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ARTICLE

First study on lipid dynamics during the female reproductive cycle of *Polybetes pythagoricus* (Araneae: Sparassidae)

S. Romero, A. Laino, F. Arrighetti, M. Cunningham, and C.F. Garcia

Abstract: Spiders are valuable to humans, not only for their role in health but also as biologic pest controllers. In oviparous species, lipids are the main energy source for embryo development and the growth and survival of larvae. Using the spider *Polybetes pythagoricus* (Holmberg, 1875) as an experimental model, we studied the fluctuations in lipids and fatty acids occurring in tissues related to vitellogenesis. Different reproductive stages (previtellogenesis, early vitellogenesis, vitellogenesis, and postvitellogenesis) were determined histologically. Gonadosomatic and hepatosomatic indices were first used in spiders. The midgut diverticula proved to be the organ with the highest lipid concentration, with triacylglycerols as the major component. Phospholipids were the principal lipids transported. In vitellogenesis, a major accumulation of lipids occurred in the ovary, principally phosphatidylethanolamine (41%); it probably synthesized in the midgut diverticula before being released into the hemolymph for transport and accumulation in the ovary. Phosphatidylethanolamine is possibly involved in maintaining membrane fluidity and in the function of the electron transport chain. The principal fatty acids in the different organs were palmitic, stearic, oleic, and linoleic acids. During vitellogenesis, the ovaries become enriched in polyunsaturated fatty acids. The lipid patterns in the male midgut diverticula, muscle, and hemolymph were similar to those of the previtellogenic or postvitellogenic females.

Key words: spider, vitellogenesis, lipid, GSI, *Polybetes pythagoricus*.

Résumé : Les araignées sont importantes pour les humains, non seulement pour leur rôle dans la santé, mais également pour celui qu'elles jouent dans la lutte contre les ravageurs. Chez les espèces ovipares, les lipides sont la principale source d'énergie pour le développement des embryons et la croissance et la survie des larves. En utilisant l'araignée *Polybetes pythagoricus* (Holmberg, 1875) comme modèle expérimental, nous avons étudié les fluctuations associées à la vitellogenèse des lipides et acides gras dans les tissus. Différents stades de la reproduction (pré-vitellogenèse, début de la vitellogenèse, vitellogenèse et post-vitellogenèse) sont définis sur une base histologique. Des indices gonadosomatiques et hépatosomatiques sont pour la première fois utilisés pour des araignées. Les glandes digestives s'avèrent être l'organe avec la plus forte concentration de lipides, les triacylglycérols en étant les plus importants. Les phospholipides sont les principaux lipides transportés. Durant la vitellogenèse, une importante accumulation de lipides se produit dans l'ovaire, principalement de la phosphatidyléthanolamine (41 %), probablement synthétisée dans les glandes digestives avant d'être libérée dans l'hémolymphe pour ensuite être transportée et s'accumuler dans l'ovaire. La phosphatidyléthanolamine participe possiblement au maintien de la fluidité des membranes et au fonctionnement de la chaîne de transport d'électrons. Les principaux acides gras dans les différents organes sont les acides palmitique, stéarique, oléique et linoléique. Durant la vitellogenèse, les ovaires deviennent enrichis en acides gras polyinsaturés. La distribution des lipides dans les glandes digestives, les muscles et l'hémolymphe des mâles est semblable aux distributions pré-vitellogenèse et post-vitellogenèse chez les femelles. [Traduit par la Rédaction]

Mots-clés : araignées, vitellogenèse, lipide, IGS, *Polybetes pythagoricus*.

Introduction

At present, spiders have gained a prominent relevance in the biologic control of the pests of different agroecosystems (Riechert and Lockley 1984; Tarabaev and Sheykin 1990). To a great extent, this significance is owing to their predatory capacity and their generalist feeding behavior (Nyffeler and Sterling 1994; Nyffeler et al. 1994). In addition, certain species of arachnids are a hazard to human health because of the harmful effect that their venom can cause (Vetter and Isbister 2008), in some instances to the extent of causing fatality (Pezzi et al. 2016). Despite the beneficial or harmful effects that spiders may have on humans, current information is scarce with respect to the biochemical and physiologic aspects of these organisms, especially those features associated with the reproductive process.

In oviparous species, the lipids present in the vitellus are the main source of energy for embryo development and the growth and survival of the larvae. In the ovaries of females in a reproductive stage, lipids are distributed among membranes, lipid droplets, and lipoproteins (Ziegler and Van Antwerpen 2006). Membrane lipids contribute to structure and are mainly formed by phospholipids and sterols. Lipid droplets are formed by triacylglycerides (TAG) and function as energy reserves. Finally, the lipovitellins are lipoproteins found in both the ovary and the eggs of oviparous animals and are mostly composed of phospholipids and TAG. In arachnids, these proteins have been characterized in *Polybetes*

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pythagoricus (Holmberg, 1875) (Sparassidae; Laino et al. 2011*b*) and in *Schizocosa malitiosa* (Tullgren, 1905) (Lycossidae; Laino et al. 2013).

During the reproductive stage, in general, the females have a high quantity of lipids in the hemolymph, ovary, and midgut diverticula (spiders), in the hepatopancreas (crustaceans and scorpions) or in the fat body (insects) (Lubzens et al. 1981; Grapes et al. 1989; Ravid et al. 1999; Warburg 2012). The synthesis of these lipids and their incorporation into the ovaries are regulated by hormones in the process referred to as vitellogenesis. These various steps are critical to those different organisms because vitellogenesis can determine embryo viability and thus species survival. Vitellogenesis has been well described in insects (Sappington and Raikhel 1998; Ziegler and Van Antwerpen 2006; Ibánez et al. 2017) and crustaceans (Muñoz et al. 1990; Vazquez Boucard et al. 2002; Shechter et al. 2005), but information of this process in the Class Arachnida is still lacking.

Although histological studies have been carried out on vitellogenesis in spiders, e.g., *Tegenaria parietina* (Fourcroy, 1785) (Andre and Rouiller 1957), *Heptathela kimurai* (Kishida, 1920) (Osaki 1972), *Cupiennius salei* (Keyserling, 1877) (Seitz 1971), and certain species of the families Lycosidae, Thomisidae, Gonyleptidae, and Sicaridae (Sotelo and Trujillo-Cenoz 1957), to date, the status of lipids during vitellogenesis has not been clarified, nor has the quantity of lipids been related to the reproductive effort.

Therefore, to understand the biochemical and physiologic mechanisms related to the vitellogenesis of spiders, a determination of the quality and quantity of lipids present in the main organs involved in lipid metabolism is necessary. In view of these considerations, in the present work using the spider *Polybetes pythagoricus* as a model, we studied the fluctuations of the different lipids and fatty acids present in muscle, midgut diverticula, ovary, and hemolymph of females during different vitellogenic stages.

Materials and methods

Ethics statement

Polybetes pythagoricus, the species used for the experiments, is neither endangered nor protected. Our research conforms to the legal requirements for the treatment and care of animals in laboratory research with respect to those prescribed accepted ethical standards.

Spider collection and rearing

Our subjects were adult females and males captured from a forest of *Eucalyptus* sp. in the Martín Rodríguez Park in the city of Ensenada (34°52′56″S, 57°56′07″W) and in Pereyra Iraola Park (Berazategui; 34°50′39″S, 58°10′55″W), Argentina, from December 2015 through December 2016. For those campaigns, we obtained capture permit No. 117/16 for Protected Areas, Province of Buenos Aires.

Spiders were housed individually in cylindrical terrariums (10 cm in diameter × 5 cm in height) without any substrate and were kept at 20 ± 1 °C under a 14 h light – 10 h dark photoperiodic cycle without food for 24 h before sacrifice under cold anesthesia.

Experimental groups

A total of 120 spiders were used in this study. They were separated into five experimental groups according to their vitellogenic stages and sex.

