Iron and Ferritin Deposition in the Ovarian Tissues of the Yellow Fever Mosquito (Diptera: Culicidae)

Dawn L. Geiser, Theresa N. Thai, Maria B. Love, and Joy J. Winzerling

Department of Nutritional Sciences, College of Agriculture and Life Sciences, The University of Arizona, Tucson, AZ 85721, and 1 Corresponding author, e-mail: dligeiser@email.arizona.edu

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Abstract

Dengue, yellow fever, and Zika are viruses transmitted by yellow fever mosquito, *Aedes aegypti* [Linnaeus (Diptera: Culicidae)], to thousands of people each year. Mosquitoes transmit these viruses while consuming a blood meal that is required for oogenesis. Iron, an essential nutrient from the blood meal, is required for egg development. Mosquitoes receive a high iron load in the meal; although iron can be toxic, these animals have developed mechanisms for dealing with this load. Our previous research has shown iron from the blood meal is absorbed in the gut and transported by ferritin, the main iron transport and storage protein, to the ovaries. We now report the distribution of iron and ferritin in ovarian tissues before blood feeding and 24 and 72 h post-blood meal. Ovarian iron is observed in specific locations. Timing post-blood feeding influences the location and distribution of the ferritin heavy-chain homolog, light-chain homolog 1, and light-chain homolog 2 in ovaries. Understanding iron deposition in ovarian tissues is important to the potential use of interference in iron metabolism as a vector control strategy for reducing mosquito fecundity, decreasing mosquito populations, and thereby reducing transmission rates of vector-borne diseases.

Key words: *Aedes aegypti*, ferritin, iron, mosquito, ovary

Mosquitoes are vectors for several diseases including malaria, dengue, yellow fever, and Zika. Over one million people worldwide die from diseases transmitted by mosquitoes every year (WHO 2013a,b, 2014). Disease transmission occurs when female mosquitoes consume a blood meal to acquire the essential nutrients for oogenesis (Clements 1992). The blood meal contains a high iron load. Although insects require iron for egg development (Pitts et al. 2014; Tang and Zhou 2013a,b; Rivera-Perez et al. 2017) and embryogenesis (Gonzalez-Morales et al. 2015), the iron load in a blood meal can increase the formation of toxic free radicals by the Fenton reaction (Walter-Nuno et al. 2017). Recent evidence indicates that lipid peroxidation as a result iron-initiated free-radical formation can initiate ferroptosis, a distinct form of cell death (Masaldan et al. 2019). Insects limit a Fenton response, in part, by sequestering iron in ferritin; therefore, ferritin and iron-related mechanisms are considered as potential targets for pest control (Ferrer et al. 2015).

Available evidence indicates that ferritin is critical for insect survival (Missirlis et al. 2007, Tang and Zhou 2013a) and serves several roles, including the absorption, storage, and transport of iron (Georgieva et al. 1999, Geiser et al. 2006, Missirlis et al. 2007, Zhou et al. 2007, Mehta et al. 2009, Tang and Zhou 2013a), in oogenesis (Walter-Nuno et al. 2018), embryonic and postembryonic development (Li 2010, Gonzalez-Morales et al. 2015, Rivera-Perez et al. 2017), in cytotoxic protection from oxidative stress (Geiser et al. 2006, 2013; Tang and Zhou 2013a), and as an immune modulator (Kremer et al. 2009, Ahmed et al. 2014, Gomes et al. 2018). We previously reported that iron from the diet is loaded into ferritin in the *Ae. aegypti* gut and iron-loaded ferritin is subsequently secreted into the hemolymph; meal iron is detected in ovaries by 24 h post-blood meal (PBM; Zhou et al. 2007). Both iron and ferritin are present in mature *Ae. aegypti* eggs, and when iron is absent from a meal, fewer eggs are oviposited (Kogan 1990, Pitts et al. 2014, Rivera-Perez et al. 2017).

