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

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1 **FIRST STUDY ON LIPID DYNAMICS DURING THE FEMALE REPRODUCTIVE CYCLE OF**
2 **POLYBETES PYTHAGORICUS (ARANEAE SPARASSIDAE)**

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22 **ABSTRACT**

23 Spiders are valuable to humans, not only for their role in health but also as biologic
24 pest controllers. In oviparous species, lipids are the main energy source for embryo
25 development, growth, and the survival of larvae. Using the spider *Polybetes pythagoricus*
26 (Holmberg, 1875) as an experimental model, we studied the fluctuations in lipids and fatty
27 acids occurring in tissues related to vitellogenesis. Different reproductive stages—
28 previtellogenic, early-vitellogenic, vitellogenic, and postvitellogenic—were determined
29 histologically. Gonadosomatic and hepatosomatic indices were first used in spiders. The

30 midgut diverticula, proved to be the organ with the highest lipid concentration, with
31 triacylglycerols as the major component. Phospholipids were the principal lipids transported.
32 In vitellogenesis, a major accumulation of lipids occurred in the ovary—principally
33 phosphatidylethanolamine (41%), it probably synthesized in the midgut diverticula before
34 release into the hemolymph for transport and accumulation in the ovary. The significance of
35 phosphatidylethanolamine possibly involves maintaining membrane fluidity and functioning of
36 the electron transport chain. The principal fatty acids in the different organs were palmitic,
37 stearic, oleic, and linoleic. During vitellogenesis the ovaries become enriched in
38 polyunsaturated fatty acids. The lipid patterns in the male midgut diverticula, muscle, and
39 hemolymph were similar to those of the previtellogenic or postvitellogenic females.

40

41 **KEY WORDS:**

42 Spider, vitellogenesis, lipid, GSI, *Polybetes pythagoricus*

43

44 INTRODUCTION

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

54 In oviparous species, the lipids present in the vitellus are the main source of energy
55 for embryo development, and growth and survival of the larvae. In the ovaries of females in a
56 reproductive stage, lipids are distributed among membranes, lipid droplets, and lipoproteins
57 (Ziegler and Van Antwerpen 2006). Membrane lipids contribute to structure and are mainly
58 formed by phospholipids and sterols. Lipid droplets are formed by triacylglycerides (TAG) and
59 function as energy reserves. Finally, the lipovitellins are lipoproteins found in both the ovary
60 and the eggs of oviparous animals and are mostly composed of phospholipids and TAG. In
61 arachnids, these proteins have been characterized in *Polybetes pythagoricus* (Holmberg,
62 1875) (Sparassidae; Laino et al. 2011b) and in *Schizocosa malitiosa* (Tullgren, 1905)
63 (Lycossidae; Laino et al. 2013).

64 During the reproductive stage, in general, the females have a high quantity of lipids
65 either in the hemolymph, ovary, and midgut diverticula (spiders) or in the hepatopancreas
66 (crustaceans and scorpions) or fat body (insects); (Lubzens et al. 1981; Grapes et al. 1989;
67 Ravid et al. 1999; Warburg 2012). The synthesis of these lipids and incorporation into the
68 ovary are regulated by hormones in the process referred to as vitellogenesis. These various
69 steps are critical to those different organisms because vitellogenesis can determine embryo
70 viability and thus species survival. Vitellogenesis has been well described in insects
71 (Sappington and Raikhel 1998; Ziegler and Van Antwerpen 2006; Ibáñez 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 the vitellogenesis in spiders, such as, *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), up to now 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 as a model the spider *Polybetes pythagoricus* 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 permits N° 117/16 for Protected Areas, Province of Buenos Aires.

99 Spiders were housed individually in cylindrical terrariums (10 cm in diameter x 5 cm in
100 height) without any substrate and were kept at 20 ± 1 °C, under a 14:10-h L:D photoperiodic
101 cycle without food for 24 h before sacrifice under cold anesthesia.

102

103 *Experimental groups*

104 A total of 120 spiders were used in this study, with those individuals being then divided
105 into five experimental groups according to their vitellogenic stages and sex.

106 Females were separated according to their reproductive status as being
107 previtellogenic (adult females before the start of vitellogenesis), early-vitellogenic, vitellogenic,
108 and postvitellogenic (females after oviposition). We based on the histological studies of the
109 three first groups (previtellogenic, early-vitellogenic, and vitellogenic) to identify the different
110 stages, while for the postvitellogenics the oviposition was taken into account, sacrificing them
111 2 days after the same.

