OVARIAN NUTRITIONAL RESOURCES DURING THE REPRODUCTIVE CYCLE OF THE HEMATOPHAGOUS *DIPETALOGASTER MAXIMA* (HEMIPTERA: REDUVIIDAE): FOCUS ON LIPID METABOLISM

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In this study, we have analyzed the changes of the ovarian nutritional resources in Dipetalogaster maxima *at representative days of the reproductive cycle: previtellogenesis, vitellogenesis, as well as fasting-induced early and late atresia. As expected, the amounts of ovarian lipids, proteins, and glycogen increased significantly from previtellogenesis to vitellogenesis and then, diminished during atresia. However, lipids and protein stores found at the atretic stages were higher in comparison to those registered at previtellogenesis. Specific lipid staining of ovarian tissue sections evidenced remarkable changes in the shape, size, and distribution of lipid droplets throughout the reproductive cycle. The role of lipophorin (Lp) as a yolk protein precursor was analyzed by co-injecting Lp-OG (where OG is Oregon Green) and Lp-DiI (where DiI is*

1,10-dioctadecyl-3,3,30,30-tetramethylindocarbocyanine) to follow the entire particle, demonstrating that both probes colocalized mainly in the yolk bodies of vitellogenic oocytes. Immunofluorescence assays also showed that Lp was associated to yolk bodies, supporting its endocytic pathway during vitellogenesis. The involvement of Lp in lipid delivery to oocytes was investigated in vivo by co-injecting fluorescent probes to follow the fate of the entire particle (Lp-DiI) and its lipid cargo (Lp-Bodipy-FA). Lp-DiI was readily incorporated by vitellogenic oocytes and no lipoprotein uptake was observed in terminal follicles of ovaries at atretic stages. Bodipy-FA was promptly transferred to vitellogenic oocytes and, to a much lesser extent, to previtellogenic follicles and to oocytes of ovarian tissue at atretic stages. Colocalization of Lp-DiI and Lp-Bodipy-FA inside yolk bodies indicated the relevance of Lp in the buildup of lipid and protein oocyte stores during vitellogenesis. © 2014 Wiley Periodicals, Inc.

INTRODUCTION

In insects, the mature egg contains all vital components to successfully support the growth of the embryo and, in some species, to sustain the first larval or nymphal instars (Swevers et al., 2005). A completely developed egg is composed of proteins, carbohydrates, and lipids as major constituents, as well as others minor components, all collectively named "yolk" (Atella et al., 2005).

The period of rapid oocyte growth and accumulation of yolk, vitellogenesis, is characterized by the extraovarian synthesis of yolk protein precursors that are taken up by the oocyte (Snigirevskaya and Raikhel, 2005). On the other hand, food deprivation as well as other unfavorable behavioral or environmental factors promote the degeneration of some follicles to an atretic stage and oocyte resorption (Bell and Bohm, 1975; Aguirre et al., 2011; Medeiros et al., 2011). Oocyte resorption (oosorption) is a reproductive strategy to preserve valuable resources to expand female lifespan and/or to secure its reproductive success (Bell and Bohm, 1975; Wang and Horng, 2004; Moore and Attisano, 2011). Although vitellogenesis has been thoroughly characterized in several insect species (Snigirevskaya and Raikhel, 2005), oocyte resorption and follicular atresia have received far less attention (Uchida et al., 2001; Kotaki, 2003; Aguirre et al., 2011, 2013; Medeiros et al., 2011).

Currently, the processes of lipid accumulation and mobilization in oocytes are poorly understood. The oocyte has a very limited capacity to synthesize lipids de novo (Ziegler and Van Antwerpen, 2006). Since lipids, mostly triacylglycerol (TAG), can account up to 40% of the dry weight of the insect egg, most of lipids found in mature eggs must be imported from circulating lipid carriers (Canavoso et al., 2001). Once in the oocyte, lipids are stored in specialized organelles or lipids droplets (Kühnlein, 2012).

Vitellogenin (Vg) is the main yolk protein precursor in most insect species (Tufail and Takeda, 2008). Vg carries small amounts of lipids (8–15%) but is taken up by developing oocytes in large quantities by receptor-mediated endocytosis (Ziegler and Van Antwerpen, 2006). Reports from *Manduca sexta* indicated that Vg contributes with 5% of the lipids found in the eggs (Kawooya and Law, 1988).

Lipophorin (Lp), the main hemolymphatic lipoprotein in insects, contains 30–50% of lipids. It cycles among peripheral tissues exchanging its lipid cargo at the cell surface, without additional synthesis or degradation of its apolipoprotein matrix (Van der Horst et al., 2009). Endocytosis of Lp has also been described as a minor pathway in the fat body and in the oocytes of some insects (Ziegler and Van Antwerpen, 2006; Van der Horst et al., 2009; Fruttero et al., 2011).

Triatomines or "kissing bugs" are hematophagous insects with relevance in public health since they are vectors of the parasite *Trypanosoma cruzi*, the etiological agent of Chagas' disease (Coura and Viñas, 2010). In triatomine females, each gonotrophic cycle is coupled to the intake of blood meal (Stoka et al., 1987). Although autogeny has been observed in some species of Triatominae, blood meal triggers vitellogenesis, which lasts until the end of the oviposition period. Thereafter, and without another blood meal, ovarian follicles enter in postvitellogenesis and degenerate (follicular atresia). In this context, *Dipetalogaster maxima* constitutes a suitable model to study follicular atresia since under standardized conditions, depriving females of blood meal during postvitellogenesis elicits gradual morphological changes in the ovarian tissue, allowing to distinguish an early and a late stage of follicular degeneration (Aguirre et al., 2011, 2013). In contrast, in most insect species previously analyzed, the onset of atresia occurs very fast (Uchida et al., 2001, 2004; Moore and Attisano, 2011).