The only insect ferritin structure currently available is that of *Triechoplusia ni* [Hubner (Lepidoptera: Noctuidae), cabbage looper; Hamburger et al. 2005]. *Triechoplusia ni* ferritin consists of 24 subunits that are homologs of the vertebrate ferritin heavy and light chains. The assembled protein has 12 heavy-chain homologs (HCHs) and 12 light-chain homologs (LCHs). The HCH and LCH1 have been characterized in several insects (*Dunkov and Georgieva 1999, Georgieva et al. 2002b, Nichol et al. 2002, Missirlis et al. 2006, Kim et al. 2009, Wang et al. 2009, Li 2010, Tang and Zhou 2013b, Gonzalez-Morales et al. 2015, Yu et al. 2017, Fei et al. 2018, Wajnberg et al. 2018). We and others have characterized expression of HCH and LCH1 in mosquito cells and tissues (Charlesworth et al. 1997; Dunkov et al. 2002; Georgieva et al. 2002a, Geiser et al. 2003, 2015, 2017). More recently, we identified a second unique LCH subunit, LCH2 (Geiser et al. 2017). Similar to the HCH and LCH1,
LCH2 is expressed in ovaries. In contrast to HCH and LCH1, LCH2 was unresponsive to iron stimulation. LCH2 protein and message levels were increased in the absence of iron, and both message and protein levels were increased in ovaries by 72 h following blood feeding.

We are interested in interference in iron metabolism as a potential strategy to reduce mosquito fecundity, decrease mosquito populations, and, thereby, lower transmission rates of vector-borne diseases. Because the LCH2 showed differences in expression from LCH1, we wondered if the three proteins (HCH, LCH1, and LCH2) would show differential location in ovarian tissues. How *Ae. aegypti* ovaries accumulate iron and the proteins involved in processing this mineral are significant to understanding the how iron influences oogenesis. We have used confocal microscopy to visualize iron and ferritin subunits in ovarian tissues. We report that iron is present in *Ae. aegypti* ovaries prior to and after a blood meal and that ferritin accumulates in the ovaries. Furthermore, we observe that ferritin also accumulates in the ovaries and that the three ferritin subunits and iron are found in similar regions of the *Ae. aegypti* ovarian tissues prior to and after a blood meal.

**Materials and Methods**

**Mosquito Rearing**

*Aedes aegypti* (Rockefeller strain) eggs, a kind gift from Dr. Michael Riehle (Department of Entomology, University of Arizona), were hatched and animals raised as previously described (Zhou et al. 2007, Corby-Harris et al. 2010). Briefly, larvae were fed on a diet of Purina Cat Chow Complete Cat Food Formula pellets (0.5 g/l ddH2O; UPC 1780046572, Nestle’ Purina PetCare Company, St. Louis, MO) and maintained in trays of approximately 125 mosquito larvae. Larvae were maintained in water-filled trays for approximately 7 d until pupation, and then pupae were transferred into water-filled cups inside small cages. From the onset of pupation, adult mosquitoes emerged approximately 1–2 d later and were provided 10% sucrose ad libitum; animals were kept at 26–28°C, 75–80% relative humidity with a photoperiod of 16:8 (L:D) h.

**Blood Feeding and Tissue Collection**

Approximately 150 mated, 8–9 d post-emergence, female *Ae. aegypti* were fasted for 12–14 h with sterile water ad libitum prior to feeding experiments. Forty-five females were sacrificed as the pre-blood fed treatment group (0 h time point), and the ovary tissues dissected. Ninety *Ae. aegypti* females were membrane-fed a 1-ml diet of warmed (37°C), defibrinated porcine blood supplemented with 5 mM ATP (Sigma, St Louis, MO) for 2 h in glass feeders (Zhou et al. 2007, Geiser et al. 2015). Engorged females were maintained on 10% sucrose solution ad libitum following feeding; 45 ovary tissues were dissected at 24 and 72 h PBM. For dissection, mosquitoes were cold anesthetized and tissues dissected into mosquito saline (125 mM NaCl, 5 mM KCl, 1.85 mM CaCl2; pH 6.5; Riehle and Brown 2002). Tissues were transferred to a 24-well plate with 300 µl of mosquito saline. Three pairs of ovaries were placed into each well. Fifteen ovary pairs were collected at each time point.