Females were separated according to their reproductive status: previtellogenic (adult females before the start of vitellogenesis), early-vitellogenic, vitellogenic, and postvitellogenic (females after oviposition). We used histological studies to identify the three first groups (previtellogenic, early-vitellogenic, and vitellogenic), while oviposition was taken into account for the postvitellogenics, sacrificing them after 2 days.

General measurements

All of the individuals were weighed on an analytical balance and the sizes of prosoma and opisthosoma were measured with a digital hardened stainless-steel caliper (0–150 mm).

Hemolymph was obtained by severing the spiders' legs and centrifuging the spiders in a tube at low speed (Cunningham et al. 1994). A dorsal incision was made in the tegument, and the midgut diverticula and gonads were carefully dissected out, as indicated in Laino et al. (2009). At the same time, muscles were removed from the legs. All of the tissues isolated (the midgut diverticula, gonads, and muscles) were weighed in a Mettler-Toledo NewClassic MS-204S analytical balance.

We measured two indices, the gonadosomatic index (GSI) and the hepatosomatic index (HSI). The first was calculated as the gonad mass (g) × 100/body mass (g). The second is normally used in different invertebrates having a hepatopancreas, an organ comparable with the midgut diverticula of spiders with respect to lipid metabolism. As no term analogous to HSI exists for spiders, we adapted the same nomenclature for the present work. Thus, the HSI here was calculated as the midgut diverticula mass (g) × 100/body mass (g).

Histological analysis

For the study of the different oocyte stages, females were dissected and their ovaries were fixed in Bouin's solution for 4 h and then washed and stored in 70% ethanol. The ovaries were dehydrated through an increasing series of ethanol concentrations and embedded in Leica Historesin®. Sections were cut at 4 μm with an electronic microtome (Leica® RM 2155), mounted on microscope slides, and finally stained with hematoxylin–eosin. The oocytes were viewed under light microscopy (AXIOPLAN 2 Zeiss®), and the diameters were measured using the AxioVision Rel. 4.6.3 software (Zeiss®) analyzing program.

Lipid extraction and analysis

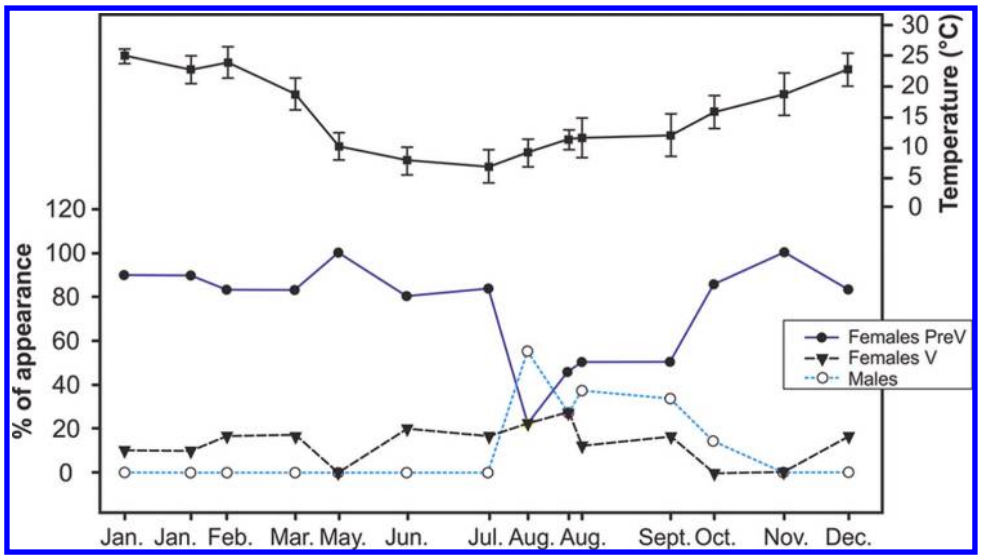
The lipids from the midgut diverticula, hemolymph, muscle, and gonad were extracted following the procedure of Folch et al. (1957). Quantitative determination of lipid classes was performed by thin layer chromatography (TLC) coupled to a flame ionization detector (FID) in an Iatroscan apparatus model TH-10 (Iatron Laboratories, Tokyo, Japan) after separation on type S-III Chromarods (Ackman et al. 1990; Laino et al. 2015). The lipid classes were quantified using monoacylglycerol as an internal standard.

Lipids were separated by a sequence of three different solvent systems. First, after development in hexane–benzene (70:30 by volume), the chromarods were dried and partially scanned to determine the apolar lipids. Second, the solvent benzene–chloroform–formic acid (70:25:1 by volume) was used to analyze the neutral lipids. Finally, the polar lipids were determined by development in chloroform–methanol–water (70:25:3 by volume). The lipid spots were quantified by comparison with curves of the following standards run under the same conditions: C24 hydrocarbons (HC), cholesteryl oleate, tripalmitin, dipalmitin, oleic acid, cholesterol (C), phosphatidylethanolamine (PE), phosphatidylcholine (PC), and sphingomyelin (SM). Three determinations from each of three independent pools were performed for each sample.

Fatty-acid analysis

Fatty-acid methyl esters from the total lipids of each of the tissues were prepared using a boron trifluoride – methanol solution according to the method of Morrison and Smith (1964). 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). The column temperature was programmed for a linear increase of 3 °C·min^{-1} from 175 to 230 °C, and the peaks

Fig. 1. Percentage of samples of *Polybetes pythagoricus* males and females in the vitellogenic and previtellogenic stages throughout a year (n = 12 per capture site). In the figure, the percentage of appearance of females (previtellogenic, PreV; vitellogenic, V) and males is plotted on the lower left ordinate and the temperature (in °C) is plotted on the upper right ordinate for each of the months during the year (denoted on the abscissa). Colour version online.



were identified by comparison of their retention times with those from a mixture of standard methyl esters.

Statistical analyses

A statistical comparison of the quantity of lipids and the percentage of different lipid classes of midgut diverticula, muscle, gonads, and hemolymph was performed by a one-way ANOVA after checking for normality and homogeneity of variances. The lipid and fatty-acid compositions were expressed as the means ± standard deviations (SDs). Significant differences ($p < 0.05$) were compared by Tukey's post hoc test and the Student's t test. Data were analyzed using GraphPad InStat 3.01 (GraphPad Software, San Diego, California, USA).

Results

After the capture of the *P. pythagoricus* spiders in the locations described in Materials and methods, we observed that the quantity of males remained constant throughout the entire year at less than 30%. Among the females, the percentage of previtellogenic individuals decreased in reciprocity to the increase in the vitellogenic females during the months of August, September, and October, reaching almost 60% at temperatures ranging from 7.8 to 16 °C (Fig. 1). In the previtellogenic stage, the length and width of the opisthosoma were 10.7 ± 1.6 mm and 8.8 ± 1.4 mm, respectively. In the early-vitellogenic, vitellogenic, and postvitellogenic stages, the respective corresponding values were 11.5 ± 1.6 and 9.4 ± 2.1 mm, 16.8 ± 1.6 and 13.3 ± 1.4 mm, and 10.2 ± 1.2 mm and 7.8 ± 0.8. The respective analogous measurements in males (9.7 ± 1.3 mm and 7.2 ± 0.5 mm) were similar to those of the previtellogenic and postvitellogenic females.

