The role of Anopheles gambiae vitellogenin in nutrient transport, fertility and Plasmodium falciparum development

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The role of *Anopheles gambiae* vitellogenin in nutrient transport, fertility and *Plasmodium falciparum* development

A dissertation presented

by

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Abstract

The female *Anopheles gambiae* mosquito, which is the main vector of the human malaria parasite *Plasmodium falciparum*, feeds on blood to develop eggs. After ingesting a bloodmeal, she produces a yolk precursor protein called vitellogenin (Vg) and deposits it into the oocyte for amino acid needs of the embryo. This dissertation shows that vitellogenin is essential to *An. gambiae* reproduction, as its depletion results in complete infertility. *Vg* silencing causes major dysregulation of amino acid and protein deposition in eggs, leading to upregulation of TOR signaling. In turn, such upregulation leads to enhanced transcription of the lipid transporter *lipophorin* (*Lp*), inducing excessive triglyceride deposition into the ovaries. Embryonic lethality occurs early during development and is likely caused by severe amino acid deprivation. Vg depletion in infected females also leads to accelerated rates of *P. falciparum* development, most likely mediated by both increased amino acid levels and Lp-transported lipids, so that mosquitoes become infectious to humans in a shorter period of time. Combined, this work reveals previously unknown dynamics between two major mosquito nutrient transporters, and shows that although Vg is an attractive target to induce sterility in field populations, preventing its expression would potentially lead to more effective transmission of malaria parasites.

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1
Introduction

Overview of *Anopheles gambiae* reproductive biology

Anopheline mosquitoes belong to the order Diptera, and contain 465 species, 70 of which are capable of transmitting *Plasmodium* parasites, the causative agents of malaria (Sinka et al. 2012). This thesis focuses on *Anopheles gambiae* mosquitoes, which are one of the deadliest species due to their efficiency of malaria transmission (Coetzee 2004; Kiszewski et al. 2004). These mosquitoes are anautogenous, meaning that the females need to consume a blood meal to obtain enough nutrients for egg development, which results in an opportunity for pathogen dissemination (Attardo, Hansen, and Raikhel 2005). The mechanisms of mosquito reproductive biology have been largely elucidated in *Aedes aegypti*, historically due to ease of rearing and their importance as arbovirus vectors. However, anophelines diverged from the lineage containing *Aedes* and *Culex* mosquitoes about 217 million years ago (Shaw, Marcenac, and Catteruccia 2022), and therefore their reproductive biology may differ from *An. gambiae*.

When a female blood feeds, the blood goes to the midgut, where it is digested by a host of enzymes (Gooding 1966; Billingsley and Hecker 1991; Vizioli et al. 2001). Nutrients extracted from the blood meal are transported into the hemolymph (open circulatory system) and then to the other tissues. Blood meal ingestion results in production of ovarian ecdysiotropic hormone (OEH) and insulin-like peptides (ILPs) in the brain (Klowden 1987; Sharma, Nuss, and Gulia-Nuss 2019). OEH and ILP signaling then lead to production of the steroid hormone precursor ecdysone in the mosquito ovarian epithelium, which travels to the fat body where it is hydroxylated to 20 hydroxyecdysone (20E) (Hagedorn et al. 1975; M R Brown et al. 1998; Mark R Brown et al. 2008). The mosquito fat body is a storage and endocrine organ that is most similar to a mammalian liver and adipose tissue. In the fat body, 20E binds its heterodimeric nuclear receptor EcR and USP, and regulates many reproductive genes, including lipophorin (Lp) and vitellogenin (Vg), which

transport lipids and proteins to the ovaries respectively (Werling et al. 2019; Ekoka et al. 2021). Of note, "transporter" in this thesis is utilized to describe a protein that travels between tissues to transport lipids or proteins, rather than a transmembrane transporter protein. The cascade following blood meal is summarized in Figure 1.1.

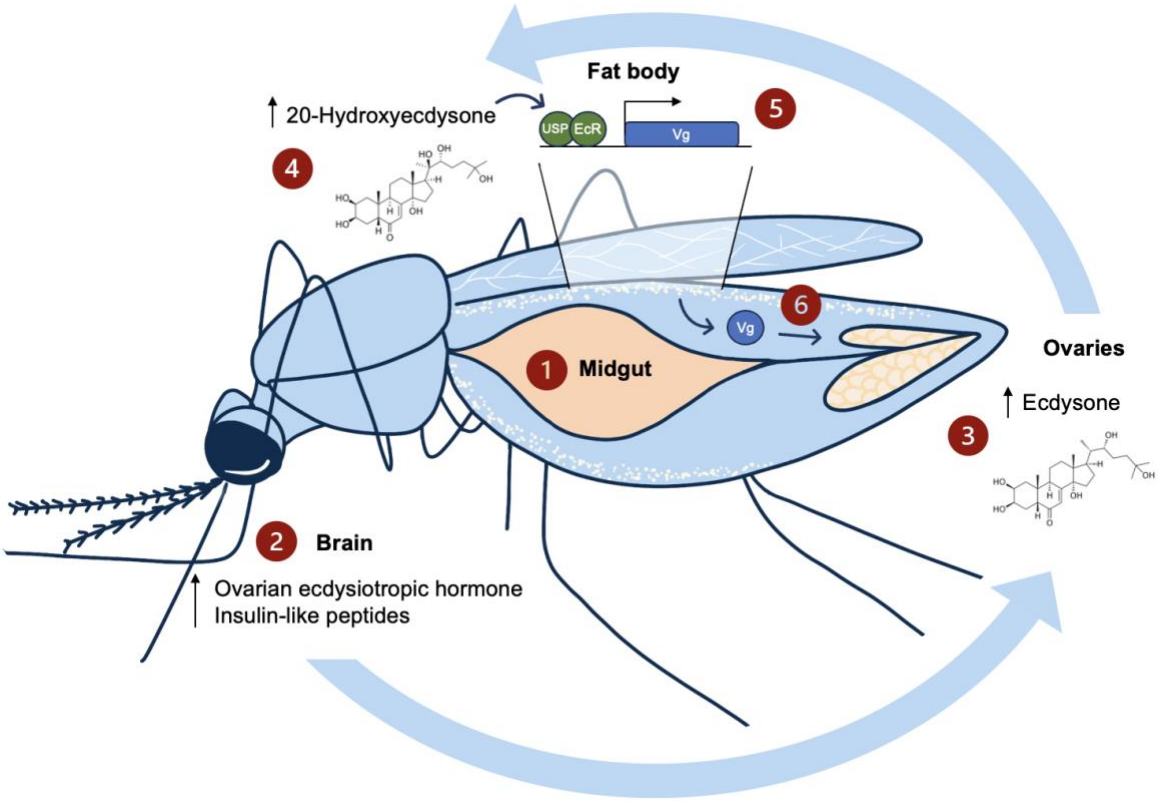

Figure 1.1. Molecular cascade following a blood meal. (1) The midgut is filled upon blood feeding. (2) Ovarian ecdysiotropic hormone (OEH) and insulin-like peptides (ILPs) are produced in the brain. (3) Ecdysone (E) is produced in the ovaries stimulated by OEH and ILPs. (4) In the fat body, E is converted to 20-hydroxyecdysone (20E). (5) 20E binds its heterodimeric receptor EcR and USP, which then turn on *Vg* transcription. (6) Vg is released from the fat body to be internalized by the ovary.

The ovaries contain many developing follicles, each composed of an oocyte, seven nurse cells and a follicular epithelium (Clements 1992). The oocytes are loaded with proteins and lipids from the hemolymph as well as ribosomes and mRNAs from the nurse cells. Egg development is complete within 2-3 days upon blood feeding. If the female is mated, she will oviposit the eggs (about 70-100), into water, at which point she is ready to take another blood meal. During

oviposition, sperm, which is stored in the female spermathecae after mating, is used by the female to fertilize the eggs as she lays them. In the embryo, the male pronucleus will fuse with the female pronucleus, which will then undergo multiple rounds of mitotic division. When the number of nuclei reaches 128, they migrate to the egg surface and line the inside of the eggshell forming a syncytial blastoderm with 3,200 nuclei at around 4h post oviposition. Cellular membranes form around the nuclei, resulting in a cellular blastoderm around 5h post oviposition. Over the next 40 hours the embryo undergoes differentiation, forming tissues, segmenting the body and depositing the cuticle. After about 2 days of development a first instar larva hatches from the embryo. Larvae undergo four rounds of molting, guided by 20E, until they become pupae. After one day, pupae will eclose into adults, which will blood feed within a few days after eclosion if they can find a host.

Vitellogenin as a yolk protein precursor

Vitellogenin (Vg) is a yolk protein precursor present in all oviparous species such as birds, fish, reptiles, monotremes and insects (Sappington and Raikhel 1998). In vertebrates it is produced in the liver while in insects it is produced in the fat body. Vg functions as the source of amino acids for the embryo during development. Mammals do not express Vg, but they have three non-functional pseudogenes (Brawand, Wahli, and Kaessmann 2008). It is thought that the evolution of lactation as an alternative nutrition source for the offspring has released evolutionary pressures from Vg, resulting in mutations. Indeed, monotremes, egg-laying mammals such as platypuses and echidnas, express one of their three Vgs and their eggs contain yolk; however, the yolk is underdeveloped compared to other egg-laying species with complete Vg expression.

Vitellogeninins are large glycolipophosphoproteins, about 200 kDa in size (Sappington and Raikhel 1998). They belong to the lipid transfer protein superfamily, and contain three conserved domains: lipid binding domain (LPD_N), a domain of unknown function (DUF1943), and a von Willebrand factor type D domain (VWD) (Hu et al. 2019; Tufail and Takeda 2009). The protein is translated in the fat body following a blood meal, cleaved at a site between two polyserine stretches into two subunits (~190 kDa and ~60 kDa in *Ae. aegypti*) and released as a complex of 400-700 kDa into the hemolymph (Sappington and Raikhel 1998; Swevers et al. 2005; Dhadialla and Raikhel 1990). The *An. gambiae* Vg (*Ag*Vg) is 2,051 amino acids in length (Vectorbase – AGAP004203), contains the three putative domains outlined above, as well as the putative cleavage site between two polyserine stretches (Uniprot – Q9NAW9; Pfam) (Tufail and Takeda 2009; Hu et al. 2019). In the hemolymph *Ag*Vg has been identified by denaturing Western blot to be above 170 kDa and below 250 kDa but no further biochemical characterization has been carried out (Rono et al. 2010). *An. gambiae* expresses two other proteins that are identical truncated versions of Vg – AGAP013109 (815amino acids spanning a portion of the LPD_N and VWN domains) and AGAP013503 (212 amino acids spanning a portion of the LPD_N domain). This thesis focuses on the longest Vg protein, or the major yolk protein (Rono et al. 2010), since it is most highly expressed (unpublished RNA sequencing) and since the other two share identical sequences but do not have all functional domains.

The structure of silver lamprey Vg was derived in 2002, yielding a molecule that shows a barrel for lipid storage. Indeed, Vg contains phospholipids and hydrocarbons within the binding cavity (Thompson and Banaszak 2002). The predicted structure of *Ag*Vg, generated by Phyre 2 using the silver lamprey Vg structure as a template, is displayed in Figure 1.2. Lipids make up 8-15% of Vg, the major types carried being phospholipids, diglycerides and hydrocarbons (Maruta

et al. 2002; Swevers et al. 2005; A S Raikhel and Dhadialla 1992). Overall, Vg is estimated to contribute 5% of lipid to the eggs, while lipophorin (Lp, the major lipid transporter, discussed below) contributes 95% (Kawooya and Law 1988).

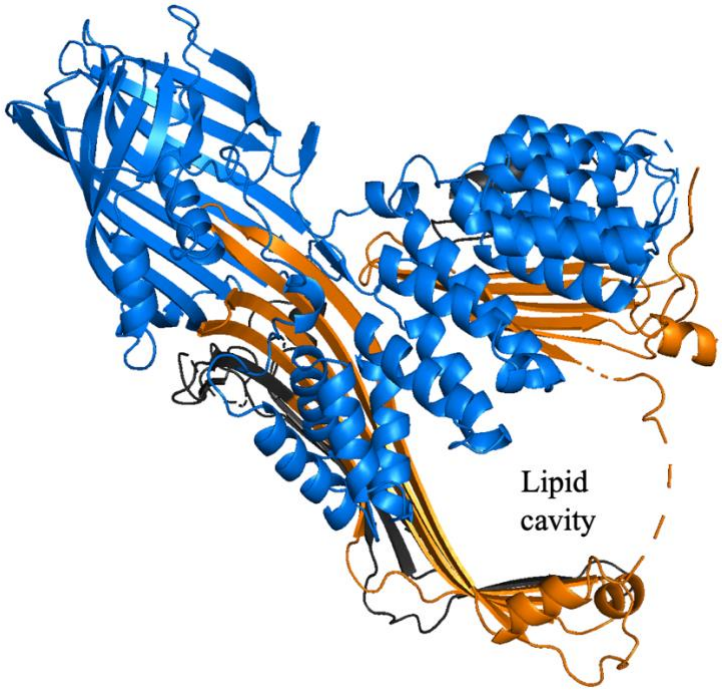

Figure 1.2. Predicted structure of *An. gambiae* Vg. The LPD_N domain is in blue while the DUF1943 domain is in orange. The VWN domain was not included in the predicted structure. Lipids are predicted to reside in the circular barrel facing the reader. This predicted structure was generated using Phyre2 because *An. gambiae* Vg is not currently available on AlphaFold.

In mosquitoes, Vg is produced in the fat body after blood meal with mRNA levels peaking at 24h post blood feeding (PBF), returning to unfed levels by 48h PBM (Rono et al. 2010). This protein travels through the hemolymph to the ovaries where it passes between follicular epithelial cells surrounding the oocyte, and is then internalized into the oocyte through receptor-mediated endocytosis utilizing the vitellogenin receptor (VgR) (Snigirevskaya, Sappington, and Raikhel 1997; A S Raikhel and Dhadialla 1992; Sappington et al. 1996). In fact, clathrin-mediated endocytosis was first observed in *Ae. aegypti* ovaries upon Vg internalization by the oocyte (ROTH and PORTER 1964). In the ovaries, Vg is crystallized into yolk granules, at which point it is called

vitellin (Vn) (Figure 1.3) (A S Raikhel and Dhadialla 1992). Many yolk granules are evenly distributed throughout the developing egg, unlike a single yolk in bird eggs. As the embryo develops, proteases such as vitellogenic carboxypeptidase and vitellogenic cathepsin B process Vg into amino acids for the nutritional needs of the embryo (Cho et al. 1999; Snigirevskaya, Hays, and Raikhel 1997).

