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First insight into the lipid uptake, storage and mobilization in arachnids: Role of midgut diverticula and lipoproteins

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ABSTRACT

The importance of midgut diverticula (M-diverticula) and hemolymph lipoproteins in the lipid homeostasis of *Polybetes phythagoricus* was studied. Radioactivity distribution in tissues and hemolymph was analyzed either after feeding or injecting [1-^{14}C]-palmitate. In both experiments, radioactivity was mostly taken up by M-diverticula that synthesized diacylglycerols, triacylglycerols and phospholipids in a ratio close to its lipid class composition. M-diverticula total lipids represent 8.08% (by wt), mostly triacylglycerols (74%) and phosphatidylcholine (13%). Major fatty acids were (in decreasing order of abundance) 18:1$n-9$, 18:2$n-6$, 16:0, 16:1$n-7$, 18:0, 18:3$n-3$. Spider hemocyanin-containing lipoprotein (VHDL) transported 83% of the circulating label at short incubation times. After 24 h, VHDL and HDL-1 (comparable to insect lipophorin) were found to be involved in the lipid uptake and release from M-diverticula, HDL-2 playing a negligible role. Lipoprotein's labelled lipid changed with time, phospholipids becoming the main circulating lipid after 24 h. These results indicate that arachnid M-diverticula play a central role in lipid synthesis, storage and movilization, analogous to insect fat body or crustacean midgut gland. The relative contribution of HDL-1 and VHDL to lipid dynamics indicated that, unlike insects, spider VHDL significantly contributes to the lipid exchange between M-diverticula and hemolymph.

1. Introduction

Insect fat body and crustacean midgut gland (sometimes termed hepatopancreas) are the organs involved in lipid metabolism that have been extensively studied in arthropods (Arrese et al., 2001; Gilbert and Chino, 1974; ÓConnor and Gilert, 1968). Crustacean midgut gland is involved in active lipid metabolism and has the combined functions of the mammalian liver, intestine, pancreas and adipose tissue (Al-Mohana and Nott, 1986; Al-Mohanna et al., 1985; García et al., 2002b; González Baró et al., 1990; González Baró and Pollero, 1993, 1988; Lavarías et al., 2006, 2007). Likewise, insects' fat body store and metabolize large amounts of lipids which support insect metabolic needs such as flight, metamorphosis and reproduction (Arrese et al., 2001; Fernando-Warnakulasuria et al., 1988; Ryan and Van Der Horst, 2000; Tsuchida and Wells, 1990; Ziegler and Ibrahim, 2001). Surprisingly, there is very little information about the analogous organ in arachnids. This is remarkable considering that lipids are also of vital importance for this class of arthropods that comprises spiders, scorpions, harvestman, mites and ticks among others. Spider, harvestman and scorpion's extensively developed intestinal system has been thought to be the lipid and glycogen (Foelix, 1996) storage site, but this conclusion was based on histological and ultrastructural observations (Foelix, 1996) only (Becker and Peters, 1985a; Becker and Peters, 1985b; Foelix, 1996; McCormick and Polis, 1990; Shultz and Pinto-da-Rocha, 2007). Spider midgut elaborate branching (henceforward termed midgut diverticula (M-diverticula) fills out most of the opisthosoma and is also present in the prosoma surrounding many organs. Two cell types have been described in M-diverticula epithelium: secretory and resorptive cells. Secretory cells contain digestive enzymes, while the resorptive cells have numerous "food vacuoles", process the nutrients further and pass them on to the underlying interstitial tissue or into the hemolymph.

Arthropod lipid circulation mechanisms are well known only in insects and crustaceans. Insect lipids are mostly carried out by the lipophorin lipoprotein in the form of diacylglycerol (Arrese et al., 2001; Blacklock and Ryan, 1994; Chino, 1985; Gonzalez et al., 1995; Soulages and Wells, 1994). In crustaceans, lipids are transported by high-density lipoproteins (HDL) mostly in the form of phospholipids (Chang and ÓConnor, 1983; García et al., 2002a,b; Lee, 1991; Lee and Puppione, 1978). Surprisingly, comparatively little information is available for arachnids where an HDL and a very high-density lipoprotein (VHDL) have been

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identified in the hemolymph of a few species (for review see Cunningham et al., 2007). In particular, the hemolymph lipid transport system of *Polybetes pythagoricus* (Araneae: Sparacidae) is composed of 3 lipoproteins: two HDL named HDL-1 (δ = 1.13 g/ml, 28% lipids by wt) and HDL-2 (δ = 1.18–1.20, 3.6% lipids) and a VHDL (δ = 1.21–1.24 g/ml, 3.5% lipids) and their hemolymph concentrations are 2.3, 23.6 and 45.4 mg lipoprotein/ml, respectively. Circulating lipids in *P. pythagoricus* (3.41 mg/ml hemolymph) are therefore carried by HDL-1 (26.2%), HDL-2 (25.1%) and VHDL (48.7%) (Cunningham et al., 1994; Cunningham and Pollero, 1996).