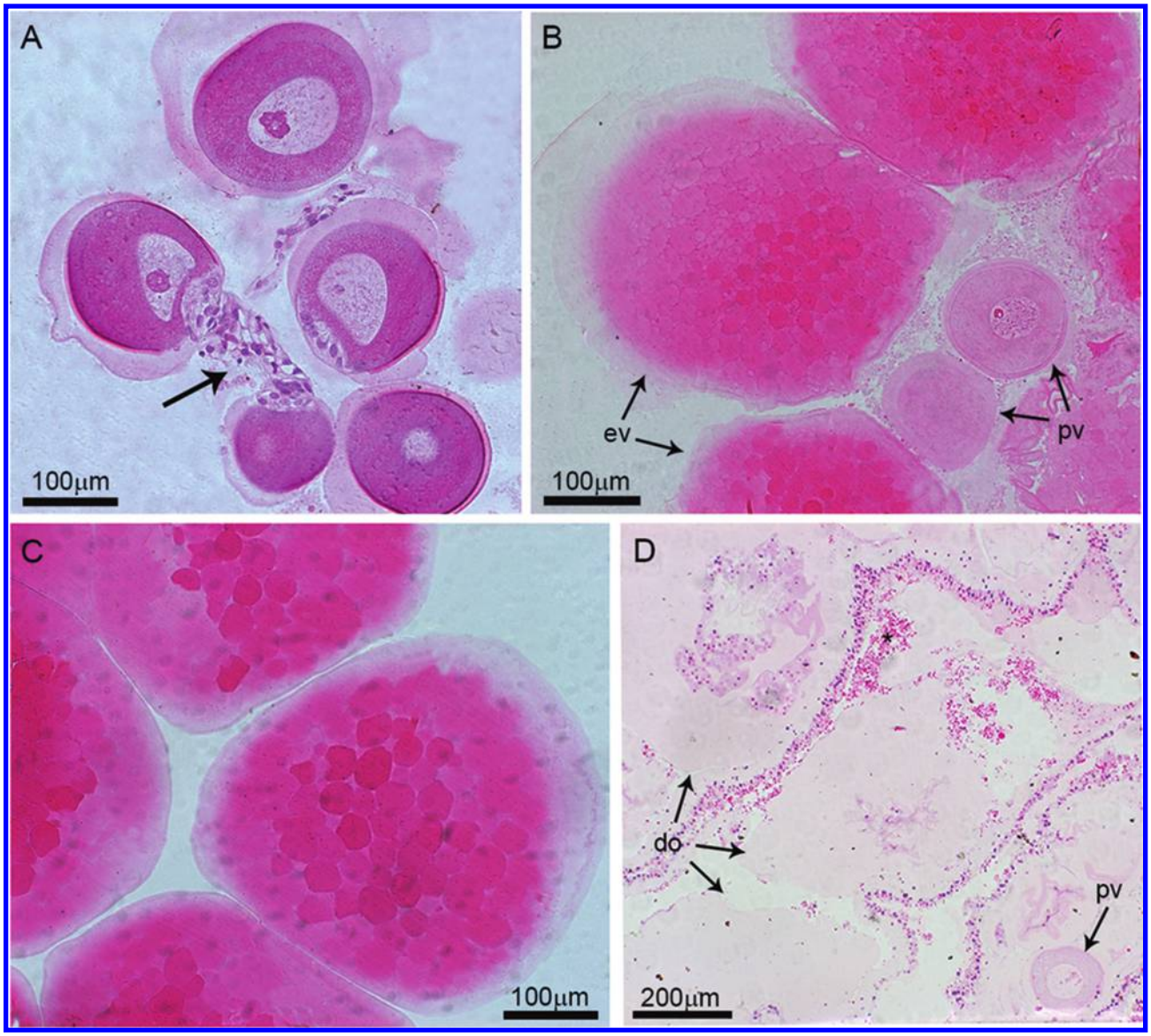
The ovaries in spiders are a pair of elongated sacs located in the ventral region of the opisthosoma. Throughout the ovary sacs, oocytes in different stages of development were present (Fig. 2): previtellogenic, early-vitellogenic, and vitellogenic oocytes along with those in the degeneration–resorption phase. The previtellogenic oocytes were connected to the ovarian wall by a pedicel possibly derived from the epithelial cells of the ovarian wall (Fig. 2A). Those oocytes were 90 to 183 μm (115 ± 39 μm) in diameter and were characterized by a large oval nucleus with a prominent spherical nucleolus and an eosinophilic gelatinous coat surrounding the cell. The oocytes increased in size at the start of yolk synthesis. The early-vitellogenic oocytes were 627 ± 86 μm in diameter and were characterized by the presence of small yolk granules (Fig. 2B). At the end of vitellogenesis, the yolk granules increased in size and filled the entire cytoplasm of the vitellogenic oocytes, which, at this stage, had diameters that measured 759 ± 60 μm (Fig. 2C). After oviposition, the remaining oocytes degenerated, their cytoplasm became distended, and the yolk granules disintegrated and were dispersed throughout the ovary (Fig. 2D). During this stage, some previtellogenic oocytes were still present.

The macroscopic groupings were corroborated with histologic patterns. In the previtellogenic group, only previtellogenic oocytes were found in the ovary, whereas in the early-vitellogenic group, the ovary contained early-vitellogenic and some previtellogenic oocytes. The ovary of the vitellogenic group then became fully filled with vitellogenic oocytes, whereas in the postvitellogenic group, the ovary finally went into a regression stage with degenerative oocytes.

Figure 3 presents the masses of the entire body, ovary, and midgut diverticula of the spiders during the four stages of the study. Although there was some increase in the total body mass during vitellogenesis, with values that reached 2500 mg, the masses of the different stages were statistically equivalent. The mass of the midgut diverticula remained constant (about 220 mg) during vitellogenesis. In contrast, the mass of the ovary was at an initial value of 30 mg in the previtellogenic females and then increased significantly in the subsequent stages: first, by a fourfold increase during the early-vitellogenic stage (to 120 mg) and finally by a further threefold increase during vitellogenesis (to 370 mg). This last rise was reflected in the comparison between the corresponding indices (GSI and HSI) illustrated in Fig. 4. Figure 4 also demonstrates that the HSI underwent no significant change during the different vitellogenic stages, but that the GSI increased 2.9-fold in the early-vitellogenic stage and a further 1.4-fold during the vitellogenic stage.

After gravimetrically quantifying the lipids of the midgut diverticula, ovary, muscle, and hemolymph, we observed significant differences in these organs during the different vitellogenic stages (Fig. 5). The total mass of lipid in those four organs and (or)

Fig. 2. Histology of the ovaries in (A) previtellogenic, (B) early-vitellogenic, (C) vitellogenic, and (D) postvitellogenic stages. Staining: hematoxylin–eosin. In (A), the thick arrow marks a pedicel; in (B), the thin arrows indicate previtellogenic (pv) and early-vitellogenic (ev) oocytes, and in (D), the thin arrows indicate previtellogenic (pv) and degenerated (do) oocytes. Colour version online.



tissues in the previtellogenic and vitellogenic stages were 42 and 199 mg·g^{-1} wet mass, respectively, representing a 4.7-fold increase above the lipid content of the previtellogenic stage, with 84.7% of that increase occurring in early vitellogenesis and the remaining 15.3% occurring in vitellogenesis. The initial value for the total lipids of the midgut diverticula (in the previtellogenic stage) was 26.1 mg·g^{-1}, with a 3.3-fold increase of 87.1 mg·g^{-1} occurring in vitellogenesis, resulting from an initial increase of 71.7% in the early vitellogenic stage followed by a final rise of 28.3% in the vitellogenic stage. In previtellogenesis, the lipid content of the ovary was 9.9 mg·g^{-1}, with a major increase in lipid content of 6.6-fold in the vitellogenic stage. This increase constituted a difference of 65.3 mg·g^{-1} of lipids, representing a 77% increase in the early-vitellogenic stage along with a further increment of 24% at the vitellogenic stage. Finally, the increase in the lipid content of the muscles throughout the four stages was from 6.3 mg·g^{-1} to 20.4 mg·g^{-1} and of the hemolymph was from 4.6 mg·mL^{-1} to 7.3 mg·mL^{-1}. The total lipid content of the different tissues in the males was 102.0 mg·g^{-1}, with 50.0 mg·g^{-1} in the midgut diverticula, 40.9 mg·g^{-1} in the testicle, and 11.1 mg·mL^{-1} or mg·g^{-1} in the hemolymph and muscle, respectively.