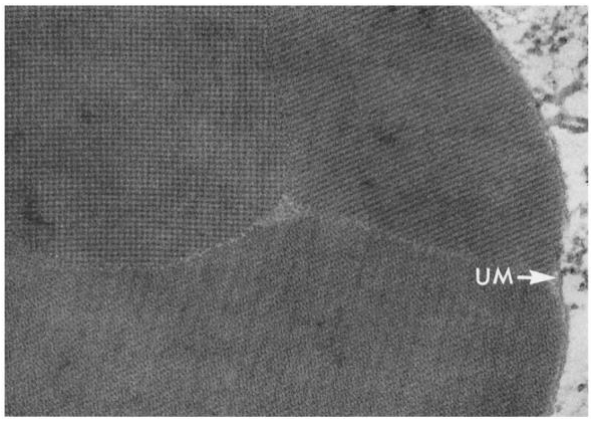

Figure 1.3. *Ae. aegypti* yolk granule containing crystallized Vg. The yolk granule was visualized by electron microscopy (from Roth and Porter, 1964). UM is a unit membrane around the yolk granule.

Vg is a significant nutritional investment made by the female. Upon blood feeding, *Vg* is upregulated several hundred fold and, along with other yolk proteins, accounts for a third of all mRNAs expressed by the fat body at 24h PBF (I. Hansen et al. 2014). Yolk production is estimated to utilize up to 30% of the total protein content from the blood meal (Briegel 1990), and Vg comprises 60-90% of proteins in the egg yolk (Hagedorn and Kunkel 1979). Thus, it is not surprising that Vg is an important player in the fecundity and fertility of many insect species. For example, in *Drosophila melanogaster* decreasing *Vg* gene copy number results in reduced fertility and fecundity (Mary Bownes, Lineruth, and Mauchline 1991), and multiple sterile yolkless mutants have been identified by impairing Vg secretion or internalization by the oocyte (DiMario and Mahowald 1987; Gutzeit and Arendt 1994; Williams et al. 1987), although the mechanisms of

infertility have not been elucidated. Altering Vg expression or internalization also leads to decreased egg numbers and decreased hatching rate in *Bombyx mori*, *Blatella germanica*, *Plutella xylostella*, *Aphis (Toxoptera) citricidus* and *Haemaphysalis longicornis* (Lin et al. 2013; Ciudad, Piulachs, and Bellés 2006; Peng et al. 2020; Shang et al. 2018; Mitchell III et al. 2007).

Other functions of Vg

Although Vg's main function is its nutritional value for the embryo, it has been shown to be involved in other processes such as ecdysteroid storage, antioxidant responses, and pathogen recognition. In locust, fruit fly and silkworm species, ecdysteroids are complexed with vitellin within the egg (Lagueux et al., 1981; Bownes et al., 1988; Yamada et al., 2005), which as mentioned above is the crystallized form of Vg within yolk granules. As vitellin is broken down by the embryo as a source of amino acids, 20E is released from the vitellin crystals and begins to regulate organ formation and cuticle deposition (Chavoshi et al., 2010; Kozlova & Thummel, 2003).

In honeybees Vg is associated with increased longevity due to its antioxidant properties (Seehuus et al. 2006; Corona et al. 2007). Indeed, *Vg* knockdown leads to lower survival when honeybees are treated with the potent oxidant paraquat (Seehuus et al. 2006). Vg is oxidized more strongly than other proteins, and it protects cells by directly shielding them from reactive oxygen species damage (H. G. Park et al. 2018; Havukainen et al. 2013). This property is of especial interest because *Vg* is also expressed in the *An. gambiae* female spermatheca after mating, which is an organ dedicated to long-term sperm storage since the female mates only once in her lifetime (Rogers et al. 2008).

Vg also plays important immune roles in many insects and fish. For example, zebrafish Vg binds to and inhibits growth of *Escherichia coli* and *Staphylococcus aureus* (Tong et al. 2010), and acts as an opsonin by recruiting macrophages (Zhang, Dong, and Cui 2015); silkworm Vg has strong antibacterial effects against *E. coli* and *Bacillus subtilis* (Singh et al. 2013); and honeybee Vg can bind bacterial pathogen-associated molecular patterns (PAMPs) and carry them into developing eggs potentially to "prime" the immune system of the offspring (Salmela, Amdam, and Freitak 2015). Importantly, Vg is one of the most abundant proteins in the hemolymph of the female *An. gambiae* mosquito after blood feeding as visualized by Coomassie staining (Rono et al. 2010), meaning that it is in an excellent position to recognize and respond to infectious agents.

Vitellogenin regulation in mosquitoes

In mosquitoes, as shown by experiments done in *Ae. aegypti*, *Vg* transcription is regulated by a complex combination of juvenile hormone (JH), 20E, ILPs and amino acid signaling (Attardo, Hansen, and Raikhel 2005; I. Hansen et al. 2014). Hormonal regulation plays an important role: indeed, knockdown of the JH receptor Methoprene-tolerant (*Met*) almost completely eliminates *Vg* expression (Wang et al. 2017), while injections of the steroid hormone 20E into mosquitoes and addition of 20E into fat body culture are both sufficient to stimulate Vg synthesis (Fallon et al. 1974), as expected given that the *Vg* promoter contains binding sites for the 20E heterodimeric receptor (EcR and USP) as well as for transcription factors activated by 20E (E74 and E75) (Alexander S Raikhel et al. 2002). ILPs have been shown to have a synergistic effect with 20E on *Vg* expression (Roy, Hansen, and Raikhel 2007), and knockdown of *FOXO*, the downstream effector of ILP signaling which has a binding site in the *Vg* promoter, results in decreased expression (I. A. Hansen et al. 2007).

Finally, amino acid signaling is also involved in *Vg* regulation. Specifically, *ex vivo* fat body culture lacking amino acids but containing 20E could not stimulate *Vg* expression, which was rescued by amino acids supplementation (I. A. Hansen et al. 2004). Amino acid signaling was dependent upon target of rapamycin (TOR) since treatment with the TOR inhibitor rapamycin or *TOR* dsRNA reduced *Vg* mRNA levels. TOR is a central kinase in cell biology pathways which integrates information on nutrient availability, such as amino acid levels, and signals through phosphorylation of downstream effectors to regulate translation and transcription resulting in growth, proliferation and autophagy (Valvezan and Manning 2019). This effect on *Vg* expression is mediated by a GATA transcription factor, which binds the *Vg* promoter and is a downstream effector of TOR signaling (J.-H. Park et al. 2006). TOR depletion also results in fewer eggs being produced by *Ae. aegypti* females as well as a decreased hatching rate (I. A. Hansen et al. 2004).

The four pathways outlined above do not function in isolation. Instead, as shown in *Ae. aegypti,* they work in unison, each regulating the other to control *Vg* transcription. For example, application of JH results in increased responsiveness of *Vg* expression to 20E (FLANAGAN and HAGEDORN 1977). JH and 20E regulate *ILP* expression (Ling and Raikhel 2021), which is also regulated by amino acid signaling along the TOR/GATA axis (Ling and Raikhel 2023). Conversely, ILPs stimulate ecdysone synthesis (Mark R Brown et al. 2008), and feed into TOR signaling to stimulate JH production (Pérez-Hedo, Rivera-Perez, and Noriega 2013).

Lipophorin as a lipid transporter

While Vg transports some lipids to the developing eggs, this role is mainly carried out by lipophorin (Lp), the major lipid transporter in insects (Gilbert and Chino 1974; Canavoso et al. 2001). Like Vg, Lp is also expressed in the fat body, as a large polypeptide that is then cleaved

intro apoI (~280 kDa) and apoII (~80 kDa) (Osvaldo Marinotti et al. 2006). Both apoI and apoII associate with lipid particles in the hemolymph, resulting in globular lipoparticles with a diameter of 13-16 nm (Chino and Downer 1982). Lipids are loaded into Lp with the help of the lipid transfer particle (LTP) at the sites of storage or absorption such as fat body or the midgut and then transported through the hemolymph to other tissues such as flight muscles and the ovaries, and structural components such as cuticle (Chino and Downer 1982; Kawooya and Law 1988; Van der Horst 1990; Arrese et al. 2001). At the tissues of delivery, Lp binds its receptor (LpR) and either deposits its cargo and is recycled back into the hemolymph or is internalized by the tissue, as observed in oocytes where it is deposited into yolk granules (Kawooya and Law 1988; Sun et al. 2000; Atella et al. 2006; Tufail and Takeda 2009).

Lipid particles transported by Lp are composed of neutral lipids, phospholipids and cholesterol. The particles contain apolipoproteins and phospholipids on the outer surface while the cores are composed of hydrocarbons (Soulages and Wells 1994). Mosquito Lp was found to transport triglycerides in *Ae. aegypti*, *Anopheles albimanus* and *Culex quinquefasciatus*, which was a surprising finding since in all other insects studied to date Lp transports diglycerides (Ford and Van Heusden 1994). The *An. gambiae* Lp complex is composed of 50% protein, 48% lipid and 2% carbohydrate (Atella et al. 2006). The Lp-transported lipids were identified to be hydrocarbons, diglycerides and triglycerides, cholesterol and cholesteryl ester, as well as phospholipids such as phosphatidyl choline and phosphatidyl ethanolamine (Atella et al. 2006).

The regulation of *Lp* expression in the mosquito remains to be fully elucidated. *Lp* is transcribed at low levels in the fat body of the mosquito pre-blood meal (Sun et al. 2000; Werling et al. 2019). Upon blood feeding, there is an increase in *Lp* mRNA and protein levels that peak around 12-18h PBM and then go down to pre-blood feeding levels at later time points. A study in

Ae. aegypti has suggested that *Lp* is upregulated by 20E, with higher mRNA and protein expression observed upon addition of increasing 20E concentrations to *ex vivo* cultures of dissected fat bodies. In *An. gambiae* 20E signalling seems to exert the opposite effect, since the knockdown of the 20E receptor *EcR* does not decrease *Lp* levels but rather results in their increase compared to controls following the peak at 12h PBM (Werling et al. 2019), causing *Lp* mRNA levels to remain high rather than returning to their pre-blood meal values. Interestingly, the *Lp* promoter contains important transcription factor binding sites such as GATA motifs and ecdysone-responsive elements, consistent with regulation by this steroid hormone (Osvaldo Marinotti et al. 2006).

Due to its essential role in lipid transport, *Lp* knockdown by dsRNA results in severely reduced oogenesis in *An. gambiae*, with Lp-depleted females developing about 0-10% of the number of eggs developed by control mosquitoes (Vlachou et al. 2005; Rono et al. 2010; Werling et al. 2019).

Mosquito stages of the *Plasmodium falciparum* lifecycle

Malaria remains a devastating disease, causing 247 million cases, and 619,000 deaths in 2021 (*World Malaria Report 2022*). Due to a breakdown in supply chains because of the COVID pandemic, malaria control took a hard hit, with cases and deaths rising in 2020, and remaining high in 2021. The vast majority of the cases, 95%, is borne by the African region, and the death burden is disproportionately shouldered by children under 5 years old. Malaria is the 6th leading cause of death in low-income countries and remains a major public health concern (WHO).

Plasmodium falciparum is the most prevalent and the deadliest malaria parasite accounting for 99.7% of all of the cases in the African region in 2018 (WHO 2019). The parasite is transmitted by mosquitoes from the *Anopheles* genus, which contains about 70 competent species (Sinka et al.

2012). As explained above, females of *Anopheles gambiae* species complex, arguably the most efficient malaria vectors (Coetzee 2004; Kiszewski et al. 2004), must blood feed to ingest the nutrients needed for egg development, and if they ingest blood of a person infected with a *Plasmodium* parasite, they will become infectious in about two weeks. It is estimated that anophelines have been transmitting ancestral forms of *P. falciparum* for 13-64 million years, with a single transfer event from gorilla *P. praefalciparum* to humans being responsible for the origin of *P. falciparum* 10,000-100,000 years ago (Ricklefs and Outlaw 2010; Silva et al. 2015; Liu et al. 2010). This long evolutionary history has allowed the parasite to shield itself from the mosquito immune system while being minimally taxing on her nutritional resources (discussed later) (Shaw, Marcenac, and Catteruccia 2022). As the two processes of mosquito egg and *Plasmodium* development are kicked off by the same blood feeding event, they happen concurrently in different tissues (Figure 1.4).