We have previously characterized the morphological and biochemical changes that take place during the transition from vitellogenesis to follicular atresia in *D. maxima*, and the role of Vg and vitellin (Vt), its storage form in the oocytes (Aguirre et al., 2011). In this work, we have analyzed the ovarian nutritional resources at different stages of the reproductive cycle of this vector, focusing on lipid metabolism. Cellular approaches demonstrated for the first time that Lp-mediated lipid transfer to oocytes occurs at all reproductive stages. The relevance of different pathways accounting for the process will be discussed in the context of triatomine physiology.

MATERIALS AND METHODS

Chemicals

Bodipy FL C16 (4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-hexadecanoic acid) and Oregon green (OG) 514 carboxylic acid (Molecular Probes, Eugene, OR); Sephadex G-25 PD-10 columns (GE Healthcare, Little Chalfont, UK); Tissue-Tek embedding medium optimal cutting temperature (OCT; Miles, Elkhart, IN); Fluor-save (Calbiochem, Darmstadt, Germany); enhanced chemiluminescence detection kit (PerkinElmer, Waltham, MA); electrophoresis protein standards (New England Biolabs, Ipswich, MA); Centricon centrifugal filter devices (Millipore, Bedford, MA); silica gel plates and solvents (J.T. Baker Chemical Co., Phillipsburg, NJ); and lipid standards (Avanti Polar Lipids, Alabaster, AL) were from indicated commercial sources. Horseradish peroxidase conjugated goat anti-rabbit IgG; N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDAC); 1,10-dioctadecyl-3,3,30,30-tetramethylindocarbocyanine (DiI), and all other chemicals were from Sigma-Aldrich (St. Louis, MO).

Insects

Experiments were carried out using insects supplied from a colony of anautogenous *D. maxima*, maintained under standardized conditions (28°C, 70% relative humidity, 8:16 h light:dark photoperiod) and fed fortnightly on hen blood (Canavoso and Rubiolo, 1995),

according to the recommendations of the National Institute of Parasitology (Health Ministry, Argentina) (Núñez and Segura, 1987).

Standardized conditions of insect rearing for the study were described elsewhere (Aguirre et al., 2011). Briefly, newly emerged females were segregated individually and placed together with two recently fed males for a period of 48 h. Mated females were maintained in individual containers until they were able to feed on blood (day 10–12 post-ecdysis). For the studies, ovaries were sampled from females at representative days of the reproductive cycle as previously described: (a) previtellogenesis (day 2 post-ecdysis, unfed period); (b) vitellogenesis (days 4–6 post-blood feeding); (c) postvitellogenesis (days 10–12 and days 30–32 after the end of oviposition for early and late atresia, respectively; Aguirre et al., 2011, 2013).

Hemolymph and Ovaries Sampling

At the sampling periods indicated above, females were immobilized and ovaries were carefully dissected out under cold phosphate buffered saline (PBS, 66 mM Na_2HPO_4/KH_2PO_4, 150 mM NaCl, pH 7.4). Only ovaries containing all the ovarioles were further processed for the experiments.

For Lp purification, the hemolymph from vitellogenic females was collected from immobilized insects with a Hamilton syringe, from sectioned legs while gently pressing the abdomen. The hemolymph was received in cold microtubes containing 10 mM Na_2EDTA, 5 mM dithiothreitol, and protease inhibitors as follows: 1 mM phenylmethyl-sulfonyl fluoride; 1 mM N-α-p-tosyl-L-lysine chloromethyl ketone; 1 mM pepstatin A and 0.3 mM aprotinin (Fruttero et al., 2009). Hemolymph was centrifuged to remove hemocytes and then subjected to a KBr gradient ultracentrifugation to isolate Lp. Final Lp purification was achieved by its fractionation on Sepharose 6B (Fruttero et al., 2009). On the other hand, Vg was purified from the hemolymph by combination of KBr gradient ultracentrifugation and ion-exchange chromatography as previously described (Aguirre et al., 2011; Fruttero et al., 2011). Lp and Vg were concentrated with Centricon 100 devices and subjected to polyacrylamide gel electrophoresis under both denaturing and nondenaturing conditions to monitor purification (Laemmli, 1970; Bollag and Edelstein, 1992).

Biochemical Assays

Lipids and glycogen from ovaries were separated according to Van Handel (1965) and then quantified by colorimetric assays (Scott and Melvin, 1953; Frings and Dunn, 1970). Proteins were measured according to Bradford (1976) processing the ovarian tissues as described previously (Fruttero et al., 2011).

Lipid composition was determined by processing the ovaries of two females at different stages of the reproductive cycle for lipid extraction according to Folch et al. (1957). Extracted lipids were dried under nitrogen and separated by thin-layer chromatography on silica gel using hexane/ethyl ether/formic acid (70:30:3, v/v/v) as developing system. Lipid fractions were detected by spraying the plates with 10% H_2SO_4/0.04M $(NH_4)_6Mo_7O_{24}.4H_2O$/3 mM $Ce(SO_4)_2$ and posterior heating. Plates were analyzed by densitometry employing standards as described elsewhere (Canavoso et al., 2003).

Tissue Sectioning and Staining

Dissected ovaries were fixed in 4% paraformaldehyde in PBS (30 min, room temperature), transferred into sucrose/PBS, embedded in OCT, and snap frozen in liquid nitrogen

(Fruttero et al., 2011). Tissue sections of 9 μm were obtained with a Leica CM1510 cryostat (Leica Microsystems, Wetzlar, Germany) and placed onto poly-L-lysine-treated glass slides.

For the histological analysis, the sections were stained with toluidine blue (0.1% in 1% acetic acid) for 1 min at room temperature. To assess the distribution of lipid droplets, tissue sections were stained with Oil Red O for 30 min at room temperature (Akiduki and Imanishi, 2007). After removal of the staining, the slides were gently washed with PBS, air-dried, and mounted. Stained sections were observed with an Eclipse TE2000-U microscope (Nikon, Tokio, Japan) and digital images were captured with a Nikon Digital Sight DS-U1 camera and processed with ACT-2U version 1.51.116.256 software.