HDL-1 is a lipophorin-like lipoprotein regarding size and subunit composition (both contain 250 and 80 kDa apolipoproteins) but, unlike insect lipophorin, it mainly transports triacylglycerol and phospholipids instead of diacylglycerol, thus being more similar to crustacean HDL (Cunningham et al., 1994; García et al., 2002a). HDL-2 and VHDL mainly transport phospholipids and triacylglycerols and have a gray and bluish coloration given by the high amount of hemocyanin associated (18.9 and 36.3 mg/ml hemolymph, respectively) (Cunningham et al., 1994; Cunningham and Pollero, 1996). This function of the respiratory pigment hemocyanin as a lipid carrier is not only found in arachnids (Cunningham et al., 2000; Cunningham and Pollero, 1996) but was also demonstrated in cephalopods (Heras and Pollero, 1990, 1992).

Considering that the lipid exchange between hemolymph lipoproteins and tissues is largely unknown in the subphylum Chelicerata and since M-diverticula could potentially be the main organ involved in lipid biosynthesis in arachnids, the present study focussed on the first characterization of M-diverticula lipid and fatty acid compositions and metabolism and the *in vivo* lipid exchange between M-diverticula and hemolymph lipoproteins.

2. Materials and methods

2.1. Biological and chemical materials

Male and non-vitellogenic females adults of *P. pythagoricus* spiders weighing 2.32 ± 0.3 g were caught from barks of eucalyptus trees 10 km NE from La Plata, Argentina. They were kept without food in glass jars until used for the experiments.

All investigations were conducted in conformity with the Public Health Service (PHS) Policy on Humane Care and Use of Laboratory Animals. Studies were approved by a committee of the Consejo Nacional de Investigaciones Científicas y Técnicas. [1-^{14}C]-palmitic acid (57.0 mCi/mmol) was purchased from Amersham. All chemicals were of analytical grade.

2.2. Feeding experiment to label spider tissue lipids

The utilization of dietary lipids was studied in adults of *P. pythagoricus* with the administration of a small ball (50 mg) of minced meat containing 2 μCi radiolabelled [1-^{14}C]-palmitic acid as ammonium salt. Though most spiders feed mostly on live prey, we tested the use of minced beef meat balls to feed *P. pythagoricus*, and found spiders accepted the "dead prey". This approach simplified the experimental design allowing the administration of the radioactive precursor to spiders without the metabolic modifications that could arise from first labelling a living prey. Spiders were maintained in individual glass jars, at room temperature until they finished the food (1–3 h). Two hours later animals were cold-anesthetized and hemolymph obtained by severing the spider's legs and centrifuging the spider in a tube at low speed (Cunningham et al., 1994). After that, a dorsal incision was made in the opisthosoma tegument and M-diverticula carefully dissected out. Subsequently, book lungs were collected and the remaining tissues, including prosoma and opisthosoma muscle, tegument and gonads, were pooled and referred as "muscle" hereafter. Tissues were weighed and kept at −70 °C until lipid class and radioactivity distribution analyses (see below). Experiments were carried out with groups of 3 spiders. The samples from each spider were individually analyzed, and the data were combined to obtain the average and SD values.

2.3. In vivo labelling tissue lipids with injected fatty acid

Radioactive palmitic acid was administered to groups of 5 spiders. Each spider was injected into the leg with 4 μCi (70 nmol) fatty acid as ammonium salt and maintained in glass jars at room temperature. After 4 h incubation, M-diverticula, hemolymph, lung and muscle were dissected (the injected leg was discarded). Total radioactivity in each tissue and radioactivity incorporated into different lipid classes was quantified as described below.

Based on this initial screening that identified M-diverticula as the major lipid metabolism organ, a second experiment was conducted where groups of 5 spiders were injected into the leg with 4 μCi fatty acid as ammonium salt and maintained in glass jars at room temperature. After 1, 5 and 24 h incubation, only M-diverticula and hemolymph were obtained, and the distribution of the label into hemolymph lipoproteins were analyzed as described in the following section.