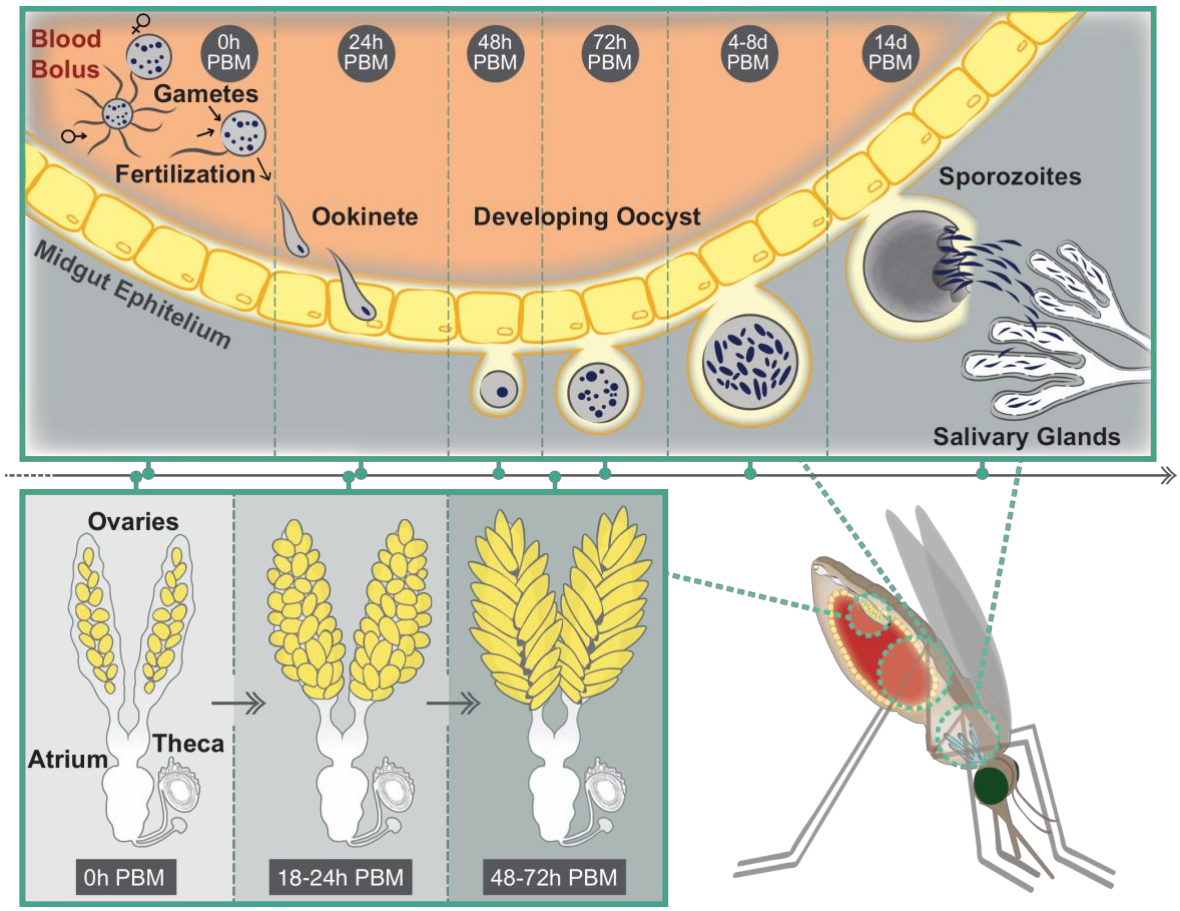

Figure 1.4. *P. falciparum* development within the mosquito midgut is temporally linked to oocyte development within the ovary. A female mosquito takes an infected blood meal, resulting in parasite gametocytes being ingested into her midgut. Following fertilization, the parasite develops into an ookinete that crosses the midgut epithelium and transforms into an oocyst. The oocyst grows and divides, eventually bursting to release thousands of sporozoites that travel to the mosquito salivary glands, at which point the mosquito becomes infectious. During the initial phases of parasite development, the mosquito is depositing nutrients into her ovaries, developing 70-100 eggs with each blood meal (Graphic by Manuela Bernardi).

The mosquito stages of the parasite life cycle begin when the female mosquito ingests gametocytes with an infected individual's blood. Within the midgut, gametocytes develop into gametes due to a drop in temperature and presence of xanthurenic acid (Billker et al. 1997; 1998). The male and female gametes undergo fertilization, forming a zygote which then develops into an ookinete (Baton and Ranford-Cartwright 2005). The ookinete crosses the midgut epithelium by traversing midgut cells from their apical to basal end, and it establishes itself beneath the basal

lamina of the midgut. There, it transforms into an oocyst, which grows dramatically within the lamina for the next 7-10 days. During this time, sporozoites develop within the oocyst undergoing nuclear division and membrane formation (Sinden and Strong 1978). This extraordinary growth process requires nutrients that the parasite scavenges from the female, and from any of her subsequent blood meals (Shaw et al. 2020). The oocyst is in direct contact with the midgut, while also being bathed by the mosquito hemolymph. At day 10-14 post infection, the oocyst releases sporozoites that travel through the hemolymph and invade the salivary glands of the female, making her infectious for the rest of her lifetime if she ingests another blood meal (Sultan et al. 1997; Graumans et al. 2020).

Mosquito antiparasitic immunity

The mosquito has evolved multiple immune mechanisms to protect itself from parasitic pathogens. When faced with mouse *Plasmodium berghei* parasites the mosquito utilizes a slew of proteins to melanize and kill the invader (Cirimotich et al. 2010). Specifically, leucine-rich repeat proteins LRIM1 and APL1C form a complex that is secreted into the hemolymph and interacts with a complement-like protein TEP1 (Povelones et al. 2009; Fraiture et al. 2009). TEP1 then binds the parasite, which underwent nitration in the midgut, and targets it for degradation (Oliveira, Lieberman, and Barillas-Mury 2012). However, the *P. falciparum* human parasites have evolved to avoid the mosquito complement-like response via the expression of Pfs47 on their surface, which suppresses the midgut nitration response (Molina-Cruz et al. 2013). Specifically, Pfs47 binds to a midgut receptor, P47Rec, and this interaction is necessary for immune evasion (Molina-Cruz et al. 2020). Different haplotypes of Pfs47 are expressed on *P. falciparum* parasites from different regions, and result in best protection from mosquitoes that form natural pairings with the

parasite, further showing that the parasite has evolved to evade the mosquito's immune system (Molina-Cruz et al. 2023; 2015).

However, the mosquito has not, in turn, evolved new methods of eliminating the *P. falciparum* parasite as the Red Queen Hypothesis would suppose. This is because a natural parasite pairing does not impose a mortality or reproductive cost unto the mosquito (Ferguson and Read 2002; Werling et al. 2019; Marcenac et al. 2020). Infected females do not die earlier, and they develop as many eggs as their uninfected counterparts. Indeed, there is actually a positive correlation between egg and parasite numbers further highlighting that an increased parasite load does not result in lower reproductive output (Werling et al. 2019). These data show that females that invest more in reproduction actually have higher parasite loads, which is a finding that persists in field mosquito experiments (Marcenac et al. 2020). The mechanism of this positive correlation has not been discovered but it seems to be regulated by 20E signaling during the oocyst-to-ookinete transition (Werling et al. 2019).

Oocyst growth

As the oocyst grows under the basal lamina of the mosquito it utilizes nutrients derived from the blood meal, the mosquito and from *de novo* synthesis. While the parasite's full nutritional requirements during this stage remain to be fully characterized, previous work highlights the importance of protein and lipids during this parasitic developmental stage. A second blood feeding provided to mosquitoes that are already infected results in faster parasite growth, increasing oocyst size quite substantially and resulting in earlier sporozoite invasion of salivary glands. The nutritional factors causing such rapid growth have not yet been identified, however evidence from some studies suggests these effects may be caused by proteins rather than lipids (Shaw et al. 2020;

Kwon et al. 2021). Indeed, when mosquitoes were fed with a BSA meal, parasite size was increased to levels that were comparable to a second blood meal (Kwon et al. 2021), while silencing the lipid transporter *Lp* did not affect oocyst size after the second blood meal (Shaw et al. 2020). However, lipids do play a role in oocyst growth. *P. falciparum* cannot undergo sporogony when its FASII pathway, which functions in lipid synthesis, is impaired (van Schaijk et al. 2014). Furthermore, *P. falciparum* upregulates *An. gambiae* adipokinetic hormones, which mobilize lipid reserves, and the knockdown of one of the hormones reduced sporozoite production (Nyasembe et al. 2023). Arguably, the parasite's lipid requirements have been best studied in relation to the Lp lipid transporter. Oocysts internalize Lp, and some studies have shown that *Lp* knockdown induces a decrease in oocyst size (Atella et al. 2009; Costa et al. 2018; Rono et al. 2010). However, these findings have not been confirmed by other studies where *Lp* silencing did not affect oocyst growth in normal conditions, although when oocyst growth was accelerated by the availability of extra mosquito nutritional resources, this enhanced growth could be attributed to Lp (Werling et al. 2019). All together, these studies suggest that the use of mosquito lipids by parasites is condition-dependent, and the role of lipids in oocyst growth still remains to be full ascertained.

The parasite appears to utilize the same nutrients that the mosquito uses to develop her eggs. Indeed, inhibiting *An. gambiae* reproductive processes, such as through knockout of a regulator of germ cell development (*ZPG*), enzymatic inhibition of the hormone 20E, or knockdown of 20E receptor *EcR*, all result in faster parasite growth (Werling et al. 2019; Shaw et al. 2020). Oocyst size and egg number are also negatively correlated (Werling et al. 2019), meaning that when a female invests highly into her reproduction, fewer nutritional resources can be utilized by the parasite, leading to slower growth rates. Although the parasite utilizes the same nutrients for growth as do reproductive processes, it does not interfere with egg development, since oocyst

growth starts at a time (2d post infection) when the female has fully developed her batch of eggs (Costa et al. 2018; Shaw, Marcenac, and Catteruccia 2022). Thus, parasites utilize leftover nutrients after egg development, and they can benefit and speed up their development if there is higher nutrient availability, as in the examples of second blood meal or lower reproductive investment.

Vectorial capacity as a framework for vector control

All of the considerations discussed above can be summarized with the vectorial capacity equation, which estimates the ability of a vector to transmit a pathogen (Brady et al. 2016; Shaw and Catteruccia 2019).

$$Vectorial\ capacity = \frac{ma^2c(p)^n}{-\ln(p)}$$

where:

m = vector density, or how much of a vector there is in a region

a = human biting rate, or how attracted a vector is to humans

c = competence, or whether the vector can support pathogen development

p = survival, or how long a vector will live

n = the number of days the pathogen needs to develop

Each factor in the vectorial capacity equation affects malaria transmission, and some are targeted by current vector interventions. Insecticide-treated bed nets, the most important tool to prevent malaria, both reduce mosquito densities and decrease the human biting rates, as they prevent contact between the mosquito and its human host. A crucial factor in transmission is the number of days the parasite needs to develop in the mosquito before it becomes infectious, also

referred to as the external incubation period (EIP). The longer the EIP, the less likely will parasites be to be transmitted, since the pathogen will only be successfully transmitted if the vector survives past the EIP. Thus, a parasite's ability to develop faster is beneficial to its spread and detrimental to control methods.

This dissertation focuses on mosquito reproduction and on pathogen development, thus providing insights into two key components of the vectorial capacity of *An. gambiae* mosquitoes as vectors of *P. falciparum* parasites.

Unanswered questions

There remain several unanswered questions in *An. gambiae* reproductive biology and *P. falciparum* development. Firstly, the function of Vg in fertility has not been assessed in *An. gambiae*, and any embryonic phenotypes upon *Vg* silencing in mothers have not been characterized. Secondly, many questions remain about how the two nutrient transporters, Vg and Lp, interact to coordinate the process leading to successful oogenesis. As *Lp* is downregulated around 24h PBM, *Vg* reaches its peak at the same time point, so that in the developing ovaries the initial lipid incorporation phase is followed by a protein deposition phase (Rono et al. 2010; Werling et al. 2019). How does a female regulate the import of lipids into oocytes to avoid toxicity caused by excess lipid accumulation and allow for the correct amount of protein to be deposited? Furthermore, Vg is one of the most upregulated proteins upon blood feeding in the mosquito, and up to 30% of the protein derived from the blood meal is estimated to be destined to Vg synthesis (Briegel 1990; O Marinotti et al. 2006). With this huge nutritional investment of the female, what metabolic effects does Vg production have?

Lastly, Vg does not only play a nutritional role, but also an immune one as well, and it is transported to the ovaries via the hemolymph that bathes the oocysts during their growth (Tong et al. 2010; Singh et al. 2013; Salmela, Amdam, and Freitak 2015). *P. falciparum* interacts with the immune system of the mosquito, yet the effects of Vg on parasite development have not been studied. Rono *et al*. showed that *Vg* knockdown in *An. gambiae* results in a lower number of murine *P. berghei* oocysts (Rono et al., 2010). They further showed that this phenotype was due to an immune response mediated by TEP1, a complement-like protein that coats and lyses ookinetes as they are crossing the midgut epithelium, suggesting that Vg may have a protective effect on the parasite by shielding it from the mosquito immune system. However, *P. falciparum* has fundamentally different interactions with the *An. gambiae* immune system. Most notably, it has a surface protein (Pfs47) that allows the ookinete to evade lysis by TEP1 (Molina-Cruz et al. 2013). Therefore, *Vg* knockdown may not result in a lower numbers of *P. falciparum* oocysts, and overall, it is unclear if *P. falciparum* survival and growth are affected by this yolk protein as it has been shown in *P. berghei*. It is also unknown whether *P. falciparum* relies on Vg for development, which is possible given that the parasite depends on the other key nutrient transporter, Lp, for faster growth when surplus lipids not required for reproduction become available (Werling et al. 2019; Costa et al. 2018). Could the parasite rely on Vg as an amino acid source? Or could it possibly rely on Vg-transported lipids, such as phospholipids for membrane formation?

These are important questions to answer because mosquito reproductive pathways have been characterized largely in *Aedes* species and it is unknown how conserved they are in the main malaria vector *An. gambiae*. From a basic biology standpoint, it is imperative to understand how a mosquito can convert blood meal resources into nutrients for a whole new generation of offspring, a process that is orchestrated by hormones and nutrient transporter proteins.

Furthermore, elucidating how the parasite can hijack the process of nutrient deposition into eggs will lead to better understanding of host-parasite interactions. Ultimately, knowledge of these processes may result in better tools for malaria control, at a time when widespread insecticide resistance in mosquitoes is threatening the successful application of current control strategies (*World Malaria Report* 2022).

Summary of aims

Chapter 2: Interplay between nutrient transporters ensures fertility in the malaria mosquito *Anopheles gambiae*

This chapter aims to characterize the interplay between Lp and Vg and their contribution to egg development and fertility. We show that defective lipid transport due to Lp depletion triggers abortive ovarian follicle development, leading to Vg accumulation within other tissues. Conversely, upon Vg depletion, Lp is upregulated after blood feeding in a TOR-dependent manner, resulting in excess lipid delivery to the developing follicles. Vg depletion also causes completely penetrant infertility, as eggs produced go on to arrest early in embryonic development. These findings show that Lp and Vg coordinate one another to achieve successful oogenesis, and both posit potent targets for vector control.

Chapter 3: The role of *Anopheles gambiae* vitellogenin in *Plasmodium falciparum* development

This chapter aims to determine the role of Vg in *P. falciparum* development. We show that while Vg depletion does not affect the number of parasites that develop within the mosquito midgut, it does increase their rate of growth, resulting in sporozoites reaching the mosquito salivary glands faster. This increase in growth rate is potentially mediated in part by *Lp* upregulation upon *Vg*

knockdown. Lipidomic and metabolomic analyses show that phospholipids are upregulated upon Vg depletion in the fat body, and there is an increase in amino acids in midgut, hemolymph and fat body, potentially providing a nutritional resource for parasites. Indeed, supplementing amino acids to females previously infected with *P. falciparum* causes an increase in parasite growth rates, suggesting that the increase observed upon *Vg* knockdown may be at least partially mediated by higher levels of amino acids in the mosquito tissues and hemolymph.