In vivo Studies With Fluorescently Labeled Lp

For the in vivo experiments, purified Lp was labeled with the nonexchangeable fluorophore DiI (Lp-DiI), which intercalates among phospholipids. DiI does not transfer due to its extremely hydrophobic nature, allowing to trace the Lp particle (Via and Smith, 1986; Fruttero et al., 2009). Lp was also labeled on its protein moiety with OG (Lp-OG) (Fruttero et al., 2009). On the other hand, to follow the fate of Lp lipid cargo, Lp was labeled on its exchangeable lipid fraction with Bodipy FL C16 (Bodipy-FA), a fluorescent palmitic acid analog (Lp-Bodipy-FA) (Martin-Nizard et al., 1987; Fruttero et al., 2011). In addition, Vg was labeled with OG (Vg-OG) using EDAC (10 mg/ml) as described elsewhere (Fruttero et al., 2011). After conjugation, fluorescently labeled probes were passed through PD-10 columns to remove free fluorophores.

In order to follow simultaneously the fate of the entire Lp particle and its exchangeable lipid moiety, females at different stages of the reproductive cycle were co-injected with Lp-DiI and Lp-OG or with Lp-DiI and Lp-Bodipy-FA (10 μl, 20 μg each). Endocytosis of Lp by vitellogenic oocytes was evidenced by co-injecting in the hemocoel Vg-OG and Lp-DiI under similar experimental conditions. In all cases, injected insects were kept at 28 or 4°C (controls) for 3 h and thereafter, ovaries were dissected out in PBS and processed for cryostat sectioning. Tissue sections were air-dried, mounted in Fluorsave, and examined with an Olympus FV300 laser scanning confocal microscope (Olympus, Tokyo, Japan), equipped with 488 and 543 nm lasers. Corresponding merged images were acquired and processed with FluoView FV1000 version 1.7.1.0 software.

Direct Immunofluorescence Assays

Tissue sections were blocked and then, subjected to permeabilization with 1% bovine serum albumin (BSA), 5% fetal bovine serum, and 0.1% Triton X-100 in PBS for 30 min. Tissue sections were incubated with the anti-Lp-FITC antibody obtained as described previously (Fruttero et al., 2011), diluted in 1% BSA in PBS (1:40; 1 h). All incubations were carried out in a humid chamber at 37°C and followed by two washes of 5 min each with PBS. The slides were processed for confocal scanning microscopy as described in the "In vivo studies with fluorescently labeled Lp" section.

Statistical Analysis

For the biochemical parameters, four independent experiments were performed and data for each point were registered by triplicate. Graphs and statistical tests were performed using GraphPad Prism and GraphPad Instat 3.0. Results were expressed as mean ± SEM.
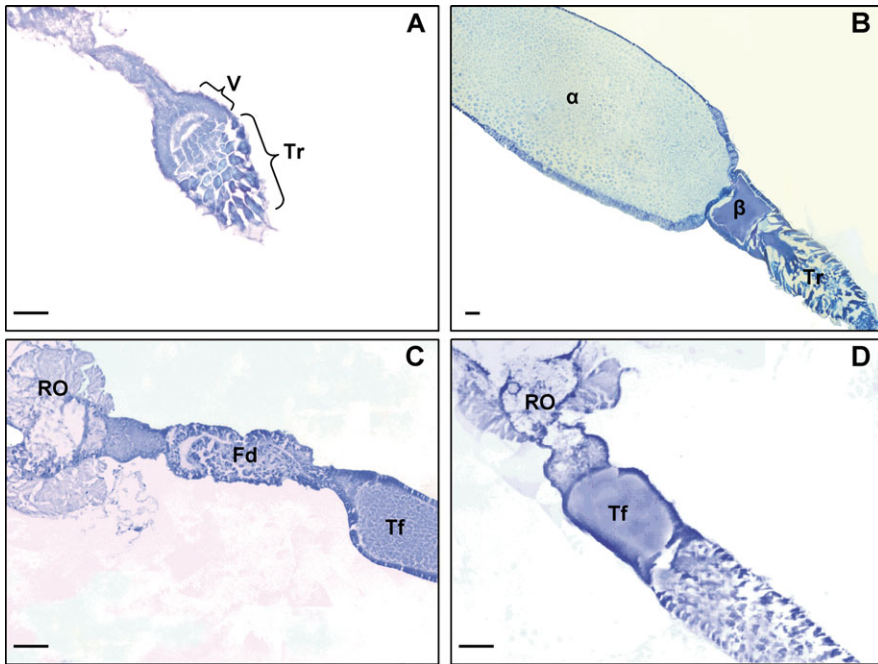
Figure 1. Histological changes in the ovary of *D. maxima*. Females at different stages of the reproductive cycle were dissected, the ovaries processed for cryostat sectioning and stained with Toluidine blue. (A) Previtellogenesis, (B) vitellogenesis, (C) early atresia, and (D) late atresia. V, vitellarium; Tr, tropharium; α and β indicate the positions of alpha and beta oocytes, respectively; RO, resorbed oocyte; Fd, follicle undergoing incipient degeneration; TF, terminal follicle. Bars: 100 μm.
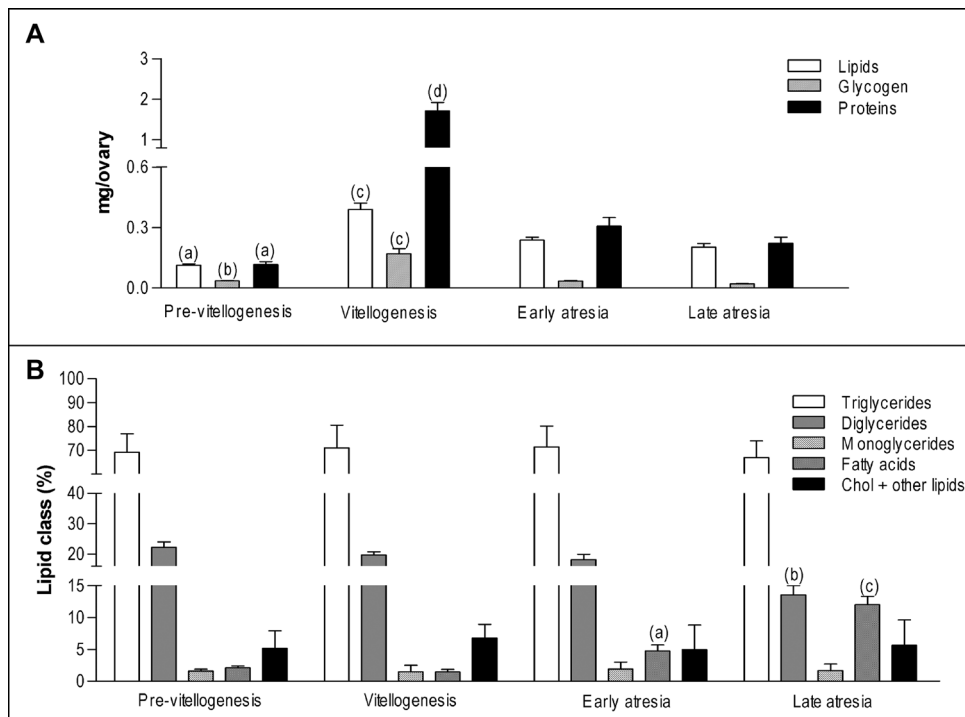
The analyses of lipid, glycogen, and protein contents were performed by one-way Kruskal–Wallis nonparametric analysis of variance (ANOVA) for comparisons between means and employing Dunn's multiple comparisons test as post-test. In addition, the determination of lipid classes was subjected to one-way parametric ANOVA for comparisons between means whereas the Student–Newman–Keuls test was applied as post-test. A P value < 0.05 was considered statistically significant.