2.4. Lipoprotein isolation

Aliquots of hemolymph were over layered on a 3 ml NaBr solution (δ = 1.26 g/ml) and centrifuged at 178,000 × *g* at 10 °C for 22 h in a Beckman L8 70 M centrifuge with a SW60Ti rotor (Palo Alto, USA). Assuming that the density of spider's hemolymph was 1.006 g/ml, a saline solution of this density was centrifugated simultaneously as blank, and its density was determined in a Bausch & Lomb refractometer. The total volume of the tubes was fractionated from top to bottom into 0.2 ml aliquots. The protein content of each aliquot was monitored spectrophotometrically at 280 nm. Radioactivity in each fraction was measured by liquid scintillation in a Wallac 1214 Rack Beta equipment (Turku, Finand), and density calculated from the control blank tube. Fractions showing an increase in A_{280} and radioactivity (δ = 1.13 and 1.21–1.24 g/ml), were pooled and their protein content determined colorimetrically (Lowry et al., 1951). Radioactive lipids distributions were analyzed as described below.

2.5. Lipid extraction and analysis

Lipids from tissues, hemolymph and isolated lipoproteins were extracted following the procedure of Folch et al. (1957). The lipid quantitative determination was performed by thin-layer chromatography (TLC) coupled to a flame ionization detector in an Iatroscan apparatus model TH-10 (Iatron Laboratories, Tokyo, Japan), after separation on Chromarods type S-III (Ackman et al., 1990; Lavarías et al., 2005). The general procedure for separation and identification of lipids was described in a previous work (Cunningham and Pollero, 1996). Total lipids were quantified gravimetrically.

Total radioactivity was measured by liquid scintillation in a Wallac 1214 Rack Beta equipment. Radioactive lipids were analyzed by chromatography on HPTLC plates (Merk, Germany) and radioactivity of each lipid quantified using a Storm 840 scanner (Amersham Biosciences, Uppsala Sweden). Lipids were separated by double development of plates. First, chloroform–methanol–acetic acid–water (65:25:4:4, v/v/v) for polar lipid development (half of the plate) followed by a second development with hexane–diethyl ether–acetic acid (80:20:1.5, v/v/v) for neutral lipids until the solvent front reached the top of the plate.

Appropriate standards, run simultaneously, were visualized by exposure to iodine vapors.

2.6. Fatty acid analysis

Fatty acid methyl esters (FAME) from total lipids from M-diverticula were prepared with BF_3–MeOH, according to the method of Morrison and Smith (1992). The analysis were performed by gas–liquid chromatography (GLC-FID) using an HP-6890 capillary GLC (Hewlett Packard, Palo Alto, CA), fitted with an Omegawax 250 fused silica column, 30 m × 0.25 mm, with 0.25 μm phase (Supelco, Bellefonte, CA). The column temperature was programmed for a linear increase of 3 °C/min from 175 to 230 °C. Peaks were identified by comparing the retention times with those from a mixture of standard methyl esters (Heras et al., 2000).

3. Results

3.1. Incorporation of fed fatty acid into midgut diverticula and other tissues

The incorporation and release of lipid *in vivo* by tissues and hemolymph of adult *P. pythagoricus* were examined in spiders with the use of radiolabelled fatty acid either fed or injected. It was shown that 2 h after feeding [1-^{14}C]-palmitic acid, nearly 80% of the label was incorporated into the M-diverticula and the rest distributed among the other tissues (Table 1). The distribution of the radioactivity among lipid classes showed that palmitic acid was incorporated into different lipids according to the tissue analyzed. M-diverticula were metabolically the most active and used the labelled precursor for the synthesis of triacylglycerol (78%), phospholipid (6%) and traces of diacylglycerol. Hemolymph circulated the label to other tissues as free fatty acid and phospholipid. "Muscle" (muscle, gonads and tegument) incorporated most of the label into free fatty acid and phospholipid and some as triacylglycerol. Interestingly, lung showed free fatty acid as the only radiolabelled lipid (Table 1). Analysis or radioactive distribution among tissues 12 h after feeding showed a very similar incorporation pattern, with more than 85% of the label found in M-diverticula, particularly into triacylglylcerols.