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2

Interplay between nutrient transporters ensures fertility in the malaria mosquito *Anopheles gambiae*

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Abstract:

Females from many mosquito species feed on blood to acquire nutrients for egg development. The oogenetic cycle has been characterized in the arboviral vector *Aedes aegypti*, where after a blood meal, the lipid transporter lipophorin (Lp) shuttles lipids from the midgut and fat body to the ovaries, and a yolk precursor protein, vitellogenin (Vg), is deposited into the oocyte by receptor-mediated endocytosis. Our understanding of how the roles of these two nutrient transporters are mutually coordinated is however limited in this and other mosquito species. Here, we demonstrate that in the malaria mosquito *Anopheles gambiae*, Lp and Vg are reciprocally regulated in a timely manner to optimize egg development and ensure fertility. Defective lipid transport via *Lp* silencing triggers abortive ovarian follicle development, leading to misregulation of Vg and aberrant yolk granules. Conversely, depletion of Vg causes an upregulation of *Lp* in the fat body in a manner that appears to be at least partially dependent on target of rapamycin (TOR) signaling, resulting in excess lipid accumulation in the developing follicles. Embryos deposited by Vg-depleted mothers are completely inviable, and are arrested early during development, likely due to severely reduced amino acid levels and protein synthesis. Our findings demonstrate that the mutual regulation of these two nutrient transporters is essential to safeguard fertility by ensuring correct nutrient balance in the developing oocyte, and validate Vg and Lp as two potential candidates for mosquito control.

Introduction:

The *Anopheles gambiae* mosquito is one of the most important vectors for the transmission of *Plasmodium falciparum*, a malaria parasite that causes remarkable morbidity and mortality in sub-Saharan Africa and other tropical and subtropical regions (World Malaria Report 2022). Transmission starts when a female mosquito takes a blood meal from a human infected with the sexual stages of *Plasmodium*. At the same time as parasite development begins in the midgut, females use blood nutrients to start their reproductive cycle, which culminates in the development of a full set of eggs in about 2-3 days. The signalling cascades triggered by blood feeding and leading to successful egg development have been largely elucidated in *Aedes aegypti* mosquitoes. In this species, the ovarian ecdysteroidogenic hormone (OEH) and insulin-like peptides (ILPs) are released from the brain upon blood feeding, stimulating the production of the ecdysteroid ecdysone (E, which is synthetized from cholesterol) by the ovarian epithelium (Brown et al., 1998; Brown et al., 2008). After transport to the fat body, E is converted to 20-hydroxyecdysone (20E, the active form of this steroid hormone), which binds to its nuclear receptor to trigger transcriptional cascades leading to the activation and repression of hundreds of genes. Among these genes is *Vitellogenin* (*Vg*), the main egg yolk protein precursor in oviparous species, which peaks at 24 hours post blood meal (PBM) (Attardo, Hansen, and Raikhel 2005). Vg is then released from the fat body into the hemolymph, from where it is taken up by the ovaries by receptor-mediated endocytosis (Roth and Porter 1964; Raikhel and Dhadialla 1992). In the oocytes Vg is crystalized into vitellin, which forms the yolk bodies that the embryo uses as a nutritional source of amino acids (Clements 1992; Li and Zhang 2017; Kunkel and Nordin 1985).

Prior to *Vg* expression, the lipid transporter lipophorin (Lp) shuttles cholesterol and neutral lipids (mostly triglycerides (Ford and Van Heusden 1994)) from the midgut to the ovaries, starting

the early phase of egg development and the synthesis of the steroid hormone E (Sun et al. 2000; Atella et al. 2006). It is unclear how *Lp* expression is regulated, although *ex vivo* experiments in *Ae. aegypti* have shown this lipid transporter to be upregulated upon fat body exposure to 20E (Sun et al. 2000). After egg development is completed, if the female is mated, she will oviposit her eggs and return to the pre-blood meal metabolic state. At this point she is ready to begin another gonotrophic cycle, consisting of blood feeding, oogenesis and oviposition.

Besides triggering the synthesis of 20E through cholesterol uptake and E release by the ovaries, blood meal digestion and the subsequent influx of amino acids and ILPs results in the activation of the target of rapamycin (TOR) signalling pathway (Attardo, Hansen, and Raikhel 2005; Hansen et al. 2014). The integration of these nutritional signals leads to a TOR-mediated global regulation of translation and transcription of specific genes that control growth and metabolism (Valvezan and Manning 2019), including *Vg* transcription in mosquitoes (Hansen et al. 2004; reviewed in Attardo, Hansen, and Raikhel 2005; Hansen et al. 2014). TOR regulates translation by directly phosphorylating S6 kinase (S6K) (Hansen et al. 2005), which in turn phosphorylates S6, thus regulating translation of ribosomal proteins and translation elongation factors (reviewed in Attardo, Hansen, and Raikhel 2005; Hansen et al. 2014). S6K also activates the translation of AaGATAa, which promotes *Vg* transcription in *Ae. aegypti* (Park et al. 2006). Due to its central role in the integration of nutritional signals post blood meal, abrogation of TOR signalling results in reduced fecundity in *Ae. aegypti* and *Anopheles stephensi* (Hansen et al. 2004; 2005; Wang et al. 2022; Feng et al. 2021).

Although successful oogenesis in mosquitoes is likely to be tightly dependent on the coordinated function of Lp and Vg, very limited information is available concerning whether and how these nutrient transporters are mutually regulated to ensure egg development and fertility. A

study conducted in *An. gambiae* showed that *Lp* knockdown results in reduced expression of *Vg* after feeding on mice infected with the rodent malaria parasite *Plasmodium berghei*, while *Vg* silencing did not affect *Lp* expression (Rono et al. 2010). No further studies have clarified the possible co-regulation of these factors in ensuring accurate nutrient deposition during oogenesis. Additionally, data concerning the mechanisms regulating egg development in *An. gambiae* are sparce despite the key importance of this species for malaria transmission. In these mosquitoes, silencing of *Lp* (whose expression peaks at 12-18h PBM) has been shown to severely hamper egg development (Vlachou et al. 2005; Rono et al. 2010; Werling et al. 2019), revealing a similar role to *Aedes*. Interestingly, *Lp* levels were upregulated upon inhibition of 20E signalling via silencing of the nuclear *EcR* receptor, suggesting that in certain conditions 20E may repress its expression (Werling et al. 2019). The role of Vg in *An. gambiae* reproduction has been studied even more marginally, with a single study reporting that its depletion results in fewer females developing mature oocytes (Rono et al. 2010).

Here we show that the functions of Lp and Vg are tightly linked in *An. gambiae*. Using functional knockdowns of these genes combined with electron microscopy, multi-omics and biochemistry analyses, we show that depletion of these factors results in profoundly deleterious effects on fecundity and fertility. While Lp is required for successful egg development, Vg is essential for fertility as its depletion leads to an early block in embryonic development. We also show that the functions of these factors are mutually co-regulated, as Lp is needed for the correct incorporation of Vg in the developing oocytes while Vg is required to terminate the Lp-mediated accumulation of lipids in the ovaries. Intriguingly, our data suggest that both induction of *Vg* after blood feeding and its downstream effects on Lp are mediated by TOR signalling, which in turn appears to be repressed by Vg following a blood meal. Our data reveal an intricate reproductive

system based on the timely and mutually coordinated function of these nutrient transporters, and identify novel potential targets to interfere with the fertility of these important malaria vectors.

Results:

***Lp* knockdown significantly impairs oogenesis and affects Vg localization**

Previous studies have shown that *Lp* silencing results in severely reduced oogenesis in *An. gambiae* females (Vlachou et al. 2005; Rono et al. 2010; Werling et al. 2019) but without characterizing the phenotype. We confirmed these findings by injecting double stranded RNA targeting *Lp* (ds*Lp*) prior to blood feeding (Supplementary Figure 1A), which dramatically reduced the median number of eggs developed by females compared to controls (injected with ds*LacZ*) (Figure 2.1A). This reduction in egg numbers was characterized by a striking decrease in triglycerides in the ovaries at different time points post blood meal (PBM), paralleled by a substantial accumulation of these lipids in the midgut, consistent with a role of Lp in shuttling these lipids from the midgut to the developing eggs (Figure 2.1B).

We next examined how *Lp* knockdown affects levels of *Vg*, the major nutrient transporter incorporated into developing eggs. Interestingly, *Lp*-silenced females had aberrant Vg production and distribution. Not only was *Vg* expressed at lower levels upon Lp depletion (Supplemental Figure 1B), consistent with previous work (Rono et al. 2010), but also this yolk protein showed atypical localization. While in control samples Vg was detected in the fat body at 24h PBM and was fully incorporated into the ovaries by 48h PBM, in *Lp*-silenced females Vg persisted in the fat body at the latter time point and its levels were reduced in the ovaries (Figure 2.1C; Supplemental Figure 1C). This was consistent with our observation that the ovaries of Lp-depleted females develop normally up to 24h PBM but degenerate by 48h PBM (Supplemental Figure 1D).

Interestingly, as observed by electron microscopy, yolk granules in the few eggs that develop after *Lp* silencing appeared larger at 24h PBM than those in control ovaries, and Vg had largely disappeared from Western blots by 48h PBM (Figure 2.1D), suggesting yolk degradation at this latter time point. Lp has been shown to transport cholesterol (Gilbert and Chino 1974), a key building block in ecdysteroid synthesis. Since *Vg* expression is regulated by the ecdysteroid 20E we measured ecdysteroid levels upon *Lp* knockdown at 26h PBM (a peak time of 20E synthesis) to determine whether this could be the mechanism for lower *Vg* mRNA levels (Supplemental Figure 1E). We did not observe a change in ecdysteroid levels.

Together, these data show that Lp-mediated shuttling of lipids from the midgut into the ovaries is an essential check point for oogenesis, as preventing this process results in misregulated vitellogenesis.

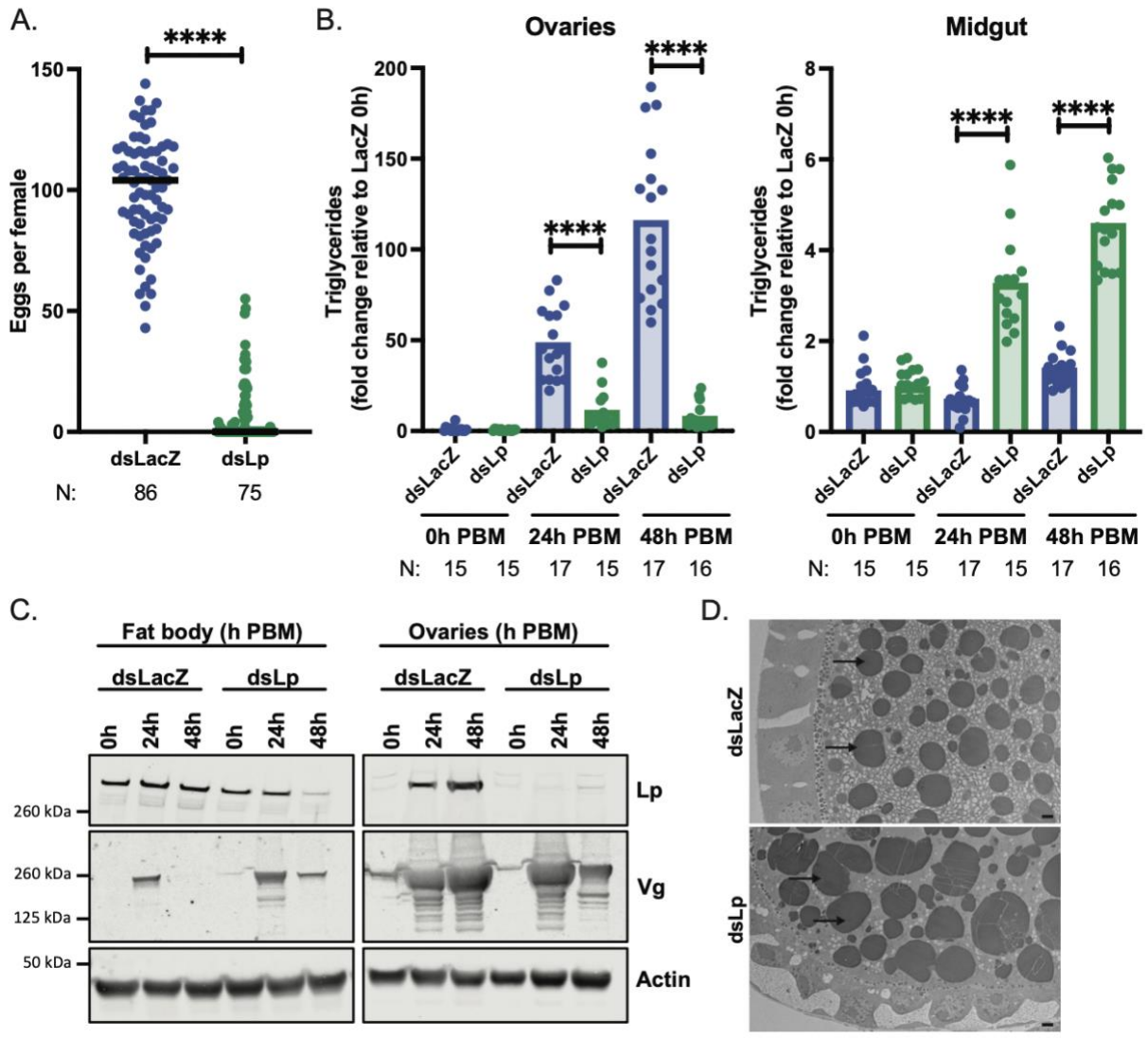

Figure 2.1. *Lp* knockdown significantly impairs oogenesis and affects Vg localization. (A) Following *Lp* knockdown, females develop a decreased number of eggs; each dot represents eggs per female; N = number of females, pooled from four biological replicates (Mann-Whitney: **** = $p < 0.0001$). (B) Triglycerides accumulate in the midgut of Lp-depleted females, and fail to accumulate in the ovaries; PBM = post blood meal; each dot represents tissues from three females; N=number of samples of three tissues, pooled from three biological replicates (REML variance component analysis by timepoint: **** = $p < 0.0001$). (C) Vg is persistently detectable in the fat body of Lp-depleted females and is decreased in the ovary at 48h PBM (three biological replicates). (D) Transmission electron microscopy showing larger yolk granules (arrows pointing at darkly staining circles) in the ds*Lp* ovary compared to ds*LacZ* at 24h PBM; scale bar = 2µm (one biological replicate).