In addition, the total volume of the hemolymph in previtellogenesis was 143 ± 78 µL per spider; while in early vitellogenesis, vitellogenesis, and postvitellogenesis, the respective values were 230 ± 80, 420 ± 56, and 211 ± 74 µL.

The lipid composition of the midgut diverticula remained similar during vitellogenesis, with the principal lipids clearly being TAG at 75%, PC and PE at approximately 7%, and the sterol esters (SE), free fatty acids (FFA), C, HC, and SM at values below 3% (Fig. 6).

In the ovary during the previtellogenic stage, PC comprised 34% and both PE and TAG together comprised 19%. In contrast to the midgut diverticula, in the ovary, a significant increase in the PE content, along with a reciprocal decrease in FFA, occurred throughout vitellogenesis to attain respective values of 41% and 2.7% in the vitellogenic stage (Fig. 7).

The analysis of the hemolymph lipid classes indicated two similarities to the ovarian lipids. First, PC was the principal lipid at 56%, and, second, PE clearly increased throughout vitellogenesis,

Fig. 3. Masses of whole spiders and their organs in different vitellogenic stages (PreV, previtellogenic; EarlyV, early-vitellogenic; V, vitellogenic; PostV, postvitellogenic). In the figure, the masses (in grams) of the spider bodies, ovaries, and midgut diverticula are plotted on the ordinate for each of the reproductive stages indicated on the abscissa. Different letters (a, b, c, and d) above the bars indicate statistically significant differences ($p < 0.05$, $n = 12$) among the four vitellogenic stages, as determined by Tukey's post hoc test.

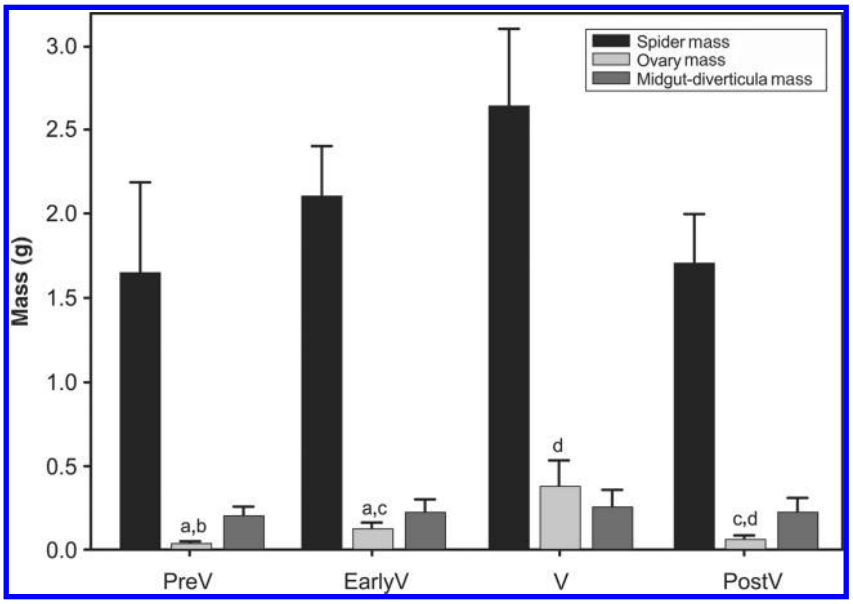
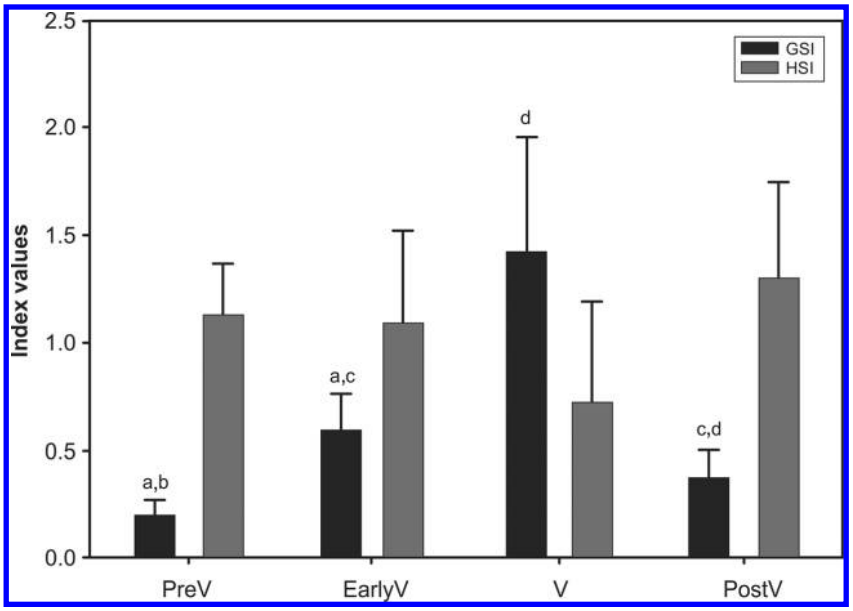


Fig. 4. Variations in the gonadosomatic index (GSI) and hepatosomatic index (HSI) during the four stages of vitellogenesis. The number of samples and the statistical analyses were similar to those of Fig. 3.



reaching a maximum of 35% in the vitellogenic stage, while PC was seen to decrease (Fig. 8). Supplementary Fig. S1[1] features a chromatography comparing the previtellogenic ovarian polar lipids with those of the vitellogenic stage.

Figure 9 illustrates the lipid classes in the muscle of females in the previtellogenic stage, indicating PC and PE as the principal classes present at 52% and 25%, respectively. The only lipid class that significantly varied throughout vitellogenesis was the FFA, with values ranging from 3% in the previtellogenic stage up to 6.7% in the vitellogenic stage.

The lipid analysis of the males (Fig. 10) indicated both similarities and differences compared with the analogous tissues analyzed in the females. In the midgut diverticula, the major lipids were TAG and FFA at 30% and 19%, respectively. In the testicle, the principal lipid was PE at 34%, being similar to the ovarian content during the vitellogenic stage, along with HC at 18%. In the hemolymph and muscle, the predominant phospholipids were PC and PE at 29.9% and 19.3% in the hemolymph and 27.8% and 42.2% in the muscle, respectively. In both females and males, diacylglycerols (DAG) were either present in trace amounts or absent.

[1]Supplementary material is available with the article through the journal Web site at http://nrcresearchpress.com/doi/suppl/10.1139/cjz-2017-0222.

Fig. 5. Quantity of lipids present in the midgut diverticula, gonad, muscle, hemolymph, and their sum in females in different vitellogenic stages and in males. Values are the means ± SDs of three pools of tissue analyzed separately (n = 12) in total mg lipids·g wet mass^{-1}; values for lipids in hemolymph are in mg lipids·mL hemolymph^{-1}). Different letters (a, b, c, and d) above the bars indicate statistically significant differences in the quantity of lipids during the four different stages at $p < 0.05$, as determined by Tukey's post hoc test, in a separate comparison with each organ.