3.2. Lipid transport in hemolymph and incorporation into tissues

To further confirm that M-diverticula was the major organ metabolizing lipids, the radioactivity distribution among lipid classes of spider tissues was studied injecting [1-^{14}C]-palmitic acid into one leg, waiting for 4 h to allow hemolymph to movilize the label to the rest of the body. Most of the label was found in the "muscle" fraction, followed by M-diverticula (Table 2). Interestingly, analysis of the incorporation into the lipid classes of each tissue showed a pattern very similar to that obtained in the feeding experiment. Thus, "muscle" has mostly the injected free fatty acid

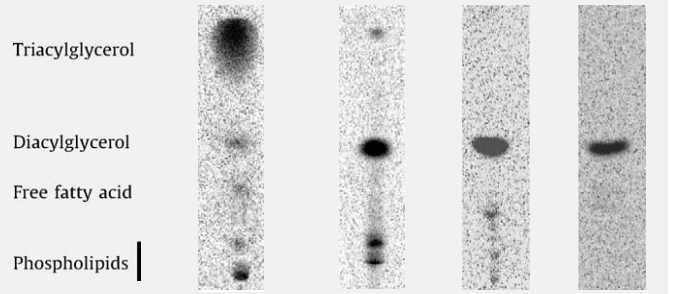
Table 1
Incorporation of radioactivity into spider tissues and lipid classes after 2 h feeding labelled palmitic acid (%)[a].

Tissue	M-diverticula	Hemolymph	Muscle	Book lung
% Radioactivity	79.3 ± 8.42	4.7 ± 1.30	14.2 ± 5.60	2.64 ± 1.6
Triacylglycerol	78.5 ± 10.67	Tr	8.6 ± 1.02	ND
Free fatty acid	15.3 ± 6.65	65.1 ± 12.63	70.4 ± 6.43	100
Diacylglycerol	Tr	ND	ND	ND
Phospholipid[b]	6.3 ± 2.15	35.7 ± 11.66	16.3 ± 2.66	ND

[a] Values are the mean ± 1 SD from the data of three individual spiders. Tr: traces; ND: not detected.
[b] Mostly phosphatidylcholine + phosphatidylethanolamine.

Table 2
Distribution of radioactivity into *P. pythagoricus* tissues and incorporation into lipid classes after injecting [^{14}C] palmitic acid (%)[a].

Tissue	M-diverticula	Hemolymph	Muscle	Book lung
% Radioactivity	11.94	12.64	71.62	2.04
Triacylglycerol	77.0 ± 20.5	3.5 ± 1.3	ND	ND
Free fatty acid	14.0 ± 7.6	62.6 ± 18.6	84.7 ± 6.7	100
Diacylglycerol	3.8 ± 1.3	Tr	8.9 ± 0.4	ND
Phospholipids[b]	5.1 ± 3.2	34.2 ± 9.8	4.4 ± 2.5	ND



Lower panel: typical radiochromatograms.
[a] Values are the mean ± 1 SD from the data of 5 individual spiders analyzed after 4 h injection of [1-^{14}C]-palmitate. ND: not detected.
[b] Mostly phosphatidylcholine + phosphatidylethanolamine.

(85%) followed by smaller amounts of phospholipid and diacylglycerol, while most of the fatty acid taken by M-diverticula was incorporated into triacylglycerol (77%) and phospholipids. After 4 h, hemolymph already circulates phospholipid (34%) besides free fatty acid and a small amounts of triacylglycerol (Table 2). Therefore, having identified M-diverticula as the most active organ regarding lipid metabolism, we focused on this organ and its interaction with the hemolymph in the second part of the study.

The lipid exchange between lipoproteins and M-diverticula were analyzed after injection of labelled palmitic acid into a spider leg following the fate of the label for 24 h. One hour after injection, the label was mostly incorporated into hemolymph, showing a constant decrease at the expense of an increase in M-diverticula along the incubation time, being more than 90% radioactivity associated with the later after 24 h (Fig. 1).

To shed insight into the hemolymphatic transport and delivery of lipids, lipoproteins were isolated by density gradient ultracentrifugation and their radioactivity measured. Fig. 2 shows a protein pattern with a well defined protein peak at 1.13 g/ml which corresponds to HDL-1, and a broad peak between 1.18 and

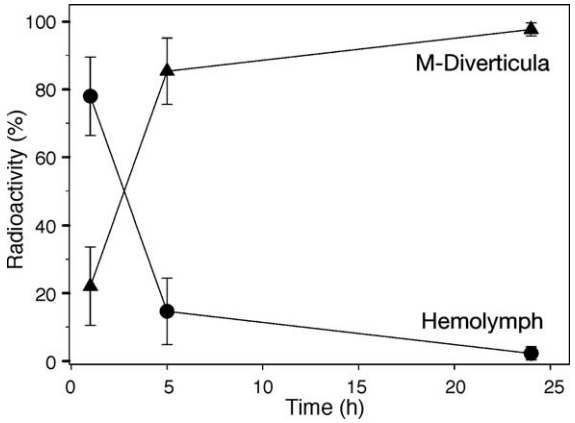


Fig. 1. Time corse of the radioactivity distribution in hemolymph and midgut diverticula of *Polybetes pythagoricus* after ^{14}C-palmitate injection into the spider leg. Error bars indicate 1 SD (n = 5).