Vg silencing upregulates *Lp* expression and enhances lipid deposition into oocytes

We next assessed the role of *Vg* in egg development and fertility by silencing this gene in females prior to blood feeding (Supplemental Figure 2A). As expected, given its main role during vitellogenesis, Vg depletion caused a significant reduction in number of eggs compared to control females (Figure 2.2A). More strikingly, however, silencing of this yolk gene induced complete infertility, with no embryos hatching from Vg-depleted mothers (Figure 2.2B). To confirm this phenotype, we tested a second dsRNA construct targeting *Vg*, and we again observed complete infertility (Figure 2.2B). No yolk bodies could be detected in ds*Vg* ovaries by electron microscopy (Figure 2.2C), and we also observed a notable decrease in total protein (Supplemental Figure 2B) and free amino acids (Supplemental Figure 2C) levels, consistent with Vg being a major amino acid source.

Upon microscopic analysis, we noticed that Vg-depleted ovaries had a remarkable accumulation of neutral lipids at both time points analyzed (24 and 48h PBM) (Figure 2.2D), a finding which was supported by an assay showing higher triglyceride levels (Figure 2.2E). Since transport of neutral lipids is mediated by Lp, we assayed *Lp* expression and found that this lipid transporter was upregulated at both transcript and protein levels (Figure 2.2F, G; Supplemental Figure 2D, E). Specifically, while *Lp* transcripts in the fat body (the tissue where Lp is synthetized) peaked at 12h PBM in controls, after *Vg* silencing they peaked later and at higher levels relative to controls (Figure 2.2F, Supplemental Figure 2D), paralleled by higher protein levels in the fat body and the ovaries (Figure 2.2G, Supplemental Figure 2E). Combined, these data suggest that Vg synthesis in the fat body and/or its incorporation into the ovaries is a signal that regulates *Lp* expression and prevents excessive Lp-mediated lipid accumulation in the ovaries, ensuring correct balance between nutrients and safeguarding fertility.

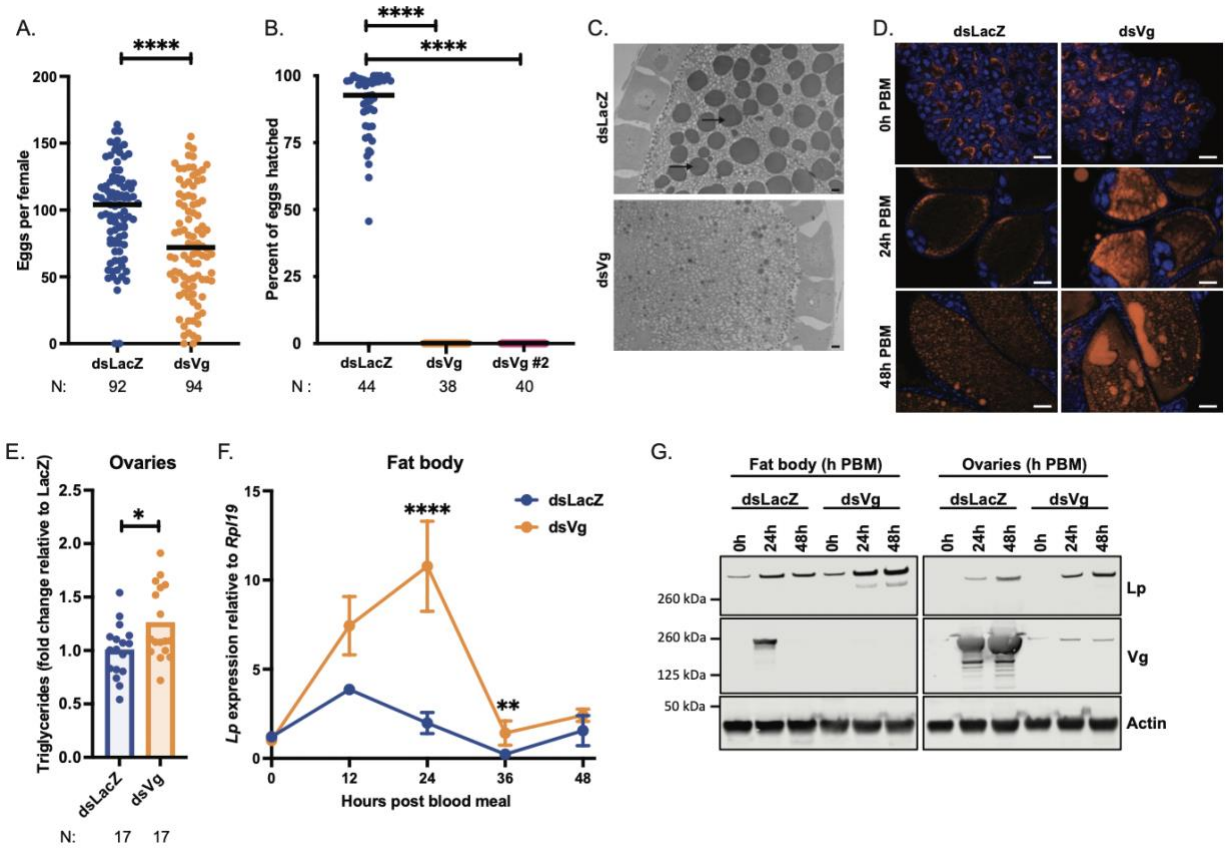

Figure 2.2. *Vg* silencing upregulates *Lp* expression and enhances lipid deposition into oocytes. (A) Following *Vg* knockdown females develop fewer eggs; each dot represents eggs per female; N = number of females, pooled from five biological replicates (Mann-Whitney: **** = $p < 0.0001$). (B) *Vg* knockdown causes complete infertility; *Vg* was targeted by two different dsRNAs; each dot represents percent hatch rate per female; N = number of females, pooled from three biological replicates (Kruskal-Wallis with Dunn's multiple comparisons test: **** = $p < 0.0001$). (C) Transmission electron microscopy showing a lack of yolk granules (arrows) upon *Vg* knockdown at 24h post blood meal; scale bar = 2µm (two biological replicates). (D) Fluorescent microscopy showing an accumulation of neutral lipids (LD540, orange) upon *Vg* knockdown; DNA (DAPI) in blue; scale bar = 50µm (three biological replicates). (E) Triglyceride levels measured in ds*LacZ* and ds*Vg* ovaries at 48h post blood meal and normalized to mean ds*LacZ* levels in that replicate; each dot is representative of ovaries from three females; N=number of samples of three tissues, pooled from three biological replicates (Unpaired t-test: * = $p < 0.05$). (F-G) *Vg* knockdown results in an increase in Lp levels in the fat body at the mRNA (REML variance component analysis: ** = $p < 0.01$; **** = $p < 0.0001$; four biological replicates) (F) and protein (three biological replicates) (G) levels in the fat body and ovaries.

Vg expression regulates Lp-mediated accumulation of lipids via TOR signaling

In *Ae. aegypti* mosquitoes, *Vg* transcription is partly regulated by TOR signalling (Attardo, Hansen, and Raikhel 2005; Hansen et al. 2014). As mentioned above, upon sensing amino acids derived from the blood meal, TOR phosphorylates S6K, which in turn activates AaGATAa, a transcription factor that binds the *Vg* promoter. We confirmed that *Vg* is regulated by TOR in *An. gambiae* by using the TOR inhibitor rapamycin, which decreased *Vg* expression in the fat body by almost 50% when applied to females at 2h post PBM (Figure 2.3A). Surprisingly, we also noticed that *Vg* silencing in turn affected TOR signaling in the fat body, mediating its increase relative to controls. Indeed, while in controls S6K phosphorylation had waned by 24h PBM, *Vg*-silenced females showed a strong signal at this time point, which corresponds to the peak of *Vg* expression (Figure 2.3B, Supplemental Figure 3A). This was paralleled by significantly higher total protein levels after blood feeding (Supplemental Figure 3B), indicating potential translational upregulation by TOR.

To determine whether TOR activation may be involved in the increased *Lp* expression observed in *Vg*-silenced females, we treated Vg-depleted mosquitoes with rapamycin and assessed *Lp* levels relative to controls. Rapamycin treatment 2h PBM reduced S6K phosphorylation at 24h PBM following Vg depletion, confirming that this phosphorylation is indeed mediated by TOR (Figure 2.3D, Supplemental Figure 3C). *Lp* upregulation was also significantly reduced (at both protein and transcript levels) by rapamycin treatment, thereby implicating TOR signaling in the Vg-mediated regulation of this lipid transporter (Figure 2.3C, E, Supplemental Figure 3D). In agreement with this observation, rapamycin treatment also slightly reduced the excess lipid accumulation observed upon *Vg* depletion (Supplemental Figure 3E). Overall, these data suggest

that in normal conditions *Vg* expression leads to repressed TOR signaling, putting a break on Lp synthesis thereby preventing excessive incorporation of lipids into the ovaries.

We hypothesized that excess amino acids not incorporated into Vg may be the triggers for activating TOR signaling. Indeed, in anopheline mosquitoes, yolk production utilizes up to 30% of the total protein content from the blood meal (Briegel 1990). Consistent with this hypothesis, we detected a modest but significant increase in amino acid levels at 24h PBM in the fat body (but not in the hemolymph) of *Vg*-silenced females, which was amplified at 48h PBM (Supplemental Figure 3F, G). TOR also incorporates inputs from ILPs to regulate metabolism (Arsic and Guerin 2008), however expression levels of 7 *An. gambiae ILPs* were similar between groups at all time points analyzed (Supplemental Figure 3H), likely ruling out insulin signaling as the cause of increased TOR signaling (note, ILP1 and 7, and ILP 3 and 6 share sequence identity, resulting in 5 RT-qPCR plots).

Based on these data, a model emerges whereby in *An. gambiae* TOR signaling both controls *Vg* expression and is in turn regulated by this yolk protein, affecting the expression of *Lp* and the timely accumulation of lipids in the developing ovaries. Fine tuning of the function of these nutrient transporters is essential to support egg development and ensure fertility, demonstrating a previously unappreciated interplay that is key to the survival of this species.

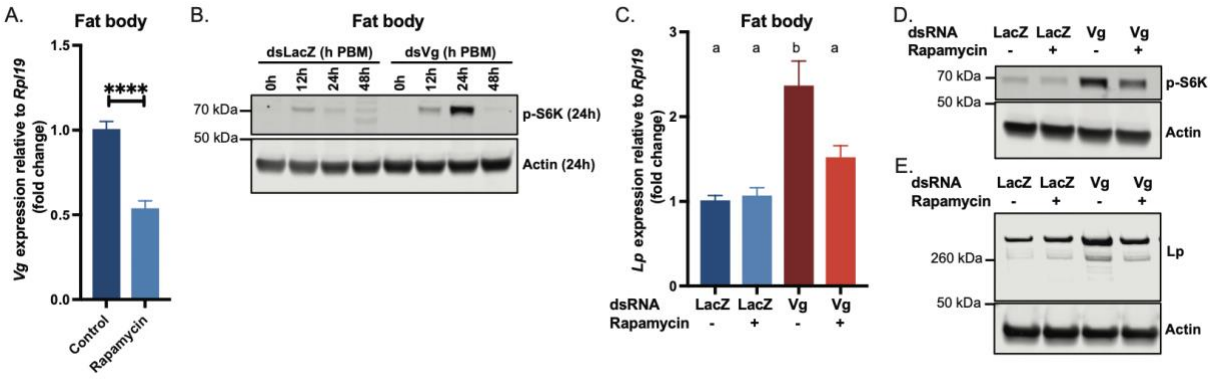

Figure 2.3. Vg regulates *Lp* levels via TOR signaling. (A) *Vg* expression relative to *Rpl19* is reduced in the fat body 24h PBM upon 0.5 µl of 40µM rapamycin treatment 2h PBM; Control is 2.4% DMSO in acetone; all values normalized to control (unpaired t test: **** = $p < 0.0001$; four biological replicates). (B) TOR signaling is induced in the fat body upon *Vg* knockdown as shown by Western blotting of phospho-S6K levels (three biological replicates). (C) The *Vg*-knockdown mediated upregulation of *Lp* at 24h PBM is suppressed in the fat body of ds*Vg* females treated with 0.5 µl of 40 µM rapamycin 2h PBM; all values normalized to ds*LacZ* without rapamycin treatment (ANOVA; four biological replicates). (D) Western blot showing phospho-S6K levels increase in the fat body at 24h PBM in *Vg*-silenced females, but this increase is repressed by rapamycin treatment (four biological replicates). (E) Western blot showing fat body Lp levels are increased in *Vg*-knockdown females at 48h PBM, but this increase is repressed by rapamycin (four biological replicates).

Vg knockdown causes early embryonic arrest

Our discovery that close cooperation between Lp and Vg is fundamental to fertility prompted us to dig deeper into the mechanisms causing infertility in embryos laid by Vg-depleted females. We found that most of these embryos were fertilized but not melanized (Supplemental Figure 4A) and did not reach the blastocyst stage, which control embryos instead reached at 3-5h post oviposition (Figure 2.4A). Some embryos were halted very early in development (Figure 2.4A, top panel), upon the first few mitotic divisions after the fusion of the gamete nuclei, while others reached the energid stage, where nuclei have divided and begin to migrate toward the outer edges (Figure 2.4A, bottom panel and zoomed-in insert). However, these nuclei displayed abnormal morphology, showing features reminiscent of apoptotic blebbing.

We did not detect any Vg in embryos deposited by Vg-depleted mothers, contrary to controls and demonstrating that in early stages of embryogenesis Vg is entirely maternally derived (Figure 2.4B). Interestingly, we detected Lp in those embryos at very early stages (0-2h) post oviposition, suggestive of maternal deposition which however did not occur in controls (Figure 2.4B). Moreover, ds*Vg*-derived embryos showed significantly reduced protein synthesis, as highlighted by undetectable actin expression over time (Figure 2.4B).

In agreement with these findings, metabolomic analysis of ds*Vg*-derived embryos (3-5 hours post oviposition) showed a depletion of amino acids, especially severe for those amino acids that are most prevalent in Vg (Figure 2.4C; Supplemental Table 1). Intriguingly, the two amino acids more strikingly depleted, phenylalanine and tyrosine, are the starting compounds in the melanization pathway (Kim et al. 2005; González-Santoyo and Córdoba-Aguilar 2012), possibly explaining the lack of chorion darkening observed in ds*Vg* embryos (Supplemental Figure 4A). Also notable was the upregulation of free nucleotides as well as products of their catabolism such as hypoxanthine and inosine, likely due to impaired DNA replication due to cell death (Figure 2.4C).