RESULTS

The Ovarian Tissue of D. Maxima and the Dynamics of Nutritional Resources

The ovaries of *D. maxima* are paired organs, each one containing seven ovarioles of the telotrophic type. At previtellogenesis (unfed period, day 2 post-ecdysis), the ovarioles presented a poorly developed vitellarium and an apical prominent tropharium (Fig. 1A). After females ingested the first blood meal, terminal oocytes (α position) became vitellogenic and enlarged rapidly due to yolk accumulation, although smaller oocytes in β position were also observed. At this stage, α oocytes were surrounded by a polarized follicular epithelium to conform a terminal follicle (Fig. 1B). During vitellogenesis, the ovarioles displayed an asynchronous development, presenting terminal follicles of different sizes (results not shown). Under standardized conditions, unless females receive a second blood meal, some follicles degenerate to an atretic stage and oocytes are resorbed. At early stages of follicular atresia, ovarioles maintained some asynchronism and showed

Figure 2. Changes in ovarian nutritional resources throughout the reproductive cycle of *D. maxima*. (A) Lipid, glycogen, and protein contents in the ovaries obtained from females in previtellogenesis, vitellogenesis, early and late atresia. Results are expressed as mean ± SEM ($n = 4$). (a) $P < 0.01$ versus vitellogenesis, early and late atresia; (b) $P < 0.05$ versus vitellogenesis; (c) $P < 0.05$ versus early and late atresia; and (d) $P < 0.01$ versus early and late atresia. (B) Total lipids from females at different stages of the reproductive cycle were fractionated by thin-layer chromatography and subjected to a densitometric analysis. Results are expressed as mean ± SEM ($n = 4$). Values are expressed as percentage. (a) $P < 0.05$ versus previtellogenesis and vitellogenesis; (b) $P < 0.05$ versus previtellogenesis, vitellogenesis, and early atresia; (c) $P < 0.001$ versus previtellogenesis, vitellogenesis, and early atresia. Chol, cholesterol.

the occurrence of well-developed terminal follicles together with follicles in incipient degeneration and resorbed oocytes (Fig. 1C). At late atresia, ovarioles lose asynchronism and only small terminal follicles and resorbed oocytes were observed (Fig. 1D).

The morphological changes of ovarian tissue of *D. maxima* throughout the reproductive cycle were in agreement with the dynamics of nutritional stores in the tissue (Fig. 2A). Thus, at previtellogenesis, the scarcely developed ovaries have small amounts of nutritional resources. As expected, at vitellogenesis, lipids, proteins, and glycogen increased significantly reaching the average of 0.39, 1.71, and 0.17 mg/ovary, respectively, at days 4–6 post-blood meal and then, decreased during follicular atresia. Nevertheless, when early and late atretic stages were compared to each other, no significant differences were observed. Interestingly, lipids and protein stores found at the atretic stages where higher in comparison to those registered at previtellogenesis.

Lipid Composition of Ovary Tissue and the Distribution of Lipid Droplets

When the composition of ovarian lipids was determined at different stages of the reproductive cycle, no substantial changes in the percentage of the main stored lipid, TAG,

were observed. However, the amounts of diacylglycerol (DAG) significantly decreased ($P <$ 0.05 for late atresia vs. previtellogenesis, vitellogenesis, and early atresia) with the progression of follicular atresia while the percentage of fatty acids amounted to 12% at late atresia. At all stages, monoglycerides as well as cholesterol and other minor lipid fractions did not change significantly (Fig. 2B).

Specific stain of lipid stores with Oil Red O showed that in previtellogenesis, ovaries had few lipid droplets, which were observed as small spherical structures mainly confined at the periphery of the oocyte (Fig. 3A). In contrast, lipid droplets in vitellogenic oocytes were more numerous, larger, and irregularly shaped. Moreover, large lipid droplets were also observed in the follicular epithelium (Fig. 3B). At early stages of atresia, lipid droplets displayed a different pattern of distribution depending on the follicle analyzed. Thus, although in those follicles maintaining some degree of development, lipid droplets were mainly observed at the cortical area of terminal oocytes, their distribution in the follicles in incipient degeneration was rather homogenous (Fig. 3C and D). As the degenerative process advanced, terminal follicles at late atresia exhibited fewer lipid droplets compared to those observed in vitellogenesis and early atresia (Fig. 3E). Unexpectedly, at this stage, large and abundant lipid droplets were observed in the tropharium of some ovarioles (Fig. 3F).