112

113 *General measurements*

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

116 Hemolymph was obtained by severing the spiders' legs, and centrifuging the spiders
117 in a tube at low speed (Cunningham et al. 1994). A dorsal incision was made in the tegument
118 and the midgut diverticula and gonads were carefully dissected out, as indicated in Laino et
119 al. (2009). At the same time, muscles were removed from the legs. All the tissues isolated (the
120 midgut diverticula, gonads, and muscles) were weighed in a Mettler-Toledo NewClassic MS-
121 204S analytical balance.

122 We measured two indices, the gonadosomatic (GSI) and the hepatosomatic (HSI).
123 The first was the gonad weight (g) x100/ body weight (g). The second is normally used in
124 different invertebrates having a hepatopancreas, an organ comparable to the midgut
125 diverticula of spiders with respect to lipid metabolism. Since no term analogous to HSI exists
126 for spiders, we adapted the same nomenclature for the present work. Thus, the HSI here was
127 calculated as the midgut-diverticula weight (g) x 100/ body weight (g).

128

129 *Histological analysis*

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

137

138 *Lipid extraction and analysis*

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

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

155

156 *Fatty-acid analysis*

157 Fatty-acid methyl esters from the total lipids of each of the tissues were prepared with
158 BF_3 -methanol according to the method of Morrison and Smith (1992). Analysis was performed
159 by gas-liquid chromatography (GLC) in an HP-6890 capillary chromatograph (Hewlett
160 Packard, Palo Alto, CA, USA), equipped with a flame-ionization detector and fitted with an
161 Omegawax 250 fused silica column of dimensions 30 m x 0.25 mm and with a 0.25- μm solid
162 phase (Supelco, Bellefonte, CA, USA). The column temperature was programmed for a linear
163 increase of 3 $^{\circ}\text{C}/\text{min}$ from 175 to 230 $^{\circ}\text{C}$ and the peaks identified by comparison of their
164 retention times with those from a mixture of standard methyl esters.

165

166 *Statistical analyses*

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

174

175 **RESULTS**

176 After the capture of the *P. pythagoricus* spiders in the locations described in Materials
177 and Methods, we observed that the quantity of males remained constant throughout the entire
178 year at less than 30%. Among the females, the percentage of previtellogenic individuals
179 decreased in reciprocity to the increase in the vitellogenic females during the months of
180 August, September, and October; reaching almost 60% at temperatures ranging from 7.8 to
181 16 $^{\circ}\text{C}$ (Fig. 1). In the previtellogenic stage, the respective length and width of the opisthosoma
182 were 10.7 ± 1.6 mm and 8.8 ± 1.4 mm. In the early-vitellogenic, vitellogenic, and
183 postvitellogenic stages the respective corresponding values were 11.5 ± 1.6 and 9.4 ± 2.1
184 mm, 16.8 ± 1.6 and 13.3 ± 1.4 mm, and 10.2 ± 1.2 mm and 7.8 ± 0.8 . The respective

185 analogous measurements in males at 9.7 ± 1.3 mm and 7.2 ± 0.5 mm were similar to those of
186 the pre- and postvitellogenic females.

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

202 The macroscopic groupings were corroborated with histologic pattern. In the
203 previtellogenic group, only previtellogenic oocytes were found in the ovary, whereas in the
204 early-vitellogenic group the ovary contained early-vitellogenic as well as some previtellogenic
205 oocytes. The ovary of the vitellogenic group then became fully filled with vitellogenic oocytes
206 whereas in the postvitellogenic group the ovary finally went into a regression stage, with
207 degenerative oocytes.

208 Figure 3 presents the weights of the entire body, ovary, and midgut diverticula of the
209 spiders during the four stages of the study. Although there was some increase in the total
210 body weight during vitellogenesis—with values that reach 2,500 mg—the weights of the
211 different stages were statistically equivalent. The weight of the midgut diverticula remained
212 constant—about 220 mg—during vitellogenesis. In contrast, the weight of the ovary was at
213 an initial value of 30 mg in the previtellogenic females, then increased significantly in the

subsequent stages: first, by fourfold during the early-vitellogenic stage (to 120 mg) and finally by a further threefold during vitellogenesis (to 370 mg). This last rise was reflected in the comparison between the corresponding indices (GSI and HSI) illustrated in Figure 4. That figure 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 vitellogenic stage.