Given the observed incorporation of Lp into the embryo, we also performed lipidomic analysis at the same time point. Although there were no differences in total lipids in ds*Vg*-derived embryos relative to controls (Supplemental Figures 4B), some minor lipid classes (phospholipids and glycerophospholipids) were decreased (Supplemental Figure 4C), while we detected a remarkable increase in triglycerides (Figure 2.4D), again consistent with the known role of Lp in transport of these lipids (Ford and Van Heusden 1994).

Therefore, when mothers are depleted of Vg, embryos cannot develop further than the first steps after fertilization, likely due to a severe drop in amino acid levels and protein synthesis.

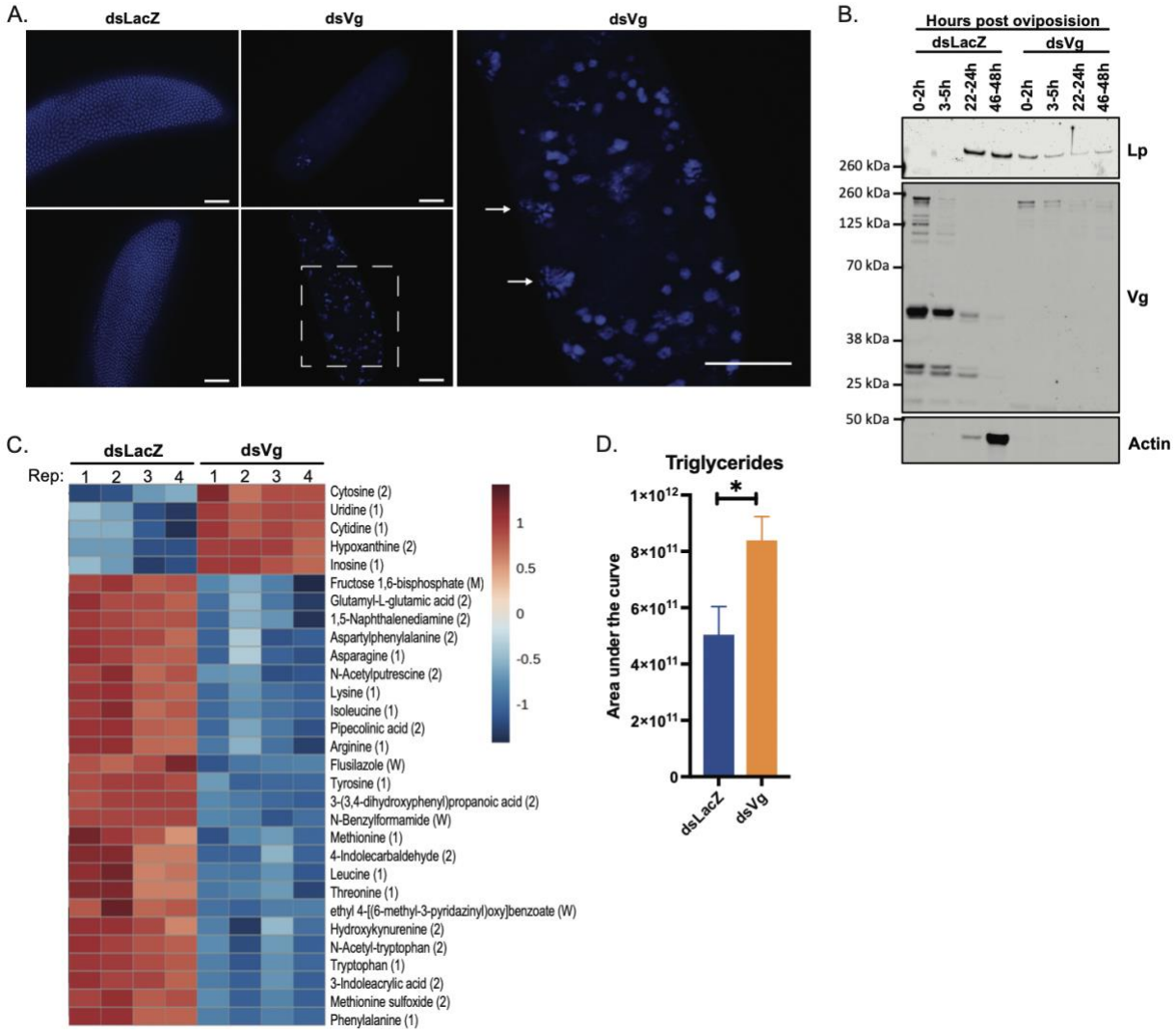

Figure 2.4. *Vg* knockdown causes early embryonic arrest likely due to limited amino acids. (A) DAPI staining of embryos from ds*LacZ* and ds*Vg* females at 3-5h post oviposition showing arrest of development before the cellular blastocyst stage in the ds*Vg*-derived embryos; scale bar = 50µm (two biological replicates); dotted box represents the field of view in the zoomed-in image to the right, and arrows point to nuclei characteristic of blebbing. (B) Lp is deposited into ds*Vg*-derived embryos and not ds*LacZ*-derived ones, as observed by Western blot at specified time post oviposition (three biological replicates). (C) Top 30 dysregulated metabolites (as determined by Metaboanalyst), of which 10 are amino acids, in embryos derived from ds*Vg* mothers compared to those of ds*LacZ* 3-5h post oviposition (each column is representative of a biological replicate; four biological replicates). ID confidence is in brackets; from strongest to weakest: (1) = Level 1 ID, (2) = Level 2 ID, (M) = MasslistRT ID, (W) = Weak/Poor ID. (D) Triglyceride levels are elevated in ds*Vg*-derived embryos 3-5h post oviposition as determined by lipidomics (unpaired t test: * = $p < 0.05$; four biological replicates).

Discussion:

Blood feeding is an essential process for the survival of mosquito species that, like *An. gambiae*, rely on blood nutrients for oogenesis. After a blood meal, different nutrient transporters start shuffling cholesterol, lipids and proteins from the midgut and fat body to the ovaries, and the individual roles of two of these factors, Lp and Vg, had been previously determined. There remained, however, important outstanding questions concerning whether and how these two essential transporters are co-regulated in order to ensure reproductive success. How do mosquitoes balance acquisition of different nutrients by the ovaries? What are the signals that limit lipid accumulation and that trigger vitellogenesis? Moreover, knowledge concerning Lp and Vg functions and their regulation in *Anopheles* as opposed to *Aedes* was only marginal. Here, we show that in these mosquitoes, oogenesis is the product of a precise and intricate interplay between these two factors. The initial phase of egg development is dominated by Lp, which incorporates lipids (mostly triglycerides) and cholesterol into ovaries triggering their growth. During this early phase, as E is converted to 20E in the fat body, this steroid hormone triggers the expression of *Vg*, which peaks at 24h PBM and provides the oocytes with considerable stores of amino acids. When *Lp* expression is impaired, egg development is strikingly reduced (Figure 2.1A), Vg localization is affected (Figure 2.1C), and yolk bodies are aberrant (Figure 2.1D). Upon Lp depletion Vg seems to be retained in the fat body, since the ovaries are degenerating and cannot accumulate further yolk. These findings suggest that correct lipid accumulation is an early check point that mosquitoes use to decide whether to proceed with vitellogenesis. Reducing *Vg* expression levels, on the other hand, leads to increased *Lp* expression (Figure 2.2F and G) and an overload of glycerides in the ovaries (Figure 2.2E), which suggests that Vg synthesis is the signal that prevents excessive lipid trafficking by Lp into the oocytes. Interestingly, in another study *Vg* silencing did not appear to

affect *Lp* expression in *An. gambiae* females fed on mice infected with *P. berghei* parasites (Rono et al. 2010). This discrepancy with our results may indicate that rodent malaria parasites, which are known to inflict severe reproductive costs in infected mosquito females (Ahmed and Hurd 2006), affect the normal accumulation of lipids during the oogenetic process, although other factors such as differences in temperature (infections with *P. berghei* are done at permissive temperatures around 20°C compared to standard mosquito rearing conditions of 28°C) cannot be excluded.

Strikingly, silencing of *Vg* led to complete infertility. This phenotype was so penetrant that we confirmed it with a dsRNA second construct targeting a different region of the gene to rule out a possible unspecific effect of the first construct. This effect is reminiscent of observations in other insects, where altering Vg gene copy number, expression or internalization leads to complete sterility or decreased hatch rate by as yet unknown mechanisms (Bownes, Lineruth, and Mauchline 1991; DiMario and Mahowald 1987; Gutzeit and Arendt 1994; Lin et al. 2013; Ciudad, Piulachs, and Bellés 2006; Peng et al. 2020; Shang et al. 2018; Mitchell III et al. 2007; Coelho et al. 2016). Understanding how embryonic lethality is induced may lead to novel ideas for the design of mosquito-targeted interventions, so we set out to determine the mechanisms behind death. As *Vg* is also expressed in the female spermatheca after mating (Rogers et al. 2008; Shaw et al. 2014) we initially thought that its depletion may have caused irreversible damage to sperm. The observation that embryos are fertilized and start undergoing nuclear division (Figure 2.4A), however, appears to discount this possibility. Based on our metabolomics analysis, the most plausible hypothesis is that infertility is a result of amino acid starvation. We show that embryos from Vg-depleted females are significantly deficient in 14 of the 19 identified amino acids, which results in a lack of building blocks for translation and thus development (Supplemental Table 1). It is plausible to

speculate that depletion in these essential nutrients may activate the amino acid response pathway, triggering a global shutdown of translation that may lead to apoptosis, compatible with our observation of nuclei blebbing in those embryos (Sikalidis 2013; Dong et al. 2000; Qin et al. 2017).

Our data using rapamycin suggest that the *An. gambiae* Vg is regulated by TOR (Figure 2.3A), as was previously shown in *Ae. aegypti* (Hansen et al. 2004). Surprisingly, however, our findings also suggest that Vg in turn suppresses TOR signaling, as upon *Vg* knockdown S6K phosphorylation was strongly upregulated possibly due to an increase in free amino acids (Figure 2.3B and Supplemental Figure 3F). This upregulation in TOR signaling also resulted in an increase in Lp transcription and translation, further shedding light on regulation of *Lp* expression. Previous studies had shown that *Lp* levels in *An. gambiae* are under steroid hormone control, as impairing 20E signaling caused an increase in *Lp* transcription at 24h, 36h and 48h post blood feeding (Werling et al. 2019). Since Vg is under 20E control, it is probable that the result observed by Werling *et al*. at 24h (increased Lp expression in ds*EcR*) is mediated by reduced Vg expression/increased TOR signaling, while additional EcR-controlled mediators, or EcR itself, are responsible for the decreased Lp expression at later timepoints. With the caveat that our current results were obtained only by using the inhibitor rapamycin rather than by also silencing key components of the TOR pathway, these combined observations may suggest that TOR and 20E signaling exert opposite effects on *Lp* expression — with 20E repressing its levels and TOR enhancing them —, an intriguing finding that deserves more thorough investigation in future studies. Compatible with our data, the Lp promoter has putative GATA transcription factor binding motifs, some of which are known to be regulated by TOR signaling (Marinotti et al. 2006; Park et al. 2006).

Does the interplay between Lp and Vg also affect the development of *P. falciparum* parasites? An earlier study showed that when *Lp* is silenced in *An. gambiae*, *P. falciparum* oocyst numbers are decreased (Werling et al. 2019). No other effects were detected on parasite development, unlike in the mouse malaria parasite *P. berghei* where Lp depletion, besides a decrease in oocyst numbers, also led to reduced oocyst growth (Rono et al. 2010). While the role of Vg in *P. falciparum* has not been directly determined, it is known that impairing 20E signaling (which in turn negatively affects *Vg* levels) has profound and opposite effects on parasites, as it reduces parasite numbers but accelerates their growth. Regardless of its impact on parasite development, our data reveal the interplay between Lp and Vg as essential for mosquito fertility, opening the possibility of targeting it to reduce the reproductive success of mosquito populations.

Materials and Methods:

Mosquito lines and rearing:

G3 *Anopheles gambiae* mosquitoes were reared at 27°C, 70-80% humidity. Adults were fed 10% glucose solution and purchased human blood (Research Blood Components, Boston, MA). Females and males were separated by pupae sexing, and females were kept separate to ensure virgin status or mixed with males at a 1:2 ratio for fertility experiments and egg collections.

dsRNA generation:

A 816bp LacZ fragment and 600bp Lp (AGAP001826) fragment were generated from plasmids pLL100-LacZ and pLL10-Lp as described previously (Blandin et al. 2004; Marcenac et al. 2020; Werling et al. 2019) using T7 primer (5'–TAATACGACTCACTATAGGG–3'). A 552 bp fragment of Vg (AGAP004203) corresponding to bases 3374–3925 of the Vg cDNA was amplified from plasmid pLL10-Vg, a gift from Miranda Whitten and Elena A. Levashina (Max Planck Institute for Infection Biology, Berlin), using a primer matching the inverted T7 promoters (same as above). To generate the ds*Vg* #2 construct, a 284bp PCR product was generated from *An. gambiae* Vg cDNA (AGAP004203) corresponding to bases 4530–4813 using forward primer ATTGGGTACCGGGCCCCCCCGCACGTCTCGATGAAGGGTA and reverse primer GGGCCGCGGTGGCGGCCGCTCTAGACCTGCCCTGGAAGAAGTAGTCC. The pLL10-Vg backbone and the PCR fragment were restriction digested with XbaI and XhoI, separated on an agarose gel and gel purified. Then, fragments were assembled using NEBuilder HiFi DNA Assembly Kit. PCR product was amplified using T7 primer. PCR for dsRNA generation was separated by gel electrophoresis for size confirmation, and transcribed into dsRNA by *in vitro* transcription Megascript T7 kit (ThermoFisher Scientific) (Werling et al. 2019). dsRNA was

purified by phenol-chloroform extraction, and diluted to 10µg/µl. To ensure accurate concentration measurement, a small aliquot of dsRNA was diluted 1:10 before using the NanoDrop.

dsRNA injections:

Females on day 1 post eclosion were injected with 69nl of dsRNA (ds*LacZ*, ds*Vg*, ds*Vg* #2, ds*Lp*) using Nanoject III (Drummond), and allowed to recover. Surviving females were fed with blood 3 days post injection. Unfed females were removed from experimental cages.