**Tissue Fixation and Storage**

Tissues were fixed in 4% fixation solution (16% formaldehyde; catalog no. 28906; Thermo Fisher Scientific, Waltham, MA) diluted in sterile Tris-buffered saline (TBS; 140 mM NaCl, 2.7 mM KCl, 24.8 mM Tris base; pH 7.4); tissues were incubated in 450-µl fixation solution per well for 30 min, rocking at RT. For short-term storage (less than a month), the fixation solution was removed after the 30-min incubation period and the ovaries were stored in 450 µl 0.02% sodium azide (NaN3) in sterile TBS per well in a 24-well plate. The plate was sealed with Parafilm to prevent evaporation and stored at 4°C.

**Tissue Permeabilization**

Prior to the staining experiments, one fixed tissue from each time point was placed into a well of a sterile 24-well plate with 450 µl of TBS. Tissues were permeabilized using a freeze–thaw method (Wasteney et al. 1997, Mardones and Gonzalez 2003, Shakes et al. 2012): the plates were placed in −80°C for 10–15 min until the tissues were completely frozen and then removed and placed at RT for 30–45 min until they were completely thawed. This freeze–thaw was repeated three times.

**Prussian Blue Staining of Tissue Iron**

To visualize the presence of iron in ovarian tissues, tissues were stained with Prussian blue that detects ferric iron under physiological conditions ferrous iron is oxidized to the ferric form, the ionic form of iron found in ferritin. Fixed tissues were rinsed 3× using 300 µl of sterile phosphate-buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, 5.4 mM Na2HPO4, 7H2O, 1.8 mM KH2PO4; pH 7.4), followed by washing 3× in 450 µl of sterile PBS for 30 min each at RT while rocking. Tissues were either incubated in 300 µl of fresh Prussian blue stain (2% K4[Fe(CN)6]; catalog no. sc-215757; Santa Cruz Biotechnology, Dallas, TX)/2% HCl in sterile PBS) or sterile PBS alone as unstained tissues for 1 h in the dark at RT while rocking. Following Prussian blue staining, tissues were rinsed 3× in 300 µl of sterile PBS and washed 3× in 450 µl of sterile PBS for 30 min each at RT while rocking.

**Slide Preparation and Visualization of Prussian Blue Stained Tissues**

Prussian blue stained tissues and unstained tissues were removed from the 24-well plate and transferred onto microscope slides with one pair of ovaries from each time point per slide. Ovary pairs were suspended in a droplet of mounting solution (3:7, sterile PBS:glycerol). Mounted tissues were then covered with 10 mm #1.5 round glass coverslips (catalog no. 260368; Ted Pella, Inc., Redding, CA). Ovaries were imaged at 10× and 40× settings of a Nikon Eclipse E600 epifluorescence microscope (Chiyoda, Tokyo, Japan). A minimum of five replicates for each time point per tissues was performed.

**Fluorescent Immunohistochemistry of Ferritin Subunits**

After permeabilization, fixed tissues were rinsed 3× using 450 µl of sterile TBS per well, then washed 3× in 450 µl of sterile TBS for 30 min each at RT while rocking. Tissues were incubated in 450 µl of antibody incubation buffer (2% IgG free bovine serum albumin [catalog no. 001-000-161]; Jackson Immuno, West Grove, PA)/1% normal donkey serum [catalog no. 017-000-121; Jackson Immuno/TBS) for 1 h at RT while rocking. The buffer was removed, and tissues were incubated in 450 µl of antibody incubation buffer with the appropriate primary antiserum overnight at 4°C while rocking: anti-*Ae. aegypti* HCH-specific rabbit serum (epitope: YNLDSIKEDKTKDL; diluted in 50% glycerol; 1:450 vol/vol;
Geiser et al. 2017), and anti-Ae. aegypti LCH2-specific rabbit serum (epitope: QPDMNKTYKQMLDK; 1:450 vol/vol; Geiser et al. 2017). The primary antiserum incubation was removed, and the tissues were rinsed 3x in 450 µl of sterile TBS per well, then washed 3x in 600 µl of sterile TBS for 30 min each at RT while rocking.