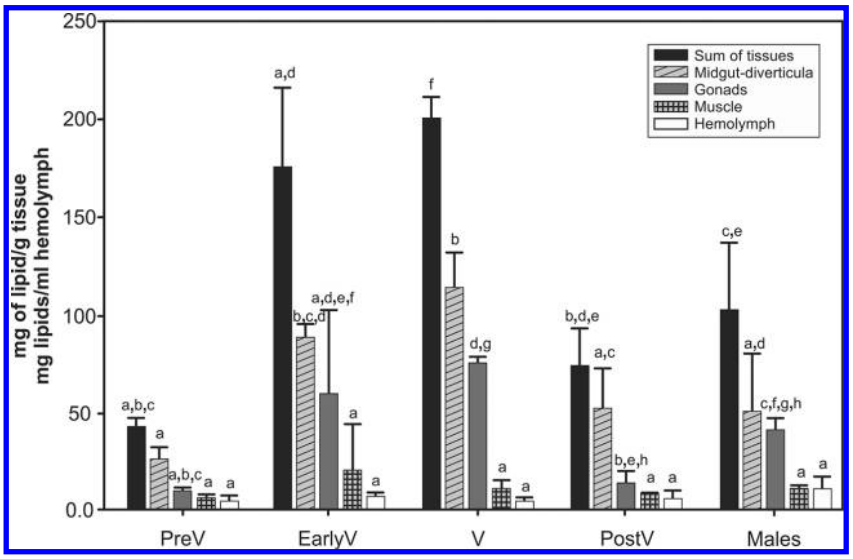
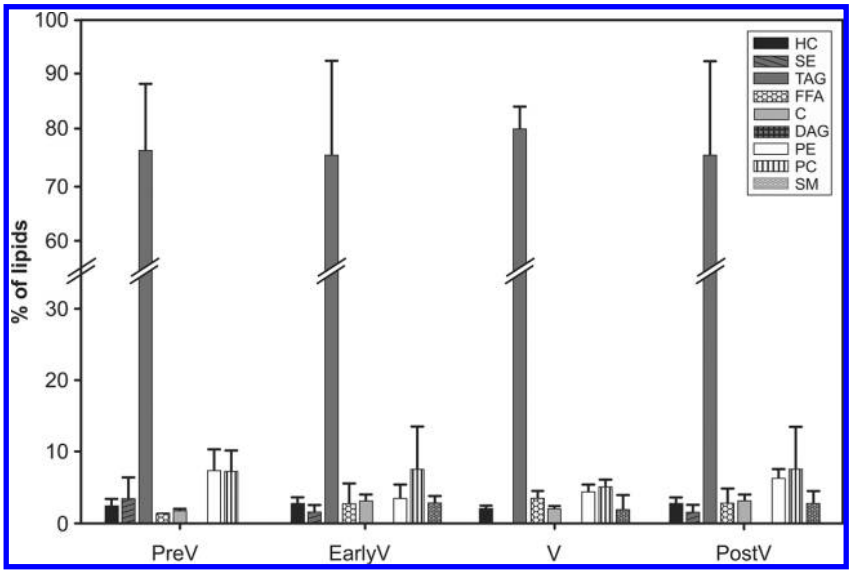


Fig. 6. Percentage of different lipid classes in the midgut diverticula of females in previtellogenic (PreV), early-vitellogenic (EarlyV), vitellogenic (V), and postvitellogenic (PostV) stages. The values are the means ± SDs of three pools, analyzed separately through chromatography coupled to a flame-ionization detector. See the text for abbreviations of the lipids.



The analysis by GLC of the total-lipid methyl esters of the hemolymph, ovary, midgut diverticula, and muscle during the four stages of vitellogenesis characterized 17 fatty acids. Among this spectrum, certain minor constituent species that would be considered of substantial metabolic significance, e.g., eicosatrienoate (20:3) or, in certain tissues, eicosapentaenoate (20:5), likewise underwent some variation throughout vitellogenesis (Supplementary Tables S1–S4)[1]. Figure 11, however, contains only the data for the four fatty acids that exhibited the greatest degree of variation, with those in combination representing from 75.6% to 95.1% of the total.

In the hemolymph, the predominant fatty acid was oleate (18:1), at 32% in the previtellogenic stage, followed by the two saturated fatty acids palmitate (16:0) and stearate (18:0) at 25.6% and 20.5%, respectively, along with linoleate (18:2) at 12.7%. Throughout vitellogenesis, linoleate became enriched along with a reciprocal decline in the two saturated fatty acids (Fig. 11A). In contrast to the pattern in the hemolymph, in the ovary (Fig. 11B), 18:1 predominated during the previtellogenic stage at 46.8%, whereas 18:0, 16:0, and 18:2 reached lower percentages of around 15%. In vitellogenesis, however, the percentages became similar to those of the hemolymph, characterized by an increase in 18:2 and a slight decrease in 18:1.

In the midgut diverticula, the organ of biosynthesis, 16:0 increased while 18:2 decreased, both significantly, throughout vitellogenesis (Fig. 11C). In contrast, in muscle during the previtellogenic stage, 18:2 reached 32% and 18:0 and 18:1 reached 20%, with 16:0 attaining only 6%. In vitellogenesis, however, 18:2 decreased markedly along with a concomitant increase in 16:0 (Fig. 11D). The Supplementary material[1]

Fig. 7. Percentage of different lipid classes in ovaries of females in the previtellogenic (PreV), early-vitellogenic (EarlyV), vitellogenic (V), and postvitellogenic (PostV) stages as determined by chromatography coupled to a flame-ionization detector. The values are the means ± SDs of three pools, analyzed separately. Different letters (a, b, c, and d) above the bars indicate statistically significant differences between the different vitellogenic stages determined by Tukey's post hoc test; same letter above or the absence of letters indicates a level of significance that is not significantly different at $p < 0.05$. See the text for abbreviations of the lipids.

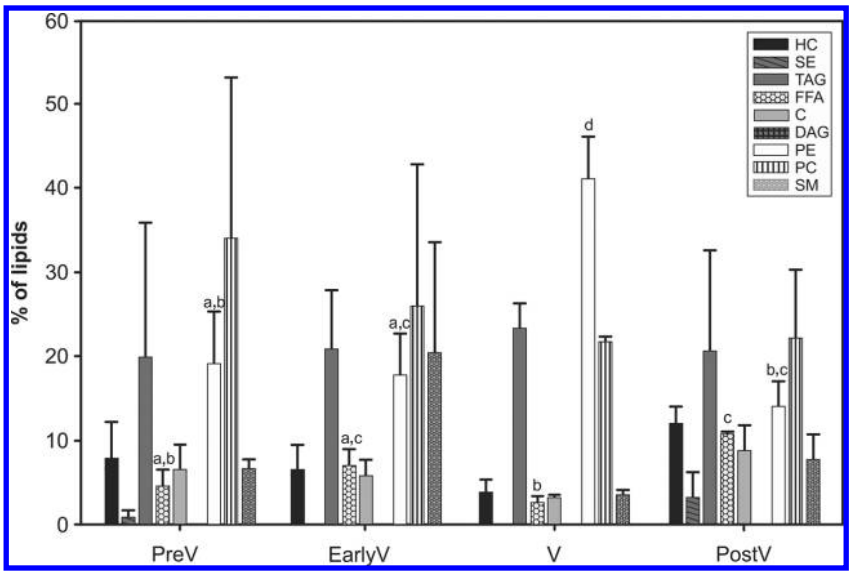
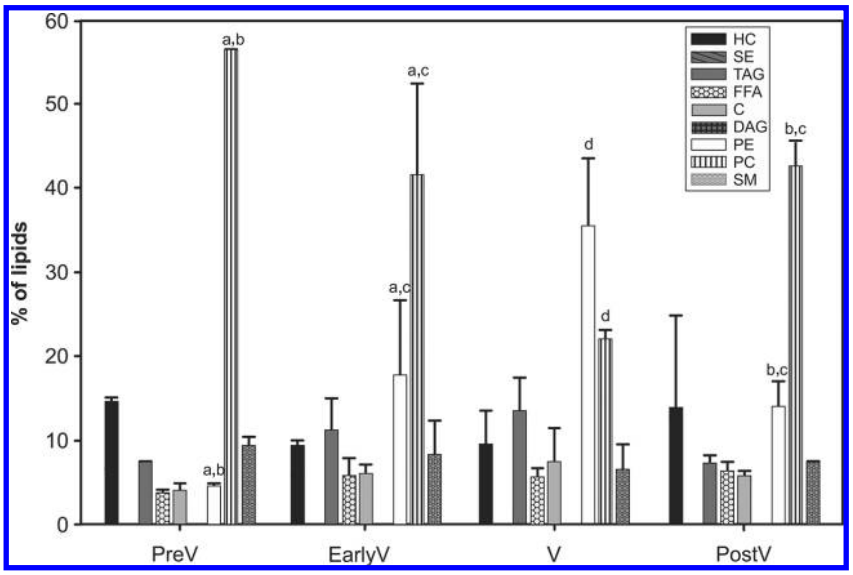


Fig. 8. Percentage of different lipid classes in the hemolymph of females in previtellogenic (PreV), early-vitellogenic (EarlyV), vitellogenic (V), and postvitellogenic (PostV) stages as determined by chromatography coupled to a flame-ionization detector. The values are the means ± SDs of three pools, analyzed separately. The statistical analyses are as in Fig. 7. See the text for abbreviations of the lipids.



summarizes the values of saturated, monounsaturated, polyunsaturated, and unsaturated fatty acids.