Egg counts:

Virgin females were dissected 3-7 d after blood feeding, and the egg clutches were counted.

Fertility assay:

Injected females were mixed with males at a 1:2 ratio immediately after injection, and blood fed three days later. One day after blood feeding, fed females were moved to individual cups with around 2cm of water at the bottom. Hatched and unhatched eggs from every cup were counted within a week.

RNA extraction, cDNA synthesis and RT-qPCR:

Fat bodies or heads (10 tissues per tube) from female mosquitoes were dissected in PBS and stored at -80^{o}C in 300µl TRI Reagent (ThermoFisher Scientific). Samples were thawed and bead beaten using 2mm beads. Then RNA was extracted using manufacturer's instructions with a modification to wash the RNA pellet using 70% ethanol. 2.5µg of RNA was aliquoted and DNase treated with Turbo DNase from the TURBO DNA-free Kit (ThermoFisher Scientific), followed by DNase inactivation from the same kit. cDNA synthesis was carried out in 100µl reactions using random primers (ThermoFisher Scientific), dNTPs (ThermoFisher Scientific), first strand buffer (VWR), RNAseOUT (ThermoFisher Scientific) and MMLV (ThermoFisher Scientific). Relative quantification RT-qPCR was carried out using SYBR-Green mix and primers from

Supplemental Table 2. Primers were designed on exon-exon junctions where possible. Quantification was performed in triplicate using the QuantStudio 6 Pro qPCR machine (ThermoFisher Scientific). *Rpl19* was used as the endogenous control for relative quantification.

Ecdysteroid level measurement

Ten females at 26h PBM were collected per sample by removing their heads and storing them in 400 µL of 100% methanol at -80°C. Ecdysteroids were measured using the 20E Enzyme Immunoassay (EIA) kit (Cayman Chemical), according to manufacturer instructions and as described in previously published work (Werling et al. 2019). Although the kit is targeted at identifying 20E, the immunoassay cross-reacts with other ecdysteroids. Hence, the measurements in Supplemental Figure 2 was labelled as "Ecdysteroid levels".

Immunofluorescent microscopy and tissue staining:

Ovaries were dissected from females at specified timepoints and incubated at room temperature in 4% paraformaldehyde (PFA) for 30 minutes, followed by 3 washes in PBS for 15 minutes. Ovaries were permeabilized and blocked with 0.1% Triton X-100 and 1% bovine serum albumin (BSA) in PBS for 1 hour followed by 3 washes in PBS for 15 minutes. Ovaries were then incubated with DAPI and LD540 (Spandl et al. 2009), both at a concentration of 1µg/ml, at room temperature for 15 minutes. After staining, ovaries were washed in PBS 3 times for 15 minutes and mounted using Vectashield mounting medium (Vector Laboratories). Images were captured on a Zeiss Inverted Observer Z1.

Embryo collections for microscopy:

An egg bowl was inserted into cages of mated females blood fed 96h before. The egg bowl was removed 2 hours later, and embryos were collected 3 hours later, resulting in a timepoint of 3-5 hours. Embryos were dechorionated and cracked as described previously (Goltsev et al. 2004).

Briefly, embryos were washed with 25% sodium hypochlorite in 1xPBS, collected into glass vials with 9% PFA and heptane, and rotated for 25 minutes. PFA was removed and replaced with deionized water twice. Vials were shaken for another 30 minutes. Then water was replaced with boiling water and incubated in a hot water bath for 30 seconds, and immediately replaced with ice cold water. Both water and heptane were removed and replaced with heptane and methanol. Embryos were swirled vigorously to crack the shell, washed 3 times with methanol and collected into methanol. Embryos were then coaxed out of eggshells (Juhn and James 2012), and stained with DAPI as described above.

Transmission electron microscopy:

Dissected mosquito ovaries were collected in 200 uL of fixative (2.5% paraformaldehyde, 5% glutaraldehyde, 0.06% picric acid in 0.2M cacodylate buffer) and spun down briefly to fully submerge the tissues in fixative. Fixed samples were submitted to the Harvard Medical School Electron Microscopy Core. Samples were washed once in 0.1M cacodylate buffer, twice in water, and then postfixed with 1% osmium tetroxide/1.5% potassium ferrocyanide in water for 1 hour. Samples were then washed twice in water followed by once in 50mM maleate buffer pH 5.15 (MB). Next, the samples were incubated for one hour in 1% uranyl acetate in MB, followed by one wash in MB, and two washes in water. Samples were then subjected to dehydration via an increasing ethanol gradient (50%, 70%, 90%, 100%, 100% ethanol) for 10 minutes each. After dehydration, samples were placed in propylene oxide for one hour and then infiltrated overnight in a 1:1 mixture of propylene oxide and TAAB 812 Resin (https://taab.co.uk/, #T022). The following day, samples were embedded in TAAB 812 Resin and polymerized at 60C for 48 hours. Ultrathin sections (roughly 80nm) were cut on a Reichert Ultracut-S microtome, sections were

picked up onto copper grids, stained with lead citrate and imaged in a JEOL 1200EX transmission electron microscope equipped with an AMT 2K CCD camera.

Rapamycin treatment:

0.5µl of 40µM rapamycin (dissolved in DMSO and acetone) was applied on ice to the posterior thoraces of females 2h post blood feeding. Rapamycin was in 2.4% DMSO in acetone. Control mosquitoes were treated with 0.5µl of 2.4% DMSO in acetone. Acetone was used as a carrier to ensure even delivery of the compound through the cuticle and because of its rapid evaporation. Mosquitoes were placed into cages with 10% glucose to recover.

Hemolymph collections:

For amino acid assay, hemolymph was collected by making a tear between the last and second-to-last segments on the abdomen, and injecting 2µl of purified deionized water into each mosquito. A drop of liquid was collected from the abdomen with a pipette. Hemolymphs from five females were pooled for amino acid assay.

Primary antibodies:

Antibody against Vg was generated with Genscript by injecting a rabbit with a Vg peptide (QADYDQDFQTADVKC). Rabbit serum was affinity purified to produce a polyclonal antibody used at 1:1000 for Western blotting.

Anti-Lp antibody was also generated with Genscript using the following peptide: FQRDASTKDEKRSGC (Werling et al. 2019). This antibody was used at 1:4000 for Western blotting.

Anti-actin antibody was acquired from Abcam (MAC237) and used at a dilution of 1:4000.

Phospho-S6K antibody was acquired from MilliporeSigma (07-018-I) and used at a dilution of 1:1000.

Western blotting:

5 tissues per sample, or 40 embryos were collected into 55 µl of PBS with protease and phosphatase inhibitors (cOmplete Mini EDTA free protease inhibitor cocktail, Halt phosphatase inhibitor). DTT (200mM) and NuPAGE LDS Sample Buffer were added. Tissues were bead beaten and boiled for 10 minutes. Then, $1/10^{th}$ of the sample was loaded onto either a NuPAGE 4-12% Bis-Tris gel or NuPAGE 3-8% Tris Acetate gel (when blotting for Lp). Gels were transferred for 10 minutes at 22V using an iBlot2 machine and iBlot2 PVDF Stacks, blocked in Intercept Blocking Buffer (LI-COR) for 1 hour, and then incubated with antibody overnight at 4ºC. Membranes were washed with PBS-T 4 times for 5 minutes, and incubated with LI-COR secondary antibodies (Goat anti-Rat 680LT; Donkey anti-Rabbit 800CW) for 1-2 hours. Membranes were washed again with PBS-T 4 times and once in PBS. Membranes were imaged using a LI-COR developer.

Triglyceride assay and Bradford assay:

Three tissues per sample were collected into NP40 Assay Reagent, and Triglyceride Colorimetric Assay was performed according to manufacturer's instructions (Cayman). Briefly, tissues were homogenized by bead beating in 32µl of the NP40 Assay Reagent, centrifuged at 10,000g for 10 minutes. 10µl of supernatant was added to 150µl of Enzyme Mixture in duplicate and incubated for 30 minutes at 37ºC. Absorbance was measured at 530nm.

Of note, the kit releases glycerol from triglycerides and measures glycerol levels, and does not measure triglyceride levels directly.

The same supernatant was also used for Bradford assay. Supernatant was diluted 1 in 10 and 4µl of diluted supernatant was added to 200µl of Bradford reagent (Bio-Rad) at room temperature, and absorbance was recorded at 595nm.

Amino acid assay:

Five tissues per sample were collected into 50µl of Ultrapure Distilled Water (Invitrogen), and amino acid assay was performed according to manufacturer's instructions (EnzyChrom L-Amino Acid Assay Kit ELAA-100). Briefly, samples were homogenized by bead beating and centrifuged at 10,000g for 15 minutes. 20µl of supernatant was mixed with Working Reagent in duplicate and incubated at room temperature for 60 minutes. Absorbance was recorded at 570nm.

Sample collection for metabolomics and lipidomics:

200 eggs were collected into 1ml of methanol and homogenized by bead beating with five 2mm glass beads, then transferred to 8ml glass vials. Tubes were then rinsed with 1ml of methanol that was pooled with the homogenized sample, and 4ml of cold chloroform was added to the glass vials, which were then vortexed for 1 minute. 2ml of water was added and glass vials were vortexed for another minute. Vials were then centrifuged for 10 minutes at 3000*g*. The upper aqueous phase was submitted for metabolomics, and lower chloroform phase was submitted for lipidomics to the Harvard Center for Mass Spectrometry.

Metabolomics mass spectrometry:

Samples were dried down under Nitrogen flow and resuspended in 25 µL of acetonitrile 30% in water. Ten microliter of each sample was used to create a pool sample for MS2/MS3 data acquisition. Samples were analyzed by LC-MS on a Vanquish LC coupled to an ID-X MS (Thermofisher Scientific). Five µL of sample was injected on a ZIC-pHILIC peek-coated column (150 mm x 2.1 mm, 5 micron particles, maintained at 40 °C, SigmaAldrich). Buffer A was 20 mM Ammonium Carbonate, 0.1% Ammonium hydroxide in water and Buffer B was Acetonitrile 97% in water. The LC program was as follow: starting at 93% B, to 40% B in 19 min, then to 0% B in 9 min, maintained at 0% B for 5 min, then back to 93% B in 3 min and re-equilibrated at 93% B

for 9 min. The flow rate was maintained at 0.15 mL min^{-1}, except for the first 30 seconds where the flow rate was uniformly ramped from 0.05 to 0.15 mL min^{-1}. Data was acquired on the ID-X in switching polarities at 120000 resolution, with an AGC target of 1e5, and a m/z range of 65 to 1000. MS1 data was acquired in switching polarities for all samples. MS2 and MS3 data was acquired on the pool sample using the AquirX DeepScan function, with 5 reinjections, separately in positive and negative ion mode. Data was analyzed in Compound Discoverer © software (CD,version 3.3 Thermofisher Scientific). Identification was based on MS2/MS3 matching with a local MS2/MS3 mzvault library and corresponding retention time built with pure standards (Level 1 identification), or on mzcloud match (level 2 identification). Compounds where the retention time and the accurate mass matched an available standard, but for which MS2 data was not acquired are labelled MasslistRT matches. Each match was manually inspected.

Metabolomics heatmap was generated using Metaboanalyst 5.0 by log 10 transforming the area under the curve values for metabolites identified as described above.

Lipidomics mass spectrometry:

Samples were dried down under Nitrogen flow and resuspended in 60 µL of chloroform. LC–MS analyses were modified from (Miraldi et al. 2013) and were performed on an Orbitrap QExactive plus (Thermo Scientific) in line with an Ultimate 3000 LC (Thermo Scientific). Each sample was analyzed separately in positive and negative modes, in top 5 automatic data dependent MSMS mode. Twenty µL of sample was injected on a Biobond C4 column (4.6 × 50 mm, 5 µm, Dikma Technologies, coupled with a C4 guard column). Flow rate was set to 100 µl min^{-1} for 5 min with 0% mobile phase B (MB), then switched to 400 µl min^{-1} for 50 min, with a linear gradient of MB from 20% to 100%. The column was then washed at 500 µl min^{-1} for 8 min at 100% MB before being re-equilibrated for 7min at 0% MB and 500 µl min^{-1}. For positive mode runs, buffers

consisted for mobile phase A (MA) of 5mM ammonium formate, 0.1 % formic acid and 5% methanol in water, and for MB of 5 mM ammonium formate, 0.1% formic acid, 5% water, 35% methanol in Isopropanol. For negative runs, buffers consisted for MA of 0.03% ammonium hydroxide, 5% methanol in water, and for MB of 0.03% ammonium hydroxide, 5% water, 35% methanol in isopropanol. Lipids were identified and integrated using the Lipidsearch © software (version 4.2.27, Mitsui Knowledge Industry, University of Tokyo). Integrations and peak quality were curated manually before exporting and analyzing the data in Microsoft excel.

Quantification and statistical analyses:

All statistical tests were performed in GraphPad Prism 9.0 and JMP 17 Pro statistical software. The number of replicates and statistical tests performed are mentioned in the figure legend. Detailed outputs of statistical models are provided in the supplementary information. Residual Maximal Likelihood (REML) variance components analysis was used by fitting linear mixed models after data transformation to resemble normality. dsRNA treatment and timepoint were included as fixed effects and replicate as a random effect. If transformation was not possible, a generalized linear model was used instead. Interaction terms were removed when not significant and models with lower AICc scores were kept. Multiple comparisons were calculated using pairwise Student's t tests at each timepoint followed by FDR correction at a 0.05 significance level.

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Declaration of interests:

The authors do not have any conflicts of interest.

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The role of *Anopheles gambiae* vitellogenin in *Plasmodium falciparum* development

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I.S. and **F.C.** designed the study. **I.S.** performed experiments with assistance from **L.d.V.** and **J.K.** on tissue collection and processing for metabolomics and lipidomics. **L.d.V.** performed the amino acid supplementation experiments. **C.V.** performed lipidomics and metabolomics. **N.S.** cultured parasites and carried out infectious blood feeds. **I.S.** analyzed data with guidance from **W.R.S.**. **I.S.** wrote the manuscript with editorial input from **F.C.**

Abstract:

The life cycle of *Plasmodium falciparum*, the main causative agent of human malaria, is dependent on its development within its vector, the *Anopheles* mosquito. The mosquito females need to ingest blood to be able to produce eggs with nutritious deposits for the developing embryos. If the mosquito bites an infected person, the parasite will be ingested within the blood bolus in the midgut. The parasite ookinete will cross the midgut epithelium and establish as an oocyst beneath the basal lamina to grow and eventually release sporozoites that travel to the salivary glands. Here, we explored whether vitellogenin (Vg, the main yolk protein precursor) influences *P. falciparum* survival and growth in the *Anopheles gambiae* female. We show that *Vg* depletion does not affect parasite numbers but results in faster oocyst development, which in turn causes sporozoites to reach the salivary glands within a shorter time frame. We assessed whether this increase in size can be attributed to an increase in lipophorin (Lp), which is a lipid shuttling protein upregulated upon Vg depletion, and observed that the number of sporozoites reaching the salivary glands earlier decreases upon double knockdown of *Vg* and *Lp*. Multiomics analyses of midgut, fat body and hemolymph show that phospholipids and amino acids are upregulated upon *Vg* knockdown, potentially providing extra building blocks for parasite lipid membranes and driving faster oocyst growth. These results are a cautionary tale for vector control methods aiming at curtailing mosquito reproductive output.