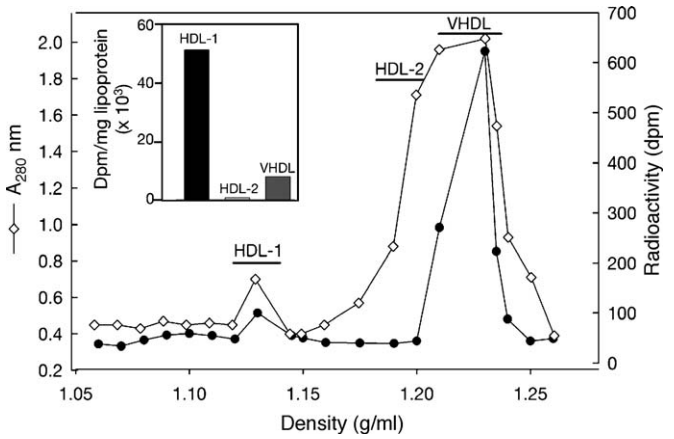


Fig. 2. Radioactivity and protein distribution in hemolymphatic fractions of *P. pythagoricus*. Spiders were injected in the leg with ^{14}C-palmitate and incubated for 5 h. Hemolymph was ultracentrifuged in a NaBr gradient and fractionated in 200 μl aliquots. Inset: specific activity of lipoproteins.

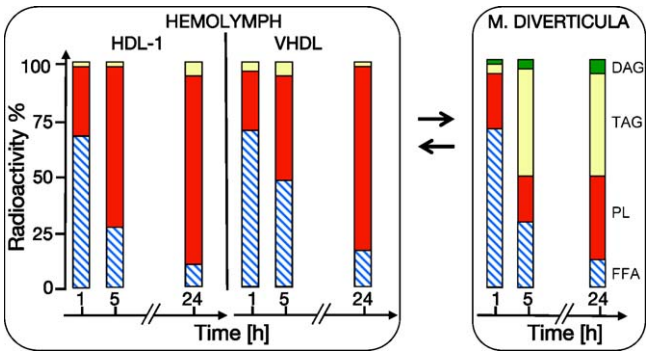


Fig. 4. Time course of the radioactivity distribution among lipid classes from lipoproteins and midgut diverticula after ^{14}C-palmitate injection in the leg of spiders. Results correspond to the mean of 3 determinations ± 1 SD (error bars omitted for clarity). (▬) diacylglycerol; (▭) triacylglycerol; (▬) phospholipid; (▨) free fatty acid. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

1.24 g/ml, which coincided with HDL-2 + VHDL densities. Interestingly, virtually all radioactivity was associated with HDL-1 and VHDL peaks, and very few with HDL-2 which was therefore not further analyzed (Fig. 2, inset).

Fig. 3 shows that most of hemolymph radioactivity was associated with VHDL at short incubation times (83 and 74% at 1 and 5 h, respectively) with a specific activity 6 times higher, while at 24 h both HDL-1 and VHDL shared the label almost in equal amounts. It is worth recalling that although VHDL has a lipid content 8 times lower than HDL-1, it is 20 times more concentrated.

The radioactivity distribution in lipid classes of M-diverticula and lipoproteins at different incubation times is shown in Fig. 4. The main labelled lipids in HDL-1 were free fatty acid (57%) and phospholipids (39%) at short incubation times. This relationship slowly changed along time and after 24 h incubation, labelled phospholipids increased to more than 85% of the radioactivity in this lipoprotein. A small amount of labelled triacylglycerol was observed at all incubation times.

VHDL displayed a pattern very similar to HDL-1 though at 5 h it had significantly higher levels of free fatty acids than HDL-1 (Fig. 4). As in HDL-1, after 24 h most of the label was associated with phospholipids (71%). Again, a small amount to triacylglycerol was also observed in this lipoprotein at all incubation times. As a whole, the time-course analysis of hemolymph labelled lipids showed that free fatty acids decreased along time, concomitantly with an increase of phospholipids, being the pattern similar for both lipoproteins.