The Role of Lp in Lipid Delivery to Oocytes

The involvement of Lp in lipid delivery to oocytes as well as its role as yolk protein precursor was analyzed in vivo by co-injecting fluorescently labeled probes to follow the fate of the entire particle (Lp-OG/Lp-DiI) or its lipid cargo (Lp-Bodipy-FA) in the hemocoel of females at different stages of the reproductive cycle. Three hours after the co-injection of Lp-OG (Fig. 4A) and Lp-DiI (Fig. 4B), both conjugates colocalized mainly in the yolk bodies of vitellogenic oocytes (Fig. 4C), indicating that either, Lp-OG or Lp-DiI, is useful to trace the entire particle in subsequent experiments. In addition, direct immunofluorescence assays using an anti-Lp-FITC antibody showed that Lp was associated to yolk bodies of vitellogenic oocytes (Fig. 4D). Altogether, these set of results strengthen the presence of an endocytic pathway for Lp during vitellogenesis.

In other set of experiments co-injecting Lp-DiI and Lp-Bodipy-FA (Fig. 5), it was observed that, 3 h after injection, the fluorescent signal for Lp-DiI was faint, being localized at the cortical area of previtellogenic follicles and in the perioocytic space of terminal follicles of ovaries at early and late atresia (Fig. 5A, C, and D). At these stages, no fluorescent signal inside the oocytes was observed implying that endocytosis of Lp does not play a relevant role. At early and late atresia, Lp-DiI was also detected in the basal region of the follicular epithelium (Fig. 5C and D). On the other hand, the fluorescent signal for the lipid analog Bodipy-FA found inside the oocytes of previtellogenic and atretic follicles was faint, pointing out that the process of lipid transfer occurs even during these stages, although at less extent than vitellogenesis (Fig. 5A–D). No specific fluorescence for Lp-DiI and Lp-Bodipy-FA was detected neither in follicles undergoing incipient degeneration nor resorbed oocytes (data not shown). Contrastingly, in vitellogenic oocytes, Lp-DiI was visualized in yolk bodies, colocalizing with Vt-OG (the storage form of Vg-OG), demonstrating the endocytosis of Lp (Fig. 5E). At 4°C, endocytosis of fluorescently labeled Lp was impaired (Fig. 5F). When Lp-DiI and Lp-Bodipy FA were co-injected in vitellogenic females, the strong signal for Bodipy-FA was found in lipid droplets and yolk bodies as Lp-Bodipy-FA, partially colocalizing with Lp-DiI in the latter structures (Fig. 5B). Interestingly, the signal for Lp-DiI also seems to be distributed at the periphery of some
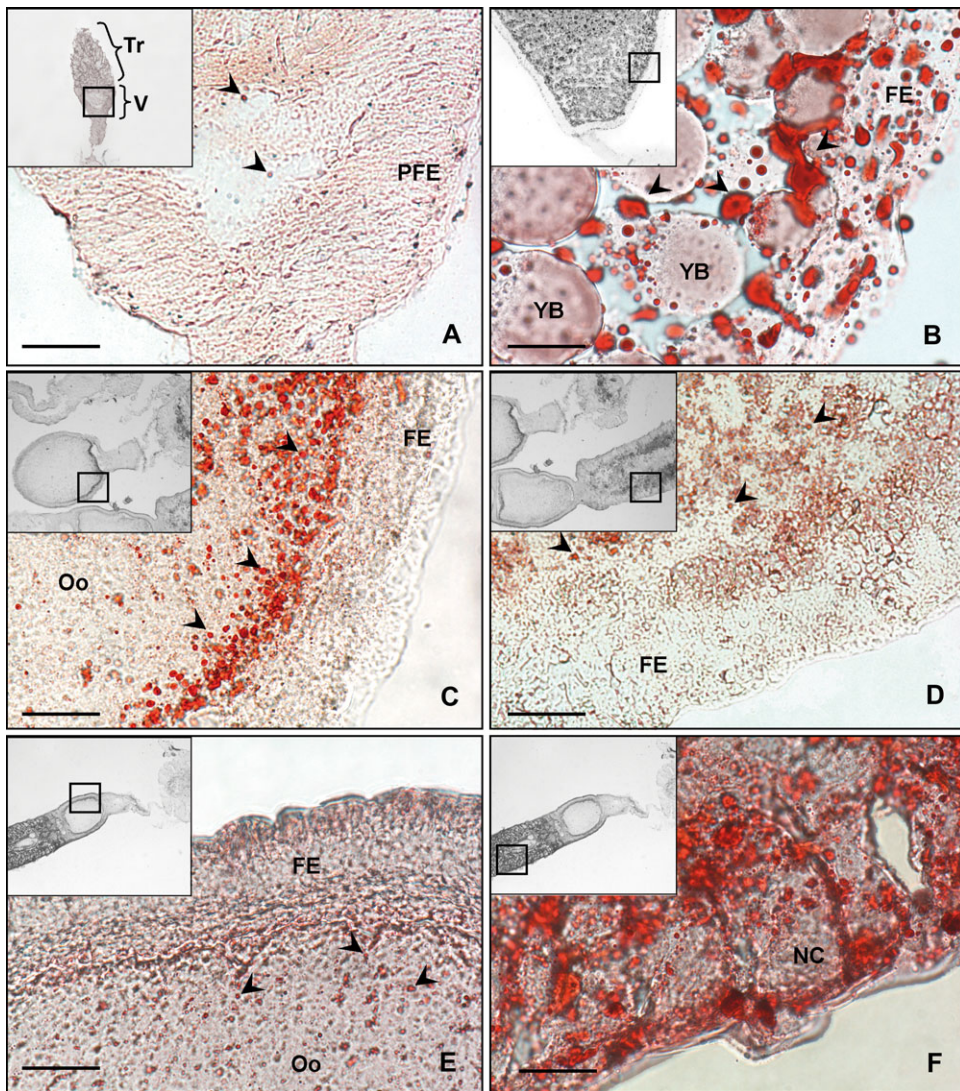
Figure 3. Distribution of lipid droplets in ovarian tissue of *D. maxima*. Tissue sections from females at different stages of the reproductive cycle were stained with Oil Red O and analyzed with light microscopy. (A) Previtellogenesis, (B) vitellogenesis, (C, D) early atresia, and (E, F) late atresia. (A), (B), (C), and (E) are micrographs of terminal follicles, (D) corresponds to a follicle undergoing incipient degeneration, and (F) is a section of the tropharium. Images show at higher magnification the area of the box indicated in the inset. Inset images are displayed in gray scale. Arrowheads indicate lipid droplets. PFE, prefollicular epithelium; YB, yolk bodies; FE, follicular epithelium; V, vitellarium; Oo, oocyte; Tr, tropharium; NC, nurse cells. Bars: 20 μm.