Tissues were incubated in 450 µl of antibody incubation buffer with the goat anti-rabbit IgG (H + L) secondary antibody, Alexa Fluor 488 conjugate (green; catalog no. A-11008; Thermo Fisher Scientific, Rockford, IL; diluted 1:450 vol/vol). Tissues were incubated overnight in the dark at 4°C while rocking. The secondary antibody incubation was removed, and the ovaries were rinsed 3x in 450 µl of sterile TBS per well, followed by washing 3x in 600 µl of sterile TBS for 30 min each at RT while rocking.

Ovary tissues were stained with 4′,6-diamidino-2-phenylindole (DAPI) to detect cell nuclei (N); 450 µl of 1 mM DAPI (catalog no. D3571; Molecular Probes, Inc., Eugene, OR) diluted 1:450 vol/vol in sterile TBS was added to each well. The ovaries were incubated for 30 min at RT while rocking. The DAPI solution was removed, and the ovaries were rinsed 3x in 450 µl of sterile TBS per well, followed by washing 3x in 600 µl of sterile TBS for 30 min each at RT while rocking. Tissues also were incubated using no primary antiserum with secondary antibody as a −/+ control or in the absence of both primary and secondary antiserum/antibody as a −/− control (Supp Fig. 1S [online only]).

Slide Preparation and Confocal Microscopy of Tissues

Fluorescent-labeled tissues were removed from the 24-well plates and transferred onto microscope slides with one pair of ovaries from each time point per slide. The slides were ‘dried’ by removing as much of the TBS as possible using micropipettes and KimWipes. Next, 15 µl of ProLong Gold Antifade Mountant (catalog no. P36930; Molecular Probes) was applied to the slide to cover the tissues, which were then covered with 10 mm #1.5 round glass coverslips (Ted Pella, Inc.). The tissues were cured flat for 24 h in the dark at RT prior to visualization. Each coverslip was sealed with clear nail polish.

Each tissue slide was imaged with the Zeiss LSM 510 Meta Confocal Microscope (Dublin, CA) at 20× and 40× in oil emersion at 488 nm. The software used to analyze images was Zeiss LSM Image Browser Version 4.2.0.121 and Fiji Version 1.52p (Schindelin et al. 2012). A minimum of five replicates for each time point per tissues was performed.

Results

Mosquito ovaries are complex tissues that change with time after a blood meal. Clements (1992) provide an extensive discussion of ovary tissues. Briefly, mosquito ovaries are composed of functional units called the ovarioles with tracheae that radiate from the calyx. An ovariolar sheath surrounds the ovary and connects with the lateral oviduct. Within the ovariole are primary (mature) and secondary follicles, and germarium where mitotic division occurs. Each follicle has one oocyte, seven nurse cells that synthesize ribosomes and mRNA for delivery to the oocyte, and an outer follicular epithelium. The nurse cells degenerate and are gone by 72 h PBM. To visualize the temporal deposition of iron in the ovarian tissues PBM, we compared control tissues with tissues stained with Prussian blue. Unstained Ae. aegypti ovarian tissues show no blue color (Fig. 1). Prussian blue staining shows that iron deposits are present in

![Fig. 1. Unstained and Prussian blue stained Aedes aegypti ovarian tissues. Females were maintained and fed, and ovaries were dissected prior to blood feeding (T0) and at 24 (T24) and 72 (T72) h PBM as described in Materials and Methods. Tissues were processed, and ferric was detected using fresh Prussian blue stain as described in Materials and Methods. Images were acquired at 10x and 40x as described in Materials and Methods. FE = follicular epithelium; G = germarium; NC = nurse cells; OLS = ovariolar sheath; OO = oocyte; OS = ovarian sheath; SF = secondary follicle; T = trachea.](image-url)
ovarian tissues prior to blood feeding (Fig. 1; T0) and localized in the ovariolar sheath that surrounds the ovary as well as in the ovarian sheath. By 24 h PBM, iron is detected in the secondary follicle (Fig. 1; T24). By 72 h PBM, iron appears primarily in the secondary follicle and follicular epithelium (Fig. 1; T72). In *Drosophila melanogaster* [Lancefield (Diptera: Drosophilidae)], the follicular epithelium is responsible for creating specific eggshell structures and establishing the ovarian follicle axes and patterning of the developing embryo (Jaglarz et al. 2008).