The metabolic fate of the labelled free fatty acid incorporated into M-diverticula is depicted in Fig. 4. After 1 h injection, 79% of M-diverticula label was associated with free fatty acids but 5.4% incorporation into triacylglycerols was already detected. At 5 h of incubation, nearly half of the label was incorporated into triacylglycerols. At 24 h the organ (that at this time holds almost all of label associated with lipids) has metabolized most of the free fatty acid, which was incorporated into triacylglycerols and phospholipids. Remarkably, there was small but significant

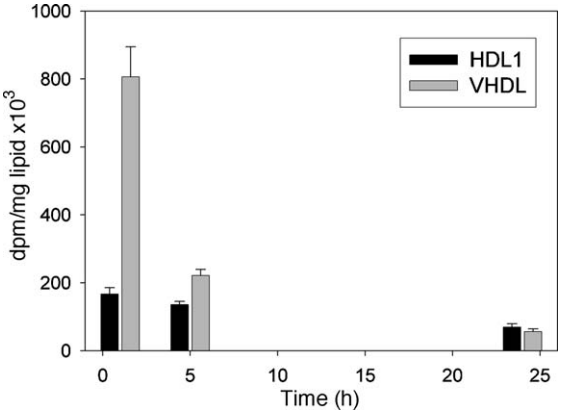


Fig. 3. Time course of the distribution of radioactivity between lipoproteins after injection of *P. pythagoricus*. Results correspond to the mean of 3 determinations ± 1 SD expressed as dpm/mg lipid. (▬) HDL-1; (▭) VHDL.

Table 3
Lipid class composition of midgut diverticula form *P. pythagoricus*[a].

Lipid class	% (w/w)
Hydrocarbons	1.68 ± 1.4
Sterols esters	1.55 ± 0.5
Triacylglycerols	74.25 ± 11.3
Free fatty acids	1.00 ± 0.10
Cholesterol	2.73 ± 1.10
Diacylglycerols	Traces
Phosphatidylethanolamine	2.73 ± 4.40
Phosphatidylcholine	13.23 ± 2.60
Sphingomyelin	1.90 ± 2.30

[a] Values are the mean of triplicate analyses ± 1 SD.

Table 4
Major fatty acids of total lipids of midgut diverticula of *P. pythagoricus*[a].

Fatty acid	% w/w
14:0	1.48 ± 0.33
16:0	15.05 ± 1.67
18:0	7.23 ± 0.99
16:1*n* − 7	10.16 ± 1.29
18:1*n* − 9	30.12 ± 2.23
18:1*n* − 7	3.76 ± 0.35
20:1*n* − 9	0.89 ± 0.11
18:2*n* − 6	23.38 ± 2.29
18:3*n* − 3	3.93 ± 0.45
20:4*n* − 6	2.25 ± 0.98
20:5*n* − 3	1.80 ± 1.00
Σ saturates[b]	23.76 ± 2.99
Σ monoenes	44.96 ± 3.98
Σ polyunsaturates	31.36 ± 4.72

[a] Values are the mean of triplicate analysis ± 1 SD.
[b] Totals include minor fatty acids not listed.

incorporation into diacylglycerols, a metabolic intermediate in acylglycerol synthesis, with no major changes in the amount of label in these lipids along the incubation time.

3.3. Lipid and fatty acid composition of midgut diverticula

The lipid content of M-diverticula is 8.08 ± 3.9% (by wt), and their lipid composition is dominated by triacylglycerols (74.2%) and phospholipids (17.8%) (Table 3). Fatty acid analysis of total lipids showed a pattern dominated by unsaturated fatty acids, being 18:1$n-9$, 18:2$n-6$ and 16:1$n-7$ quantitatively the most important, while the major saturated fatty acid was 16:0 (Table 4).

4. Discussion

4.1. Midgut diverticula as a lipid storage and delivery organ

Feeding adult spiders labelled fatty acid showed that M-diverticula is able to absorb and incorporate free fatty acids from food and it uses them to synthesize complex lipids, mostly triacylglycerol and phospholipids. Lipid metabolic features of the M-diverticula are similar to features of the crustacea midgut gland and insect midgut gland-fat body which have been extensively studied since the 1960s (Atella et al., 2000; Gilbert and Chino, 1974; Kallapur et al., 1984; Kanazawa and Koshio, 1994), unlike arachnids, where there is virtually no information on the major lipid metabolic organ. Diacylglycerol was also labelled in this organ, indicating that this intermediate may be involved in acylglycerol synthesis. Therefore, dietary lipids (or at least the free fatty acids, usual products of the digestive process) seem to be employed by midgut diverticula for *de novo* synthesis, *via* the phosphatidic acid or the monoacylglycerol (MAG) pathways, similarly to what happens in insect midgut (Arrese et al., 2001; Canavoso et al., 2001) though the exact pathway cannot be disclosed with this experimental design. Assuming spiders have an intermediate metabolism similar to other arthropods, some palmitate was probably oxidized into Acetyl CoA and incorporated into other non-lipid molecules or lost as CO_2. Though this was not analyzed, these pathways can be inferred by the amount of radioactivity recovered in the lipid fraction.