yolk bodies, being not possible to rule out if such an observation is due to a particular Lp segregation in the oocyte structures or more likely, to a difference in brightness of Lp-DiI and Lp-Bodipy-FA.

Taken together, the results showed that in the ovarian tissue of *D. maxima*, provisioning of lipids to oocytes is accomplished by Lp-mediated lipid transfer at the cell surface as well as by endocytosis of Lp particle. It was also shown that depending on the stage of the
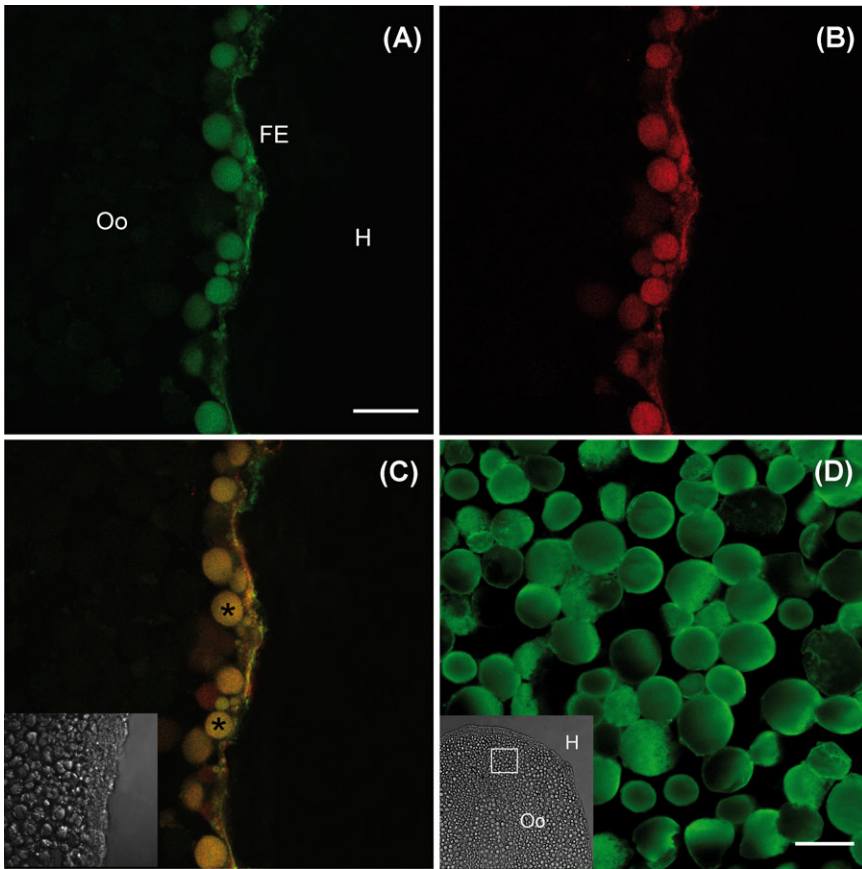
Figure 4.   Lipophorin (Lp) localization in vitellogenic oocytes of *D. maxima* by in vivo assays and immunofluorescence. For in vivo assays, purified Lp was fluorescently labeled with Oregon Green (Lp-OG, green) or with DiI (Lp-DiI, red) to trace the lipoprotein particle. Vitellogenic females were co-injected with Lp-DiI and Lp-OG, dissected 3 h later, and processed for confocal laser microscopy. (A, B) The pattern of fluorescence for Lp-OG and Lp-DiI, respectively. (C) A merged image of (A) and (B). Asterisks show the colocalization of Lp-OG with Lp-DiI in yolk bodies. The inset shows the corresponding DIC image for (A) to (C). For direct immunofluorescence assays, ovaries from vitellogenic females were dissected out and processed for cryostat sectioning. Tissue sections were incubated with anti-Lp-FITC and analyzed by scanning laser confocal microscopy. (D) The fluorescence signal for Lp in yolk bodies. The image in (D) displays at higher magnification the area of the box indicated in the inset. FE, follicular epithelium; Oo, oocyte; H, hemolymph. Bars: 20 μm.

reproductive cycle, the two pathways can converge more likely to maximize the storage of lipid resources.