After quantifying the lipids gravimetrically of the midgut diverticula, ovary, muscle, and hemolymph we observed significant differences in those organs during the different vitellogenic stages (Fig. 5). The total weight of lipid in those four organs and/or tissues in the respective previtellogenic and vitellogenic stages were 42 and 199 mg/g wet weight, 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% in vitellogenesis. The initial value for the total lipids of the midgut diverticula (in the previtellogenic stage) was 26.1 mg/g with a 3.3-fold increase of 87.1 mg/g occurring in vitellogenesis, there 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 with a major increase in the content of 6.6-fold in the vitellogenic stage. This increase constituted a difference of 65.3 mg/g of lipids, representing a 77% increase in the early-vitellogenic stage along with a further increment of 24% at vitellogenic stage. Finally, the increased in the lipid content of the muscles throughout the four stages was from 6.3 mg/g to 20.4 mg/g, and of the hemolymph was from 4.6 mg/ml to 7.3 mg/ml. The total lipid content of the different tissues in the males was 102.0 mg/g, with 50.0 mg/g, in the midgut diverticula, 40.9 mg/g in the testicle, and 11.1 mg/ml or mg/g in the hemolymph and muscle.

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

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

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

248 The analysis of the hemolymph lipid classes indicated two similarities to the ovarian
249 lipids. First, PC was the principal lipid at 56%; and, second, PE clearly increased throughout
250 vitellogenesis, reaching a maximum of 35% in the vitellogenic stage, while PC was seen to
251 decrease (Fig. 8). Figure. S1¹ of the supplementary material features a chromatography
252 comparing the previtellogenic ovarian polar lipids to those of the vitellogenic stage.

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

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

265 The analysis by GLC of the total-lipid methyl esters of the hemolymph, ovary, midgut
266 diverticula, and muscle during the four stages of vitellogenesis characterized 17 fatty acids.
267 Among this spectrum certain minor constituent species that would be considered of

¹ Supplementary material: figure S1

substantial metabolic significance, such as eicosatrienoate (20:3) or in certain tissues eicosapentaenoate (20:5), likewise underwent some variation throughout vitellogenesis (supplementary tables S1–S4)². Figure 11, however, contains the data for only 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. 11, Panel A). In contrast to the pattern in the hemolymph, in the ovary (Fig. 11, Panel B), 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. 11, Panel C). In contrast, in muscle during the previtellogenic stage, 18:2, reached 32% and 18:0 and 18:1, 20%, with 16:0 attaining only 6%. In vitellogenesis, however, 18:2 decreased markedly along with a concomitant increase in 16:0 (Fig. 11, Panel D). The supplementary material summarizes the values of saturated, monounsaturated, polyunsaturated, and unsaturated fatty acids.

288

289 **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

² Supplementary materials: table S1, S2, S3 and S4.

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 weight of the organ is a simple, rapid, and accessible measurement for estimating the reproductive condition in arachnids.

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 weight. This role has been substantiated by the observations of Laino and collaborators through the use of *in-vivo* and *in-vitro* assays with radiolabelled lipids (Laino et al. 2009; Laino et al. 2011a). 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).

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

330 The increase in the lipids of the midgut diverticula during vitellogenesis is probably
331 related to their subsequent accumulation in the ovary, as has been described for other
332 arthropods (Teshima and Kanazawa 1983; Castille and Lawrence 1989). We would expect
333 that if these lipids had an ovarian destiny, the lipid concentration in the hemolymph would rise
334 in the early-vitellogenesis or vitellogenesis, since this tissue is the only vehicle of lipid
335 transport. Contrary to expectations, however, we did not observe such an increase in the lipid
336 concentration of the hemolymph. In spite of this, the constant concentration of lipids was
337 accompanied by an increase in the total volume of the hemolymph, a greater quantity of lipids
338 could be delivered to the ovary by that tissue, thus explaining that apparent contradiction.
339 Accordingly, in the present work, we observed that the quantity of hemolymph increased
340 100% during early-vitellogenic stage and a further 100% during vitellogenesis above the
341 values in that second stage.

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

352 where the authors suggested that this lipid reserve would be necessary to support energetic
353 requirements during and after oviposition.

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

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

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

376 It is important to highlight that during early vitellogenesis in the ovary the PE content,
377 measured at 19%, was significantly different from the value of 41% found in vitellogenesis,
378 with that class then becoming the principal lipid. This observation is also consistent with the
379 lipids present in the lipovitellin of the eggs of *P. pythagoricus*, where PE was reported to be
380 the major lipid (Laino et al. 2011b). 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 physical-chemical 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 believed to occur in neither arachnids nor crustaceans (Zandee 1967; Teshima and Kanazawa 1971; Teshima and Kanazawa 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 vitellogenic stage occurs, representing a 7.9-fold enrichment in that lipid class. This content of PE is probably destined for the ovary as a target organ, since in the postvitellogenic stage, the hemolymph PE decreases down to 15.8%. The midgut diverticula, in their role as the preeminent biosynthetic organ, likely generate the PE since 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 in order 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 since interchangeable apolipoproteins (Soulages and Brenner 1991) have been previously reported to be absent in spiders (Cunningham et al. 1994; Cunningham et al. 2007).