Discussion

Vitellogenesis is the main functional event taking place in the oocyte and is characterized by the accumulation of nutrients for subsequent vitellus formation. In the literature, ovarian maturity during the vitellogenesis process in spiders occurs in the following stages: previtellogenic, early-vitellogenic, and late vitellogenic stages (Sotelo and Trujillo-Cenoz 1957; Osaki 1972; Trabalon et al. 1992; Pourie and Trabalon 2003). In the present work on vitellogenesis, we correlated histological analyses with indices calculated in spiders that could be considered analogous to the standard indices GSI and HSI in crustaceans (Millamena and Pascual 1990), at the same time dividing the entire cycle into the four stages described throughout this report. The GSI constitutes the most useful mathematical expression of gonadal development. Having been originally used in fish (Dadzie and Wangila 1980; Fatima et al. 2013), the GSI was subsequently adapted successfully to molluscs (Di Cosmo et al. 2001; Iyapparaj et al. 2013), crustaceans (Vazquez Boucard et al. 2002; Garcia and Heras 2012), and insects (Yuan et al. 2013). The HSI, for its part, is analogous to GSI but applies to arthropod hepatopancreas (midgut diverticula in spiders). To our knowledge, this is the first study in which GSI and HSI are used in spiders. Although some authors currently prefer to measure ovarian volume, as an alternative possibility, the mass of the organ is a simple, rapid, and accessible measurement for estimating the reproductive condition in arachnids.

Fig. 9. Percentage of different lipid classes in muscle of females in the previtellogenic (PreV), early-vitellogenic (EarlyV), vitellogenic (V), and postvitellogenic (PostV) stages as determined by chromatography coupled to a flame-ionization detector. The values are the means ± SDs of three pools, analyzed separately. The statistical analyses of the lipids are as in Fig. 7. See the text for abbreviations of the lipids.

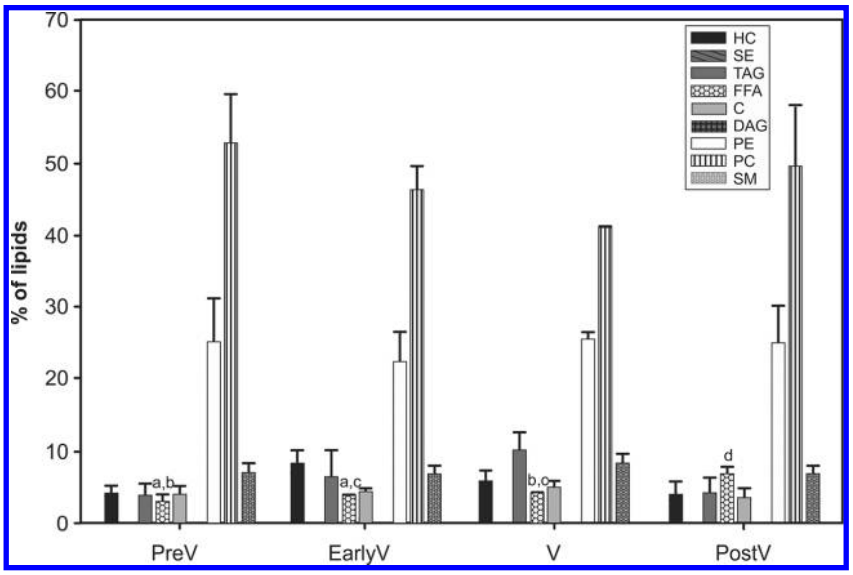
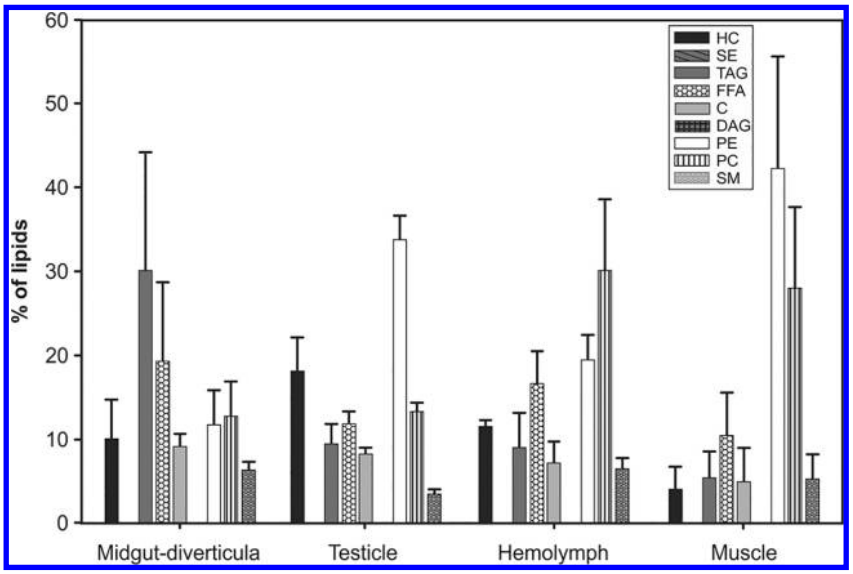


Fig. 10. Percentage of different lipid classes in the midgut diverticula, testicle, hemolymph, and muscle of the males as determined by chromatography coupled to a flame-ionization detector. The values are the means ± the SDs of three pools, analyzed separately. The statistical analyses of the lipids are as in Fig. 7. See the text for abbreviations of the lipids.