DISCUSSION

In this work, we have determined the changes of ovarian nutritional resources throughout the reproductive cycle of the hematophagous *D. maxima*, focusing on lipid metabolism. As it was observed for Vt stores (Aguirre et al., 2011), under standardized insect rearing conditions, ovarian proteins, lipids, and glycogen changed although showing similar profiles: being very low at previtellogenesis, increasing remarkably during vitellogenesis,
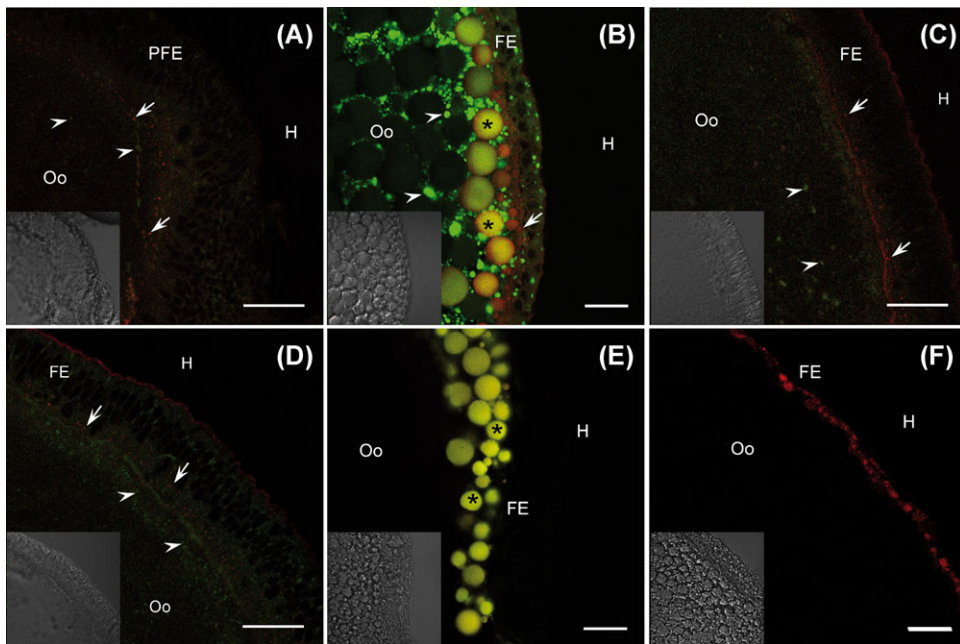
Figure 5. The role of lipophorin (Lp) in lipid delivery to oocytes. Purified Lp was fluorescently labeled with DiI to trace the particle (Lp-DiI, red) or with Bodipy-FA, a fatty acid analog, to follow its lipid cargo (Lp-Bodipy, green). Females at different stages of the reproductive cycle were co-injected with Lp-DiI and Lp-Bodipy, dissected 3 h later, and processed for confocal laser microscopy. The micrographs show merged images of red and green channels of terminal follicles for insects in previtellogenesis (A), vitellogenesis (B), early (C) and late atresia (D). Arrows indicate the signal for Lp-DiI and arrowheads the signal of Bodipy-FA in lipid droplets (A–D). Asterisks show the colocalization of Lp-DiI with Lp-Bodipy in yolk bodies (B). (E) Purified vitellogenin was conjugated with OG (Vg-OG, green) and co-injected with Lp-DiI in the hemocoel of vitellogenic female (endocytic control of Lp). Asterisks show the colocalization of Lp-DiI with Vg-OG in yolk bodies. (F) Terminal follicle of a vitellogenic female injected with Lp-DiI and kept at 4°C (Control). The insets show the corresponding DIC images (A–F). PFE, prefollicular epithelium; FE, follicular epithelium; Oo, oocyte; H, hemolymph. Bars: 20 μm.

and decreasing significantly during follicular atresia. The increases of lipids, proteins, and/or glycogen from previtellogenesis to vitellogenesis were also reported in two vectors of Chagas' disease (Santos et al., 2008, 2011; Fruttero et al., 2011). In the Dermapteran *Labidura riparia* (Sayah, 2008), the profile of ovarian lipids varied during the reproductive cycle, similarly to that observed in *D. maxima.* On the other hand, it has been proposed that resources provisioned during vitellogenesis can be mobilized by oocyte resorption to either increase the lifespan of the female or secure its reproductive success (Bell and Bohm, 1975; Wang and Horng, 2004). The decrease in ovarian nutritional resources from vitellogenesis to follicular atresia in *D. maxima* would reflect the adjustments of insect metabolism to the physiological needs of the females during postvitellogenesis. Since at early atresia some ovarioles showed well-developed terminal follicles, it seems likely that mobilization of ovarian resources would contribute to resume vitellogenesis if a female receives another blood meal. However, at late atresia, ovarioles were small and all of them presented resorbed oocytes, suggesting that mobilization of resources would be directed to sustain female lifespan.

In insects, as well as in other animal groups, lipids have several biological roles (Fahy et al., 2011). In the reproductive physiology of insects, lipids are vital because they supply energy to the developing embryo (Ziegler and Van Antwerpen, 2006). In *Rhodnius prolixus*, labeled DAG and free fatty acids were taken up by the ovaries at different rates depending on the time after blood meal (Santos et al., 2011). In *D. maxima*, TAG and DAG were the major lipid classes found in ovarian tissues sampled at all reproductive stages, with amounts found at vitellogenesis comparable to those reported for ovarian follicles of *R. prolixus* (Santos et al., 2011). However, the highest amounts of free fatty acids were detected at both stages of atresia. Taking into account that follicular atresia involves remarkable changes in ovarian morphology as well as in oocyte resorption (Bell and Bohm, 1975), it is likely that free fatty acids detected at atretic stages arose from the breakdown of TAG reserves. To what extent such free fatty acids provide energy to support the instauration and progression of the degenerative process or recycle to circulation remains to be established.

The intracellular storage of lipids occurs in specialized organelles, the lipid droplets, conformed by a neutral lipid core, surrounded by a monolayer of phospholipids and cholesterol with specific proteins embedded or associated to this outer layer (Arrese and Soulages, 2010; Kühnlein, 2012). Currently, it has been described that the abundance, size, and shape of lipid droplets vary considerably depending on the requirements of the milieu and the cell types (Walther and Farese, 2012). In insect ovaries, the dynamic of lipid droplets has been scarcely studied (Huebner and Anderson, 1972a,b; Liu and Davis, 1972; Wiemerslage, 1976; Ziegler and Antwerpen, 2006). Previtellogenic ovaries of *Aedes aegypti* maintained with high sucrose concentrations contained high amounts of neutral lipids, probably to support the energetic demands of the growing oocyte at least during the beginning of vitellogenesis (Clifton and Noriega, 2012). In *D. maxima*, the scarce amounts of ovary lipids found at the previtellogenic stage were distributed in few and small lipid droplets. Likewise, relatively few lipid droplets were found in the prefollicular tissue of *R. prolixus* (Huebner and Anderson, 1972a). Moreover, in agreement with the findings reported by Huebner and Anderson (1972b) during oocyte differentiation in *R. prolixus*, in vitellogenic oocytes of *D. maxima* lipid droplets were abundant, large, and homogeneously distributed, occupying the spaces left by yolk bodies, a pattern expected for this phase of lipid stores buildup. Interestingly, during vitellogenesis, large lipid droplets were also detected in the follicular epithelium, similarly to those reported in *R. prolixus* (Huebner and Anderson, 1972a). In nonadipocytes, lipid droplet stores have generic functions directed to cover the physiological cell's own needs (Suzuki et al., 2011). Taking into account that throughout vitellogenesis oocytes of *D. maxima* have large lipid stores and considering that follicular epithelium is undergoing a dynamic metabolism (Swevers et al., 2005), it is likely that follicular cells use lipid reserves to fulfill their own needs, rather than for exporting them to the oocyte.