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

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

431 Although the literature describes how different diets affect the composition of FFAs in
432 the muscle and the hepatopancreas of arthropods (Kucharski and Da Silva 1991; Vinagre and
433 Da Silva 1992; Carvalho et al. 2012), in the present work we observed that the FFA content of
434 those organs during the previtellogenic stage coincided with that reported in the paucity of
435 literature available in related species (scorpion, tarantula, and other spiders), suggesting that
436 any variations that might be generated by the different diets of those other arachnids are
437 minimal. The main fatty acids were 18:1; 18:2; and, to a lesser extent, 18:0 and 16:0—which
438 same pattern had also been 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 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 in the present work the fatty acids of the phospholipids and the TAG were not analyzed separately, the logic is that in muscle—whose lipid profile is more than 50% phospholipids—that higher percentage occurs. 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. 2011b; Laino et al. 2013).

Finally, with respect to the structural lipids, we can conclude that arachnids have developed functional membranes whose individual components are strongly preserved (PC and SM) but whose design in terms of the relative proportions 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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LEGENDS TO THE FIGURES

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

Figure 2. Histology of the ovaries in previtellogenic (Panel A), early-vitellogenic (Panel B), vitellogenic (Panel C), and postvitellogenic (Panel D) stages. Staining, hematoxylin-eosin. The thick arrow marks a pedicel in Panel A; the thin arrows previtellogenic (pv) and early-

vitellogenic (ev) oocytes in Panel B, and previtellogenic (pv) and degenerated (do) oocytes in Panel D.

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Figure 3. Weights of whole spiders and their organs in different vitellogenic stages (previtellogenic, PreV; early-vitellogenic, Early V; vitellogenic, V; postvitellogenic, PostV). In the figure, the weights 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, 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.

505

Figure 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 Figure 3.

509

Figure 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 total mg of lipids per wet weight expressed as the means $\pm$ the SDs of three pools of tissue analyzed separately ($n = 12$). 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 a $p < 0.05$, as determined by Tukey's *post-hoc* test, in a separate comparison with each organ.

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Figure 6. Percentage of different lipid classes in the midgut diverticula of females in previtellogenic (PreV), early-vitellogenic (Early V), vitellogenic (V), and postvitellogenic (PostV) stages. The values are the means $\pm$ the SDs of three pools, analyzed separately through chromatography coupled to a flame-ionization detector. Different letters (a, b, c, d) above the bar indicate statistically significant differences between the different vitellogenic stages determined by Tukey's *post hoc* test, same letter above or absence of letters are not a significantly different level of significance $p < 0.05$.

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Figure 7. Percentage of different lipid classes in ovary of females in the previtellogenic (PreV), early-vitellogenic (Early V), vitellogenic (V), and postvitellogenic (PostV) stages as determined by chromatography coupled to a flame-ionization detector. The values are the means $\pm$ the SDs of three pools, analyzed separately. The statistical analyses, abbreviations of the lipids, are as in Figure 6.

Figure 8. Percentage of different lipid classes in the hemolymph of females in previtellogenic (PreV), early-vitellogenic (Early V), vitellogenic (V), and postvitellogenic (PostV) stages as determined by chromatography coupled to a flame-ionization detector. The values are the means $\pm$ the SDs of three pools, analyzed separately. The statistical analyses and abbreviations of the lipids are as in Figure 6.

Figure 9. Percentage of different lipid classes in muscle of females in the previtellogenic (PreV), early-vitellogenic (Early V), vitellogenic (V), and postvitellogenic (PostV) stages as determined by chromatography coupled to a flame-ionization detector. The values are the means $\pm$ the SDs of three pools, analyzed separately. The statistical analyses and abbreviations of the lipids are as in Figure 6.

Figure 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 $\pm$ the SDs of three pools, analyzed separately. The statistical analyses and abbreviations of the lipids are as in Figure 6.