The distribution of radioactivity among the lipid classes was close to the mass distribution of the M-diverticula lipid subspecies, further supporting that it is an important site for the uptake, storage and synthesis of lipids. This is in agreement with histological descriptions of spider midgut diverticula which suggested it was involved in lipid storage (Foelix, 1996).

A comparison of the lipid composition of fat body, midgut gland and M-diverticula indicated they shared a similar pattern. *P. pythagoricus* M-diverticula triacylglycerols constitute the main lipid storage form, representing about 74% of the total lipid, as occur in the insect fat body (70–90%) (Bailey, 1975; Cvacka et al., 2006; Sobotnik et al., 2006) and crustacean midgut gland (around 68%) (González Baró and Pollero, 1998). *P. pythagoricus* M-diverticula fatty acid composition, is dominated by 16 and 18 carbon fatty acids, namely oleic, linoleic and palmitic acids, the same fatty acid reported for the scorpion *Buthus quinquestriatus* "hepatopancreas" (El-Salhy et al., 1981). The spectrum corresponds, in general to a "terrestrial pattern" where fatty acids of the $n-6$ family prevail over those of the $n-3$ family. This is also similar to the fatty acid composition of fat body of many insects, while crustacean storage organ has different proportions of unsaturated fatty acids, in agreement with its aquatic environment (Beenakkers and Gilbert, 1968; Cvacka et al., 2006; Sobotnik et al., 2006).

In adult spiders, after a meal, the newly synthesized phospholipids and traces of triacylglycerols were transferred from the M-diverticula to the hemolymph, together with free fatty acids, contrasting with insects whose midgut and fat body transfer mostly phospholipids and diacylglycerols (Atella et al., 1995; Coelho et al., 1997). After feeding ^{14}C-palmitate we observed that, whereas FFA represents 17% of the hemolymph lipid mass (Cunningham and Pollero, 1996), about 62% of the radiolabelled lipids are represented by free fatty acids. At present there is no information on arachnids lipoprotein synthesis pathways to explain these differences, but if we consider that total hemolymph radioactivity is less than 4.7% of the 35 nmol of the ^{14}C-palmitate fed, the mass of radiolabelled free fatty acid represents a very small fraction of the mass of circulating free fatty acid in this species, similarly to other invertebrates and vertebrates where the amount of circulating free fatty acids is low.

The amount associated with M-diverticula accounts for nearly 80% of the total radioactivity fed, while other tissues have the remaining 20% of the label, mostly distributed as FFA and PL. If we consider that food is first taken up and processed by M-diverticula, the presence of labelled lipids in hemolymph and in other tissues supports the hypothesis that they were transferred from M-diverticula and delivered through hemolymph to the rest of the tissues, as happens in vertebrates and other arthropods.

In addition, when free fatty acids were injected directly into hemolymph, there was a different uptake of the label by tissues: instead of being taken up by M-diverticula, most of the free fatty acid was incorporated by "muscle", that showed only a minor incorporation into complex lipids, suggesting that these tissues may use the small amount of radiolabelled fatty acid available for other purposes. Remarkably, after either feeding or injecting radioactive palmitate, about 2% of the label was found associated with book lung but entirely as free fatty acids, i.e. without incorporation into other lipids. Though there is no published data on the lipid composition of spider book lung, we could assume it is probably dominated by structural lipids. We do not know why only free fatty acid is present, though one explanation would be that lung lipid metabolism is rather slow and the time after feeding or injecting the radiolabelled palmitate was not enough for its incorporation into other lipids, or that there is a lipoprotein lipase in book lungs that degrades hemolymph acylglycerides before incorporation into lungs and after that, they are not reesterified into complex lipids and only employed for β-oxidation, or a combination of both hypothesis. At present there is no information on which lipid source spider tissues utilize for energy, an interesting avenue of research. Unlike these tissues, M-diverticula displayed a very active lipid metabolism. In fact was able to incorporate fatty acids from hemolymph into triacylglycerols and phospholipids, in a ratio very similar to that observed in the feeding experiment. The incorporation into M-diverticula, resembled what happens in insect fat body or crustacean midgut gland (García et al., 2002a; Gilbert and Chino, 1974; Soulages et al., 1988). If we recall that spiders can survive for a long time without eating (up to 200 days in black widows (Foelix, 1996)), the triacylglycerols and phospholipids stored in the extensively developed and metabolically very active M-diverticula are most probably playing an important function in the energy delivery to the rest of the body.