We expected that the iron present in the *Ae. aegypti* ovarian tissues would be associated with ferritin. Fluorescent immunohistochemistry (IHC) imaging shows that ferritin is differentially localized in the ovarian tissues. The ferritin HCH is localized primarily in the ovarian sheath, ovariolar sheath, follicular epithelium, and nurse cells prior to a blood meal (Fig. 2 and Supp Fig. 2S [online only], T0). At 24 h PBM, the HCH is still detected in the nurse cells and follicular epithelium (Fig. 2 and Supp Fig. 2S [online only], T24). By 72 h PBM, the HCH subunit is localized mainly in the follicular epithelium (Fig. 2 and Supp Fig. 2S [online only], T72).

Like HCH, LCH1 is detected in the ovarian sheath, ovariolar sheath, and nurse cells prior to blood feeding. It also shows diffuse fluorescent IHC staining in the oocyte (Fig. 3 and Supp Fig. 3S [online only], T0). By 24 h PBM, LCH1 is still detected in the oocyte and nurse cells and is present in the follicular epithelium, secondary follicles, and germarium (Fig. 3 and Supp Fig. 3S [online only], T24). By 72 h PBM, we continue to observe LCH1 primarily in the follicular epithelium and secondary follicle (Fig. 3 and Supp Fig. 3S [online only], T72).

Similar to LCH1, LCH2 is detected in the ovarian sheath, ovariolar sheath, and nurse cells, with diffuse fluorescent IHC staining in the oocyte prior to blood feeding (Fig. 4 and Supp Fig. 4S [online only], T0). Following a blood meal, LCH2 is detected in the follicular epithelium and secondary follicle at 24 and 72 h PBM (Fig. 4 and Supp Fig. 4S [online only], T24 and T72).

**Discussion**

We observed iron in the ovarian and ovariolar sheaths prior to blood feeding supporting that females retain iron in this tissue that is acquired in larval stages (Zhou et al. 2007). Iron was accumulated and differentially localized to the secondary follicles and follicular epithelium at 24 and 72 h PBM. These results, taken together with our previous work (Zhou et al. 2007, Geiser et al. 2017), indicate that meal iron is transported to and accumulates in the ovaries with time post-feeding and that the location of iron accretion in ovaries shifts during oogenesis implying that iron is important to this process in

![Confocal detection of the *Aedes aegypti* ferritin HCH subunit in ovaries. Females were maintained and fed, and ovaries were dissected prior to blood feeding (T0) and at 24 (T24) and 72 (T72) h PBM as described in Materials and Methods. Tissues were processed and the ferritin HCH subunit was detected using HCH-specific antiserum with Alexa Fluor 488 conjugate secondary (green), and cell nuclei were visualized with DAPI (blue) as described in Materials and Methods. Images were acquired at 20× and 40× as described in Materials and Methods. FE = follicular epithelium; N = nucleus; NC = nurse cells; OLS = ovariolar sheath; OO = oocyte; OS = ovarian sheath; T = trachea.](image-url)
these animals. The work of others has demonstrated that the regulation of intracellular iron is critical for optimal egg development in insects (Gill et al. 2014). Retention of iron in ovaries and the increase in iron and ferritin seen in ovarian tissues following a blood meal probably reflect the requirement for iron for the proliferation and differentiation of cells, and its involvement in many cellular processes including cellular respiration, DNA synthesis, and macromolecule biosynthesis (Heath et al. 2013). Changes in intracellular iron availability can have significant effects on cell division and mitogenesis (Heath et al. 2013). How iron is moved among the various ovarian tissues is unknown. It is probable that iron deposition and recovery from ferritin for use in various cellular and growth process involves chaperone proteins (Philpott and Jadhav 2019). Proteins that could serve as iron transporters in mosquitoes that are expressed in ovaries (Tsujimoto et al. 2018) and Malpighian tubules (Martinez-Barnetche et al. 2007) have recently been identified.