Studies carried out in eukaryotic cell cultures with limited availability of nutrients demonstrated that lipid droplets can shrink rapidly (Walther and Farese, 2012). When follicular atresia in *D. maxima* was induced by depriving females of blood meal during postvitellogenesis, not only oocyte lipid droplets became remarkable smaller compared to those found at vitellogenesis but also high amounts of free fatty acids were detected through the degenerative process, suggesting the lipolysis of lipid stores. At early atresia, the small lipid droplets were located at the periphery of most developed oocytes, although large lipid droplets were observed in the tropharium of late atretic ovarioles. In the endoparasitoid *Pteromalus puparum*, the transition from vitellogenesis to oosorption proceeded with a diminution in size of lipid droplets, which in turn were also found at the

periphery of the oocytes. Such an organization of the lipid store was attributed to a transfer of the ovarian nutrients to the hemolymph, thus providing resources to enhance the survival of the insect (Guo et al., 2011). Although we do not present evidences supporting ovarian lipid recycling through follicular atresia in *D. maxima*, a similar mechanism could be operating in this species.

It had been demonstrated that insect ovaries or follicles had a very limited capacity to synthesize lipids de novo (Kawooya et al., 1988; Ziegler, 1997). In addition, endocytosis of Vg and Lp are minor pathways contributing to the total lipid content stored in the oocytes, which are mainly supplied by Lp via its classic docking, nonendocytic mechanism (Ziegler and Van Antwerpen, 2006). In Triatominae, studies about Lp-mediated lipid delivery to developing oocytes were undertaken only in *R. prolixus* and *Panstrongylus megistus*. In *R. prolixus*, Lp proved to be an important source of phospholipids and fatty acids for developing oocytes, although in vitro and immunohistochemistry studies indicated that endocytosis of Lp was not operative in this species (Gondim et al., 1989; Machado et al., 1996; Santos et al., 2011, Entringer et al., 2013). On the other hand, in vivo assays using fluorescently labeled Lp and Vg probes demonstrated that vitellogenic oocytes of *P. megistus* were able to take up lipids by both a major, nonendocytic pathway, and by an endocytic pathway (Fruttero et al., 2011).

As determined in this work, in *D. maxima*, the oocytes recruit lipids by different pathways according to the stage of the reproductive cycle. Thus, in addition to the expected lipid transfer to oocytes by a nonendocytic mechanism, we showed that Lp is endocytosed during vitellogenesis. This conclusion is supported by the finding of the Lp signal inside oocytes observed when Lp-DiI/Lp-OG were co-injected as well as by the pattern displayed by the anti-Lp-FITC antibody in the immunofluorescence assays. The process of Lp endocytosis is further reinforced by the results that showed the colocalization of Lp-DiI with Vg-OG in yolk bodies and the absence of label inside the oocytes when the females were subjected to control conditions. Besides, no intracellular fluorescent signal for Lp compatible with endocytosis was observed in previtellogenesis and follicular atresia. It is expected that during vitellogenesis, when the oocyte needs to secure large quantities of nutrients in a short time, it does so by maximizing the uptake of lipids by converging the classic docking, nonendocytic pathway and the endocytic one. It should be noted that during vitellogenesis, follicular epithelium displayed a remarkable patency. As reported for other species, the endocytic receptors for Vg and Lp belonging to the low-density lipoprotein receptor (LDLR) superfamily are highly expressed in oocytes membranes (Ziegler and Van Antwerpen, 2006; Tufail and Takeda, 2009). However, Aguirre and co-workers (2011) showed in *D. maxima* that Vg endocytosis occurs not only during vitellogenesis but also at early atresia, although at low rates. At present, the differences in the endocytotic pattern of Lp and Vg in oocytes are still not understood.

One important finding reported in this work was the fact that although the transfer of the fatty acid analog, Bodipy-FA, from Lp to the oocytes takes place throughout reproductive cycle, the process varied according to reproductive stages. In *R. prolixus*, the transfer of radiolabeled fatty acids to the ovaries increased soon after a blood meal (Santos et al., 2011). In *D. maxima*, the transfer of lipids to vitellogenic oocytes was also expected, but the transfer of Bodipy-FA at the atretic stages was surprising if considering that ovarian lipid stores dropped significantly at early and late follicular atresia. It can be speculated that if lipids are exported from the oocyte to the hemolymph during atresia, this Lp-mediated transfer would be bidirectional and part of the lipids will pass from Lp to the oocyte. This mechanism of lipid transfer from Lp to the cell by passive diffusion following a concentration gradient was previously proposed (Soulages and Wells, 1994;

Arrese et al., 2001). To clarify this issue, it would be important to understand the roles of Lp receptors in the oocytes. Preliminary results suggest that oocyte plasma membrane proteins, unrelated to LDLR, can function as nonendocytic Lp receptors to regulate the lipid transfer process.

In this work, in addition to the dynamics of ovarian nutritional resources, we demonstrated that the supply of lipids from Lp to the oocytes takes place at all reproductive stages. It was also shown that such lipid provisioning can be accomplished by nonendocytic and/or endocytic pathways depending on the female metabolic requirements at specific stages. All these aspects are of relevance for the understanding of the reproductive biology of Chagas' disease vectors, deserving further exploration.

ACKNOWLEDGMENTS

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