acids 18:1 and 18:2, and saturated fatty acids 16:0 and 18:0. These major fatty acids were reserved in the ovaries during vitellogenesis (Romero et al. 2018), to later become part of yolk, thus coinciding with the general pattern of fatty acids of their LVs that are components of yolk (Laino et al. 2011b). This pattern of fatty acids was also observed in two members of the family Lycosidae, *S. malitiosa* and *P. saltans*, with 18:2, 18:1, 16:0 and 18:0 as major fatty acids (Laino et al. 2013, Trabalon et al. 2018).

Although it is described that in some insects the composition of fatty acids remains constant throughout embryonic development (Hoppe et al. 1975), in other arthropods consumption does not seem to be equal, since some fatty acids are consumed and others produced during embryogenesis (Figueiredo et al. 2012). In the case of spiders, the total percentage of SFA, MUFA, and PUFA remained constant until the gregarious stage. Then, the percentage of MUFA decreased with the concomitant increase of PUFA during dispersion, along with a specific change in fatty acid composition inside these groups. The impoverishment of 16:0 was offset by the enrichment of 18:0, which explains the absence of SFA, mainly in dispersion stages where the consumption of TAG was evident. Apart from being an important source of energy, it was observed that derivatives of 16:0 —as 14-methylhexadecanoic acid— can be found in the silk and have important antimicrobial activity provided by the protective effect of the methyl group (Heimer 1988, Tahir et al. 2017), which is an important fact considering that active silk production already takes place in these dispersed stages. In the case of unsaturated fatty acids, there was an impoverishment of 18:1 and a major enrichment of 18:2, which play an important role in functions such as cell physiology, immunity, and reproduction (Malcicka et al. 2018). Fatty acid species present in phospholipids and TAG appear to be similar in both types of lipids. It is assumed that energetic lipids mostly contain SFA and that phospholipids have a higher amount of PUFA (Innis 1991). In *P. pythagoricus,* phospholipids do not seem to have the majority of polyunsaturated fatty acids or else these do not correlate with the amount of PE used. Even so, 20:4 and 20:5 fatty acids in the dispersed juveniles increased six times more than in embryonic stages, which are considered to be essential fatty acids for arthropods. The importance of fatty acids with C20 lies in the fact that they are precursors of eicosanoids (prostaglandins, leukotrienes) (Meijer et al. 1986, Petzel 1993, Stanley-Samuelson et al. 1991, Stanley-Samuelson & Pedibhotla 1996). Several studies on arthropods describe the function of eicosanoids in the defence against microorganisms through immune reactions (Morishima et al. 1997, Park et al. 2003, Stanley-Samuelson et al. 1991), something that would be of vital importance for the survival of just dispersed juveniles.

As a conclusion, we can highlight in the development of *P. pythagoricus* spider that: 1) carbohydrates are gradually consumed after hatching, but they represent less than 1% of the reserves; 2) apolipoproteins of the LV in these spider species are gradually and differentially consumed, being first consumed the higher-molecular-weight polypeptides (120 and 75 kDa) and then the lower-molecular-weights polypeptides (46 and 30 kDa); 3) while LV amount and the total protein content decrease, there is an important increase of Hc content from post-embryonic instars to dispersion instars, which allows us to consider that the Hc synthesis of the offspring during development is late; 4) the main energy resources during development are represented by LV and phospholipids, but TAG surprisingly do not represent the majority energy source being consumed in advanced stages; and finally 5) the use of phospholipids (PE and PC) and unsaturated fatty acids (especially MUFA) gains great importance in the post-embryonic and emerged juveniles respectively. It is clear that, although there is a need to generate new research to better understand the metabolism of the development of spiders, the present work is the first one to have obtained a continuous and complete perspective of the biochemical changes generated during spider development.

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