Because iron is potentially toxic by formation of free radicals through Fenton chemistry (Bou-Abdallah 2010), and iron is located in different areas of the ovary at various time points following a blood meal, we anticipated the presence of the ferritin in similar locations. Although iron deposition shows temporal variation, the regions where iron is present, the ovarian sheath, ovariolar sheath, follicular epithelium, and nurse cells are also regions where we detected the HCH, LCH1, and LCH2. It is worth noting that our antisera were previously shown to detect an epitope on the specific subunits (Geiser et al. 2017) whether it is detecting the assembled particle we cannot say, although it has been shown that Drosophila ferritin assembles in the Golgi apparatus (Missirlis et al. 2007) and fully assembled, iron-loaded ferritin has been previously detected the vacuolar system of insects (Locke 1991, Nichol and Locke 1999). When the particle is assembled, the ferritin heavy chain has a ferroxidase site that allows ferrous to ferric conversion and initiates the iron sequestering in the molecule, whereas the light chain imparts structural integrity and is thought to be predominate when longer-term iron storage is required (Lawson et al. 1989, Bertini et al. 2012, Bernacchioni et al. 2015). Prior to feeding, iron and all three ferritin subunits are localized in the ovarian and ovariolar sheaths and the nurse cells. These findings suggest that iron acquired prior to a blood meal can be stored in the sheaths and that these tissues could be the port of entry for ferritin delivering iron from the blood meal. We think it likely that the ferritin present in the nurse cells pre-blood meal reflects synthesis to prepare for the iron load delivered from the blood meal and could imply that these cells function to receive iron from hemolymph or sheath ferritin and could deliver it to the developing oocytes. It is worth noting that ferritin observed in ovarian tissues could come from the hemolymph, be synthesized within a tissue or be synthesized in one region and transported to another (Gonzalez-Morales et al. 2015). Because messages for all three subunits are present in ovarian tissues (Geiser et al. 2003, 2017), it is probable that ferritin comes from both hemolymph and within tissue synthesis.

**Fig. 3.** Confocal detection of the *Aedes aegypti* ferritin LCH1 subunit in ovaries. Females were maintained and fed, and ovaries were dissected prior to blood feeding (T0) and at 24 (T24) and 72 (T72) h PBM as described in Materials and Methods. Tissues were processed, and the ferritin LCH1 subunit was detected using LCH1-specific antiserum with Alexa Fluor 488 conjugate secondary (green), and cell nuclei were visualized with DAPI (blue) as described in Materials and Methods. Images were acquired at 20x and 40x as described in Materials and Methods. FE = follicular epithelium; G = germarium; N = nucleus; NC = nurse cells; OLS = ovariolar sheath; OO = oocyte; OS = ovarian sheath; SF = secondary follicle; T = trachea.
The HCH was detected in the follicular epithelium prior to feeding suggesting that it was produced in anticipation of the need for rapid sequestering of iron by this tissue. All three ferritin subunits are present at 24 and 72 h PBM, and iron accumulated in the follicular epithelium with time post-feeding. These observations support that iron from the meal is held here probably for transfer to the developing oocyte. Ferritin of the follicular epithelium protects the surrounding tissues and oocyte from an iron load. In eukaryotic tissues, ferritin serves as an iron depot, sequestering ferric iron as a mineral complex and thereby preventing radical formation, while allowing access to iron as needed by cells (Arosio et al. 2015). Ferritin probably serves a similar role in blood-feeding insects.

We observed only the HCH in the follicular epithelium prior to feeding, and only the LCH subunits were seen in the secondary follicles (24 and 72 h PBM) and the oocytes (prior to feeding). These findings agree with work in Drosophila that shows that a single ferritin subunit can be found in some tissues in the absence of iron stimulation (Mehta et al. 2009, Rosas-Arellano et al. 2016). Available evidence also supports that in insects the ferritin subunits are synthesized in the endoplasmic reticulum and separately targeted to different Golgi-like vesicles (HCH) or other vesicles (LCH) that with time following iron stimulation assemble to form the functional ferritin molecules (Nichol and Locke 1990, Locke et al. 1991, Rosas-Arellano et al. 2016). Both HCH and LCH subunits are required for functional particle formation. Iron loading occurs only with the assembled particle inside the Golgi-like vesicles and zinc-regulated and iron-regulated transporter 13 (Zip13) imports iron into the vesicles (Xiao et al. 2014, Rosas-Arellano et al. 2016, Tsujimoto et al. 2018). Iron-loaded ferritin is retained in a separate Golgi compartment in cells or secreted (Geiser et al. 2009). The importance and fate of the LCH subunits in the absence of the HCH remains to be evaluated. Active transport of ferritin subunits when necessary for the assembly of functional ferritin has been suggested (Rosas-Arellano et al. 2016).