Figure 11. Composition of the major fatty acids of the hemolymph (Panel A), ovary (Panel B), midgut diverticula (Panel C), and muscle (Panel D). The data on the fatty-acid classes are expressed as the weight percentage as quantified by GLC. The values are the means $\pm$ 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 (Early V), vitellogenic (V), and postvitellogenic (PostV) stages at a p

554 <0.05, as determined by Tukey's *post-hoc* test. In the four panels, the percent of each of the
555 four principal fatty acids—palmitate (16:0), stearate (18:0); oleate (18:1), and linoleate
556 (18:2)—is plotted on the *ordinate* for each of the reproductive stages indicated on the
557 *abscissa*.

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Figure 1. Percentage of samples of *Polybetes pythagoricus* males and of females in the vitellogenic and previtellogenic stages throughout a year ($n = 12$ per capture site). In the figure, the percentage of appearance of the females (previtellogenic, PreV; vitellogenic, V); and males is plotted on the lower left ordinate and the temperature in $^{\circ}\text{C}$ on the upper right ordinate for each of the months during the year denoted on the abscissa.

166x93mm (299 x 299 DPI)

Figure 2. Histology of the ovaries in previtellogenic (Panel A), early-vitellogenic (Panel B), vitellogenic (Panel C), and postvitellogenic (Panel D) stages. Staining, hematoxylin-eosin. The thick arrow marks a pedicel in Panel A; the thin arrows previtellogenic (pv) and early-vitellogenic (ev) oocytes in Panel B, and previtellogenic (pv) and degenerated (do) oocytes in Panel D.

127x114mm (299 x 299 DPI)

Figure 3. Weights of whole spiders and their organs in different vitellogenic stages (previtellogenic, PreV; early-vitellogenic, Early V; vitellogenic, V; postvitellogenic, PostV). In the figure, the weights 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, 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.

160x112mm (299 x 299 DPI)

Figure 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 Figure 3.

159x112mm (299 x 299 DPI)

Figure 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 total mg of lipids per wet weight expressed as the means $\pm$ the SDs of three pools of tissue analyzed separately ($n = 12$). 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 a $p < 0.05$, as determined by Tukey's post-hoc test, in a separate comparison with each organ.

176x113mm (299 x 299 DPI)

Figure 6. Percentage of different lipid classes in the midgut diverticula of females in previtellogenic (PreV), early-vitellogenic (Early V), vitellogenic (V), and postvitellogenic (PostV) stages. The values are the means $\pm$ the SDs of three pools, analyzed separately through chromatography coupled to a flame-ionization detector. Different letters (a, b, c, d) above the bar indicate statistically significant differences between the different vitellogenic stages determined by Tukey's post hoc test, same letter above or absence of letters are not a significantly different level of significance $p < 0.05$.

170x113mm (299 x 299 DPI)

Figure 7. Percentage of different lipid classes in ovary of females in the previtellogenic (PreV), early-vitellogenic (Early V), vitellogenic (V), and postvitellogenic (PostV) stages as determined by chromatography coupled to a flame-ionization detector. The values are the means $\pm$ the SDs of three pools, analyzed separately. The statistical analyses, abbreviations of the lipids, are as in Figure 6.

170x113mm (299 x 299 DPI)

Figure 8. Percentage of different lipid classes in the hemolymph of females in previtellogenic (PreV), early-vitellogenic (Early V), vitellogenic (V), and postvitellogenic (PostV) stages as determined by chromatography coupled to a flame-ionization detector. The values are the means $\pm$ the SDs of three pools, analyzed separately. The statistical analyses and abbreviations of the lipids are as in Figure 6.

170x113mm (299 x 299 DPI)

Figure 9. Percentage of different lipid classes in muscle of females in the previtellogenic (PreV), early-vitellogenic (Early V), vitellogenic (V), and postvitellogenic (PostV) stages as determined by chromatography coupled to a flame-ionization detector. The values are the means $\pm$ the SDs of three pools, analyzed separately. The statistical analyses and abbreviations of the lipids are as in Figure 6.

170x113mm (299 x 299 DPI)

Figure 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 $\pm$ the SDs of three pools, analyzed separately. The statistical analyses and abbreviations of the lipids are as in Figure 6.

170x113mm (299 x 299 DPI)

Figure 11. Composition of the major fatty acids of the hemolymph (Panel A), ovary (Panel B), midgut diverticula (Panel C), and muscle (Panel D). The data on the fatty-acid classes are expressed as the weight percentage as quantified by GLC. The values are the means $\pm$ 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 (Early V), vitellogenic (V), and postvitellogenic (PostV) stages at a $p < 0.05$, as determined by Tukey's post-hoc test. In the four panels, the percent 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.

139x244mm (299 x 299 DPI)

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