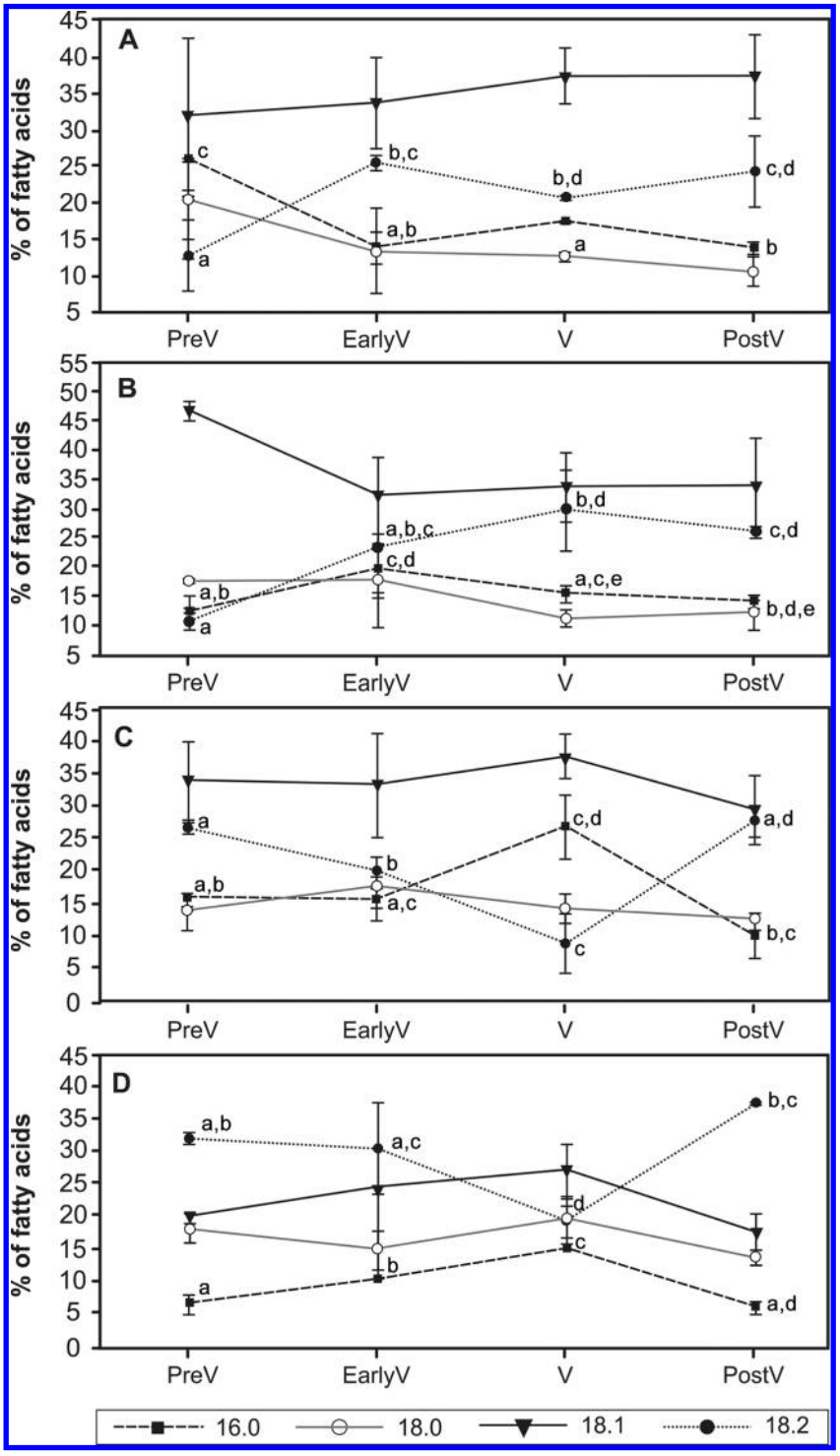
Although most authors have studied vitellogenesis through an analysis of the apolipoprotein vitellogenin, this reproductive phase is a multifaceted process that must be understood in terms of the lipids involved as well. In fact, vitellogenin is a particle composed of lipids, proteins, carbohydrates, and, in most instances, pigments, among other biomolecules; in addition, a considerable number of lipids not bound to proteins accumulate for storage to form the vitellus. In the spider *S. malitiosa*, the lipovitellin was found to contain only 24% lipids (Laino et al. 2013). Moreover, vitellogenin, like all lipoproteins, is a molecule that functions as a lipid transporter. Vitellogenesis, however, has not been extensively studied in arachnids.

As expected, the midgut diverticula, having been described as the main organ of lipid metabolism and storage in spiders, had a high concentration of lipids per wet mass. This role has been substantiated by the observations of Laino and collaborators through the use of in vivo and in vitro assays with radio-labelled lipids (Laino et al. 2009; Laino et al. 2011*a*). A similar function has been described for the fat body of insects (Canavoso et al. 2001) and the hepatopancreas of crustaceans (Kanazawa and Koshio 1994; Garcia et al. 2002) and scorpions (Warburg et al. 2002; Laino et al. 2015).

The variation in lipid dynamics in the midgut diverticula and the ovary throughout the four reproductive stages takes place mainly at the beginning of vitellogenesis (previtellogenic stage). Furthermore, the rise in lipid concentration greatly contributes to the increase in size of the oocyte (Andre and Rouiller 1957; Warburg et al. 1995). Vitellogenesis is characterized by a major accumulation of lipids in the ovary (Charniaux-Cotton 1976; Galois 1984), a process that also occurs in organisms that utilize proteins or glycogen as energy sources to meet the metabolic needs of the tissues (Pollero and Iribarne 1988).

The increase in the lipids of the midgut diverticula during vitellogenesis is probably related to their subsequent accumulation in

Fig. 11. Composition of the major fatty acids of (A) hemolymph, (B) ovary, (C) midgut diverticula, and (D) muscle. The data on the fatty-acid classes are expressed as the mass percentage as quantified by GLC. The values are the means ± SDs of three independent analyses from three pools. Different letters (a, b, c, d, and e) indicate statistically significant differences in the fatty-acid percentages among the previtellogenic (PreV), early-vitellogenic (EarlyV), vitellogenic (V), and postvitellogenic (PostV) stages at a $p < 0.05$, as determined by Tukey's post hoc test. In the four panels, the percentage of each of the four principal fatty acids — palmitate (16:0), stearate (18:0), oleate (18:1), and linoleate (18:2) — is plotted on the ordinate for each of the reproductive stages indicated on the abscissa.



the ovary, as has been described for other arthropods (Teshima and Kanazawa 1983; Castille and Lawrence 1989). We would expect that if these lipids had an ovarian destiny, the lipid concentration in the hemolymph would rise in early vitellogenesis or vitellogenesis, as this tissue is the only vehicle of lipid transport. Contrary to expectations, however, we did not observe such an increase in the lipid concentration of the hemolymph. In spite of this, the constant concentration of lipids was accompanied by an increase in the total volume of the hemolymph; a greater quantity of lipids could be delivered to the ovary by that tissue, thus explaining that

apparent contradiction. Accordingly, in the present work, we observed that the quantity of hemolymph increased 100% during the early-vitellogenic stage and a further 100% during vitellogenesis above the values in that second stage.

Another hypothesis that can be alternative and (or) complementary is that the increase in the lipids in the midgut diverticula occurs to support the metabolism carried out by females during vitellogenesis to prevent a metabolic exhaustion that would otherwise result during oviposition and ootheca maintenance. Ruhland et al. (2016) determined the great caloric content that mothers of the Lycosidae family devote to oviposition and the care of their young. *Polybetes pythagoricus* is a species that does not feed during the postvitellogenesis, and although these spiders do not carry their young on their back, they fiercely defend their offspring (Galiano 1971). This dynamic in which the greatest state of ovarian development coincides with a high accumulation of lipids in the hepatopancreas has also been described for the giant tiger shrimp *Penaeus monodon* Fabricius, 1798 (Millamena and Pascual 1990), wherein the authors suggested that this lipid reserve would be necessary to support energetic requirements during and after oviposition.

The increase in the lipid concentration in the ovary may be caused, in part, by endogenous synthesis, which occurs in certain arthropods such as crustaceans (Vazquez Boucard et al. 2002) and insects (Lubzens et al. 1981; Ferenz 1985; Ziegler and Van Antwerpen 2006). Future studies on lipid dynamics with radioisotopes are necessary to be able to confirm this relationship.

In males, lipid concentrations of the midgut diverticula, gonad, muscle, and hemolymph, as well as the total of those lipids, were similar to the values found in previtellogenic and postvitellogenic females. That similarity in lipid content is possibly simply a reflection of the lipidic homeostasis necessary to meet basal metabolic requirements.