Why mosquitoes express two light-chain subunits is unknown. Because mammals express a single light chain, isoforms of ferritin are determined by the numbers of each subunit (heavy or light chain) present in the molecules. Modeling of LCH2 indicated that it could substitute for the LCH1 in ferritin particles (Geiser et al. 2017). We observed that all three ferritin subunits are present in the nurse cells and in the follicular epithelium; this suggests that ferritin particles could contain HCH and either LCH1 or LCH2 or both and that isoforms of ferritin in mosquitoes could reflect the numbers of each type of light chain present. Work in Drosophila supports this notion as Rosas-Arellano et al. (2016) showed that ferritin particles that were iron loaded could be formed in vivo containing four different subunits. These authors also reported that iron loading is responsive to the type of subunits present in the particle (Rosas-Arellano et al. 2016). Although we do not yet know how the LCH2 influences the

Fig. 4. Confocal detection of the Aedes aegypti ferritin LCH2 subunit in ovaries. Females were maintained and fed, and ovaries were dissected prior to blood feeding (T0) and at 24 (T24) and 72 (T72) h PBM as described in Materials and Methods. Tissues were processed, and the ferritin LCH2 subunit was detected using LCH2-specific antiserum with Alexa Fluor 488 conjugate secondary (green), and cell nuclei were visualized with DAPI (blue) as described in Materials and Methods. Images were acquired at 20× and 40× as described in Materials and Methods. FE = follicular epithelium; N = nucleus; NC = nurse cells; OLS = ovarian sheath; OO = oocyte; OS = ovarian sheath; SF = secondary follicle.
structure of the ferritin molecule, we hypothesize that it might be present in the particle when it is desirable to hold iron yet make it readily available. Our recent work indicates that, in contrast to other ferritin subunits, LCH2 expression is not increased by iron exposure, but is responsive to development signals as the message levels of LCH2 in ovaries increase dramatically 72 h PBIM (Geiser et al. 2017). Our observations here suggest that LCH1 is the primary subunit found in oocytes; quite possibly, this could imply that LCH1 is particularly important in oocyte development for long-term storage of iron in eggs.

We were surprised that we did not see ferritin and iron in eggs at 72 h PBIM. Possibly the volume of the oocyte might limit ferritin and iron detection due to dilution. Our previous work established that more than half of the iron delivered to ovaries is recovered in eggs (ng levels) and that iron in eggs is stored in ferritin (Zhou et al. 2007). Our work relied on detection of iron and ferritin using Fe59, iron ICP-MS, and western blotting. Perhaps our staining methods were not sufficiently sensitive to show iron and ferritin in the eggs. One molecule of ferritin can store up to 4,500 atoms of iron. Recent work in ants indicates that insect ferritin is loaded with higher amounts of iron than that anticipated for mammalian ferritins (Wajnberg et al. 2018). Alternatively, ferritin particles could form in the oocyte in such a way that the subunit epitopes for our antisera are masked. It is also possible that iron or iron-loaded ferritin might be transferred to the oocyte before chorion formation is complete.

In summary, we observed that iron from the blood meal is increased in ovaries with time post-feeding and that ferritin is found where ferric iron is observed. The changes seen in iron location over-time PBIM are consistent with the changes in the ovarian follicle anatomy during oogenesis, and support that iron plays a role in the process of oogenesis. Female mosquitoes require essential nutrients in a blood meal in order for egg development to occur (Uchida et al. 2001, Geiser et al. 2007). The work of others has demonstrated that in insects the ovaries take up nutrients and proteins during egg development (Anderson and Spielman 1971, Ziegler and Van Antwerpen 2001, Geiser et al. 2007). The importance of eukaryotic ferritins in iron handling and cytoprotection. Biochem. J. 472: 1–15.

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