Based on the above results from the short-term study, we followed the ability of M-diverticula to take up, metabolize and release *in vivo* free fatty acids into lipoproteins along a 24-h period (see below). Nearly 90% of the injected label was cleared from the hemolymph by M-diverticula after 5 h incubation. These absorbed fatty acids were rapidly incorporated into phospholipids and triacylglycerols, resembling that observed in the feeding experiment, though with less labelled neutral lipids (Table 1 and Fig. 4). The presence of labelled diacylglycerols further supports that acylglycerols synthesis in arachnids would proceed like insect conventional pathways.

As a whole, these experiments demonstrate that M-diverticula is capable to take up circulating lipids from hemolymph and to metabolize and deliver lipids toward hemolymph from the ingested food.

4.2. Role of the different lipoproteins in spider lipid transport

The role of lipoproteins in the lipid exchange between M-diverticula and hemolymph was studied following the changes in lipoprotein labelled lipid composition for 24 h after an injection of free fatty acid as a precursor lipid. As mentioned above, hemolymph fatty acids were taken up M-diverticula and incorporated into triacylglycerols and phospholipids. Later on these acylglycerols were released to hemolymph lipoproteins. Regardless of the incubation time, HDL-1 and VHDL were always found to be involved in the lipid uptake and/or release to or from M-diverticula. The fact that HDL-2 (that, like VHDL, contains hemocyanin as apolipoprotein) only took up very small amounts of labelled lipids was unexpected. These two lipoproteins have differences in the ratio between their 105 and 120 kDa apolipoprotein subunits, as well as in their hydration densities and lipid compositions that could be responsible for the observed differences in radioactive lipid uptake (for details on lipid and apolipoprotein composition please refer to Cunningham and Pollero, 1996). Moreover, it could be speculated that its lipids may have only a structural function, similar to the crab *Carcinus maenas* in which phospholipids associate to hemocyanin and stabilize the protein structure (Zatta, 1981). The possibility that VHDL lipids are only structural was discarded in a previous work that demonstrated that the particle neither loses its stability, nor undergoes structural alterations after delipidation (Cunningham et al., 2006). The presence of lipids associated with hemocyanin has also been shown in another spider (Jaenicke et al., 1999), but more knowledge on spider lipoprotein structure and loading capacity is needed to better understand this finding.

The radioactivity distribution in hemolymph at short times showed that VHDL transported significantly more label than HDL1. Furthermore, it was observed that *in vitro* delipidated VHDL from *P. pythagoricus* has great capacity to bind lipids, and consequently to transport them, being the free fatty acids the lipid preferentially taken up (Cunningham et al., 1999). This feature probably explains the increased specific activity of VHDL shortly after injecting palmitic acid, exceeding that of HDL-1 by a factor of 4 and agrees with the labelling pattern of *E. californicun* VHDL that incorporated mostly free fatty acids and minor amounts of phospholipid and triacylglycerol from an *in vitro*-labelled "hepatopancreas". Unlike *P. pythagoricus*, *E. californicun* VHDL does not contain hemocyanin as apolipoprotein (Stratakis et al., 1993).

In spite of the uneven label distribution between HDL-1 and VHDL at 1 h, radiolabelled lipid composition changes along time were similar for both lipoproteins, indicating they have a similar role in spider lipid dynamics. HDL-1, seems to transport lipids among the sites of absorption, storage and utilization playing a role analogous to insect lipophorin (Downer, 1985). Nevertheless, unlike insects, phospholipids are the major components of the lipid moiety in arachnid lipoproteins (Cunningham et al., 1994, 2000; Cunningham and Pollero, 1996). After challenging lipid homeostasis with a direct free fatty injection into hemolymph, the system seems to approach equilibrium after 24 h when M-diverticula-synthesized phospholipids and triacylglycerols were transferred back to hemolymph and lipoprotein radiolabelled lipid distribution resembled that of their reported lipid composition, dominated by phospholipids, free fatty acids and triacylglycerols (Cunningham and Pollero, 1996). Moreover, both lipoproteins carried the label in equal amounts, as reflected by their comparable specific activity. This is similar to the metabolism of crustacean midgut gland that takes up free fatty acids and release to hemolymph phospholipids placing arachnids' lipid metabolism closer to crustaceans' (García et al., 2002a; Lee and Puppione, 1988).

The results presented here show for the first time that the M-diverticula of arachnids is a primary storage site and a major lipid metabolic center involved in the uptake and mobilization of lipids. It also clearly demonstrates that two lipoproteins participate in the lipid transport from and towards tissues.

The similarity between crustacean and arachnid lipid storage and movilization organs as well as their lipoprotein system is remarkable, considering that spiders are ecologically more closely related to insects. More work is still needed in the comparative aspects of this diverse group.

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