During vitellogenesis, the ovaries must incorporate all of the nutrients necessary to meet the energetic and structural requirements for organogenesis; for this reason, during vitellogenesis, the ovarian tissue is in close proximity to the eggs, as observed in Fig. 2C. The lipid composition of vitellogenic ovaries is strongly correlated with the only lipid analysis available in the literature for spider eggs (Laino et al. 2013). In the ovary, the main energy-associated lipid was TAG, present at 23%, with the eggs, for their part, containing some 25%. This lipid is certainly essential for meeting the energetic requirements of embryonic development. As to the structural lipids PC + PE + SM, the ovary contains 65.6% in comparison with the eggs, which contain 64.0%. This high percentage is caused by an enhanced demand for the constituents of plasma membranes and endomembranes. The same lipidic profile occurs in the arthropod *P. monodon*, where the polar lipids become the main components responsible for the increase in the lipids of the ovary during maturation (Millamena and Pascual 1990).

It is important to highlight that during early vitellogenesis in the ovary, the PE content, measured at 19%, was significantly different from the value of 41% found in vitellogenesis, with that class then becoming the principal lipid. This observation is also consistent with the lipids present in the lipovitellin of the eggs of *P. pythagoricus*, where PE was reported to be the major lipid (Laino et al. 2011*b*). One hypothesis is that the PE, in addition to fulfilling its structural role as a component of the plasma membrane, may constitute a signature mitochondrial lipid in the embryos. In the inner mitochondrial membrane, this lipid class has been described as representing about 34% of the total lipids. The presence of this phospholipid in the inner mitochondrial membrane appears to produce a major curvature for the structure and an enhancement in the functioning of the electron-transport chain (Tasseva et al. 2013; Teague et al. 2013; Ikon and Ryan 2017). At the same time, the distinctive physicochemical characteristics of PE serve to modulate the viscosity of the membranes of eukaryotic cells. Dawaliby and collaborators (2016), working with insect cells, observed that the percentage of PE increased with changes in temperatures because those cells were almost devoid of sterols (Dawaliby et al. 2016). At the present time, a de novo synthesis of C is not believed to occur in either arachnids or crustaceans (Zandee 1967; Teshima and Kanazawa 1971, 1983; Kean et al. 1985). Unfortunately, the lack of information with respect to these considerations impedes the establishment of any conclusion concerning the functioning of PE. Future studies will be necessary to formulate a viable hypothesis regarding the role of this lipid class during ovarian maturation in arachnids.

In the hemolymph, an increase in PE from 4.5% in the previtellogenic stage to 35.6% in the vitellogenic stage occurs, representing a 7.9-fold enrichment in that lipid class. This PE content is probably destined for the ovary as a target organ, as in the postvitellogenic stage, the hemolymph PE decreases to 15.8%. The midgut diverticula, in their role as the preeminent biosynthetic organ, likely generate the PE because the phospholipid does not accumulate there but rather is released into the hemolymph to be transported to the ovary for endocytosis and accumulation in the oocytes. The quantity of PE in the egg of the spider *S. malitiosa* would appear to confirm the vitellus as the final destiny of PE (Laino et al. 2013).

In contrast, the percentage of PC in the hemolymph of spiders decreases significantly during vitellogenesis. This decline probably occurs to maintain the balance of polar lipids in the circulating lipoproteins. The quantity of lipid that can be transported in the lipoprotein particles of arthropods is restricted as interchangeable apolipoproteins (Soulages and Brenner 1991) have been previously reported to be absent in spiders (Cunningham et al. 1994; Cunningham et al. 2007).

Finally, the muscle undergoes no changes in lipid concentration or profile throughout the four stages analyzed. The lipid content is lower in that tissue than in the organs of lipid metabolism and storage (midgut diverticula and ovary), thus correlating with what has been previously described for scorpions (Laino et al. 2015). This constancy probably results because muscle tissue is not in any way linked to vitellogenesis. The majority of the lipids found in the four reproductive stages are PC, PE, and SM, with total values ranging from 73.9% to 84.0%. These classes, as structural lipids, are less variable in muscle. The only example of a significant enrichment in a lipid species in that tissue is in the content of FFAs during the postvitellogenic stage, suggesting that after laying the ootheca, an energy demand likely results to meet the physiologic needs of that moment until the females can receive nourishment again.

A comparison of the lipid profiles between males and females revealed (*i*) that the lipid contents of the midgut diverticula, muscle, and hemolymph of the males had similar patterns to those of the females and (*ii*) that quantitative differences between the ovary and the testicle were evident, no doubt resulting from the separate reproductive functions that those two organs perform in the two sexes. In all of the tissues analyzed, the males had a higher FFA content than the females (midgut diverticula, 15%; hemolymph, 10%; and muscle, 4%). These differences might possibly arise as a result of the differing general metabolic needs between the sexes or from the discrepant energetic burden associated with different reproductive roles (Herrmann and Roberts 2017). Laino et al. (2009), using [1-^{14}C] palmitate, documented the major contribution of FFA to the lipid dynamics of *P. pythagoricus*.

Although the literature describes how different diets affect the composition of FFAs in the muscle and the hepatopancreas of arthropods (Kucharski and Da Silva 1991; Vinagre and Da Silva 1992; Carvalho et al. 2012), in the present work, we observed that the FFA content of those organs during the previtellogenic stage coincided with that reported in the paucity of literature available in related species (scorpion, tarantula, and other spiders), suggesting that any variations that might be generated by the different

diets of those other arachnids are minimal. The main fatty acids were 18:1, 18:2, and, to a lesser extent, 18:0 and 16:0, which is the same pattern also observed in whole-body preparations and the midgut diverticula of three other labidognath spider species (Uscian and Stanley-Samuelson 1994; Laino et al. 2009) and in the hepatopancreas of scorpions (El-Salhy et al. 1981; Uscian and Stanley-Samuelson 1994; Laino et al. 2015). We need to emphasize that the midgut diverticula has an enrichment of 18:2 and an impoverishment of 16:0 relative to the respective levels of those fatty acids in the hemolymph during the previtellogenesis. This pattern coincides with that described by El-Salhy et al. (1981), where the authors suggested that the hepatopancreas contained more than one pool of fatty acids having different degrees of interchange with the hemolymph. A similar situation was subsequently observed in scorpions (Laino et al. 2015).

The eicosanoid precursor polyunsaturated fatty acid 20:5 was either minimal or absent in most tissues, being found only at a percentage greater than 4% (6%) in muscle in the previtellogenic stage. This level coincides with that determined by Uscian and Stanley-Samuelson (1994), who reported a percentage greater than 4% in the PC of orthognath spider tissues and in the whole body of labidognath spiders and scorpions. Although the fatty acids of the phospholipids and the TAG were not analyzed separately in the present work, the logic is that a higher percentage occurs in muscle, which has a lipid profile that is more than 50% phospholipids. The presence of this fatty acid would appear to be exclusive to a specific number of predatory species (Uscian and Stanley-Samuelson 1994).

We found that the ovary was enriched in 18:2 and impoverished in 18:0 throughout the vitellogenesis. The same pattern was observed in the hemolymph, suggesting that the former fatty acid is being specifically transported by that tissue. The significant decrease in 18:2 in the midgut diverticula and muscle reinforces this hypothesis. The lipid dynamics in *P. pythagoricus* clearly features the incorporation of polyunsaturated fatty acids into the ovary — those being essential for the subsequent structural demands associated with organogenesis. The similar percentage of unsaturates present in the lipovitellins of *P. pythagoricus* and the eggs of *S. malitiosa* supports this hypothesis (Laino et al. 2011*b*; Laino et al. 2013).

Finally, with respect to the structural lipids, we can conclude that arachnids have developed functional membranes with individual components that are strongly preserved (PC and SM) but a design, in terms of the relative proportions, that is highly dissimilar (featuring a high content of PE). How the different combinations of lipids are selected to guarantee the integrity of the membrane and how the lipid components affect the functioning are essential questions that will have to be answered in future studies. Characteristic lipid patterns occur in the vitellogenesis of spiders that in the future will definitely generate the basis for a greater understanding of the biochemical processes that underlie the preparation that these arachnids need for reproduction and that, as such, are fundamental for the development and survival of oviparous species.

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