Introduction:

Malaria is a deadly disease resulting in 247 million cases and 619,000 deaths worldwide in 2021 (*World Malaria Report* 2022). Most infections are caused by the *Plasmodium falciparum* parasite which is transmitted by the *Anopheles* mosquitoes. The females of the major malaria vector *Anopheles gambiae* feed on human blood to obtain nutrients necessary for successful oogenesis, the process leading to egg development (Clements 1992). Incidentally, they may feed on an infected person and consume parasite gametocytes, which are ingested into the midgut of the mosquito along with the blood (Shaw and Catteruccia 2019). There, the parasite undergoes fertilization and crosses the midgut epithelium as an ookinete, subsequently developing into an oocyst underneath the basal lamina surrounding the midgut epithelium. After 10-14 days of growth and DNA replication, the oocyst releases thousands of sporozoites into the haemolymph (open circulatory system) of the mosquito, which then travel to the salivary glands where they infect a new human host when the mosquito feeds again.

Blood meal ingestion causes a metabolic storm within the female, with a large transcriptional response leading to a switch in metabolism to blood meal digestion and egg production, which is completed by 48-72 hours (h) post blood meal (PBM) (Marinotti et al. 2006). Upon blood feeding, ovarian ecdysteroidogenic hormone (OEH) and insulin-like peptides (ILPs) are released from the brain, stimulating the production of ecdysone (E) by the ovarian epithelium (Brown et al., 1998; Brown et al., 2008). Concurrently, the major lipid transporter lipophorin (Lp) shuttles cholesterol and lipids from the midgut and fat body to the ovaries, starting oogenesis. E is then converted to 20-hydroxyecdysone (20E), which is an insect ecdysteroid hormone that, upon binding to its nuclear receptor EcR, acts as a transcription factor activating and repressing genes. This transcriptional cascade results in the production of vitellogenin (Vg), the main egg yolk

protein precursor in oviparous species, at 24h PBM (Attardo, Hansen, & Raikhel, 2005; Li & Zhang, 2017). Vg is then released into the haemolymph, where it travels to the ovaries to be taken up by receptor-mediated endocytosis into the oocytes (Swevers et al., 2005). In the oocytes it is crystalized into vitellin, which forms the yolk bodies that the embryo uses as a nutritional source of amino acids (Clements 1992; Li and Zhang 2017; Kunkel and Nordin 1985). As shown in the previous chapter, Vg is essential for *An. gambiae* fertility as its knockdown is lethal for embryos (Stryapunina et al. 2023). Vg depletion also results in increased *Lp* expression and, subsequently, higher lipid accumulation into the ovaries. This Lp upregulation was mediated by increased target of rapamycin (TOR) signaling.

While egg development is underway, ookinetes are crossing the midgut epithelium, establishing themselves as oocysts beneath the basal lamina. Although the processes of oogenesis and parasite development happen in different tissues, they are temporally and molecularly linked. Indeed, there exists a positive correlation between egg and oocyst numbers meaning that conditions that are beneficial to egg development are also beneficial to the parasite (Werling et al. 2019). Furthermore, impairing egg development leads to fewer oocysts. For example, reducing egg numbers by EcR depletion leads to fewer *P. falciparum* oocysts, due to parasite death during the ookinete to oocyst transition (Werling et al. 2019). A similar reduction in oocyst numbers was observed upon depletion of Vg (which is induced by 20E) in the mouse parasite *Plasmodium berghei* (Rono et al., 2010). In these parasites, ookinete death was triggered by an immune response mediated by the mosquito thioester-containing protein (TEP1), a complement-like protein that coats and lyses ookinetes as they are crossing the midgut epithelium, suggesting that Vg may have a protective effect on the parasite by shielding it from the mosquito immune system. However, *An. gambiae* are not the natural vectors of *P. berghei*, and *P. falciparum* has

fundamentally different interactions with the *An. gambiae* immune system. Most notably, *P. falciparum* expresses a surface protein (Pfs47) that allows the parasite to evade lysis by TEP1 (Molina-Cruz et al. 2013; Canepa, Molina-Cruz, and Barillas-Mury 2016; Molina-Cruz et al. 2020). Since *P. falciparum* has never been tested under the conditions of Vg depletion, it is unclear whether oocyst numbers will be affected.

In addition to oocyst numbers, the rate of oocyst growth is also affected by reproductive processes. Oocyst size and egg number are inversely correlated, meaning if the female develops fewer eggs she has more nutritional reserves that the parasite can hijack for its own growth (Werling et al. 2019). Indeed, impairing egg development by *EcR* silencing or other means results in faster oocyst growth in *P. falciparum* (Werling et al. 2019). This phenotype has been linked to an increase in *Lp* expression, and upon *Lp* knockdown the increase in oocyst size observed in females with reduced number of eggs disappears. In other *Plasmodium* species, Lp appears to be accumulated by oocysts (Atella et al. 2009; Costa et al. 2018), and its contributions to growth rates underscore the importance of lipids during sporogonic development.

While oocysts are growing from day 3-12 post infection, mosquitoes have laid their eggs and are ready for the next oogenetic cycle, which begins with a second blood meal. An additional blood meal post infection results in remarkably faster oocyst growth (Shaw et al. 2020), likely due to additional protein that parasites can use for growth. Indeed, when mosquitoes were provided BSA during oocyst development, oocyst growth increased to levels comparable to oocysts exposed to a second blood meal (Kwon et al. 2021). These findings show that nutritional availability, as directed by reproductive processes of the mosquito, drives parasite growth rates.

Overall, although egg development and parasite development are happening in two distinct tissues of the mosquito, both processes appear to utilize the same nutrient and nutrient transporters,

with the parasite being able to benefit from any surplus resources. In the previous chapter we showed that Vg depletion results in higher amino acid levels in the mosquito fat body due to a lack of incorporation into the protein itself (Stryapunina et al. 2023). However, it is unknown whether Vg depletion will influence *P. falciparum* oocyst growth or nutrient distribution in the midgut and hemolymph. This study aims to characterize whether Vg plays a protective role in *P. falciparum* infection and whether it is important for parasite growth. Ultimately, knowledge of these processes may lead to better tools for malaria control, at a time when widespread insecticide resistance in mosquitoes is threatening the successful application of current control strategies (*World Malaria Report* 2022).

Results:

***Vg* knockdown increases the speed of *P. falciparum* oocyst development**

We analyzed the role of Vg in the development of the human malaria parasite *P. falciparum*, the initial phases of development of which temporally coincide with Vg incorporation into the eggs. To this end, we injected dsRNA targeting *Vg* (ds*Vg*) into 1 day old virgin females (Figure 3.1A). Three days post injection, females were fed on a *P. falciparum*-infected blood meal. Seven days post infection midguts and ovaries were dissected to yield paired oocyst and egg data per female.

As expected, Vg depletion resulted in a decrease in egg numbers, confirming our previous work (Stryapunina et al. 2023) (Figure 3.1B). It however did not affect oocyst prevalence or intensity (Figure 3.1C), contrary to similar experiments with *P. berghei* which had demonstrated a decrease in oocyst numbers (Rono et al. 2010). There was a positive correlation between egg and oocyst numbers highlighting the non-competitive nature of *P. falciparum* infections in *An.*

gambiae mosquitoes (Figure 3.1D), as has been previously shown (Werling et al. 2019). This positive correlation was not impacted by *Vg* knockdown, suggesting that Vg does not impact the equilibrium between parasite infection and mosquito fitness.

Although there was no difference in oocyst numbers, we noticed that oocysts were larger in size (Figure 3.1E and F), suggestive of increased growth rates, a phenotype not detected in *P. berghei* (Rono et al. 2010). This increase in growth rate resulted in sporozoites reaching the salivary gland of the mosquito sooner, making them infectious earlier upon *Vg* knockdown (Figure 3.2). Indeed, at day 10 post infectious blood meal (pIBM), an early timepoint for sporozoite invasion of the glands, there were more sporozoites in the salivary glands of Vg-depleted mosquitoes than in controls (ds*Lacz* injected) (Figure 3.2A), and at 12d pIBM 84% of ds*Vg* females had sporozoites in their salivary glands compared to 57% of the controls (Figure 3.2B). By 14 d pIBM there was no difference between the two groups (Figure 3.2C). Combined with the observation that there was no difference in oocyst numbers but only in oocyst size, these results demonstrate that parasites are growing faster in *Vg*-silenced females.

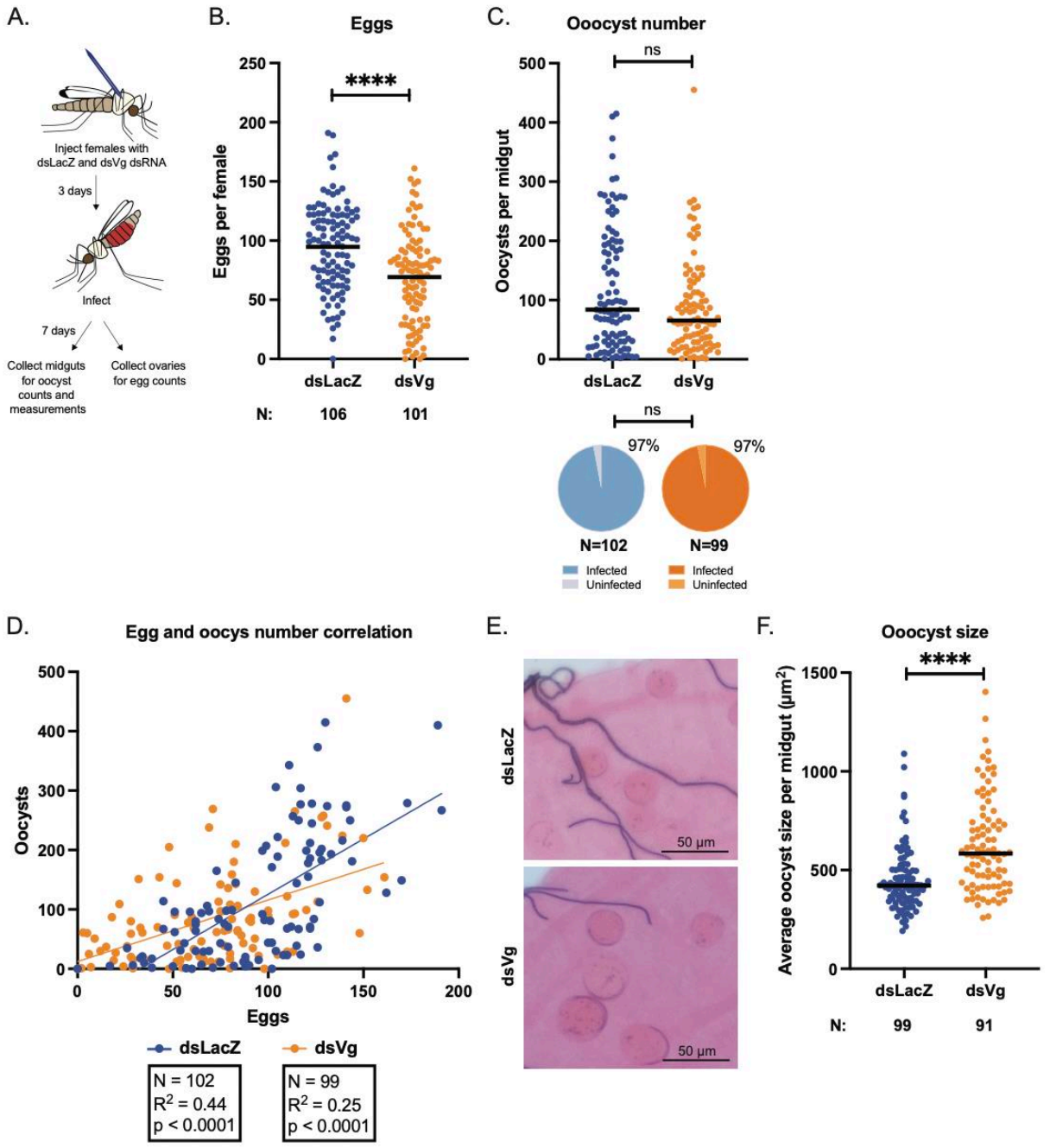

Figure 3.1. *Vg* knockdown increases the speed of *P. falciparum* oocyst development. (A) Experimental scheme: one day old virgin females were injected with dsRNA against *LacZ* or *Vg*. After 3 days they were given an infectious blood meal, and at day 7 post infectious blood meal (pIBM) midguts and ovaries were dissected to assess oocysts and eggs. (B) The number of eggs in infected females is lower in the ds*Vg* group; each dot represents eggs per female; N = number of females, pooled from four biological replicates (Unpaired t test: **** = $p < 0.0001$). (C) Following *Vg* knockdown there is no difference in oocyst intensity or prevalence; each dot represents the number of oocysts per female; the pie charts show the prevalence (percent of infected females); N = number of females, pooled from four biological replicates (Mann-Whitney for intensity and

Fisher's exact test for prevalence: ns = not significant). (D) *Vg* knockdown does not affect the positive correlation between egg and oocyst numbers; each dot corresponds to the number of oocysts and eggs that a single female developed (Simple linear regression; four replicates). (E and F) Oocyst size is increased upon *Vg* knockdown as measured at 7d pIBM; (E) representative oocyst images taken using light microscopy of mercurochrome-stained midguts; (F) quantification of average oocyst size; each dot is the average size of all oocysts in a midgut; midguts with fewer than 3 oocysts are excluded; N = number of females, pooled from four biological replicates (Mann-Whitney: **** = $p < 0.0001$).

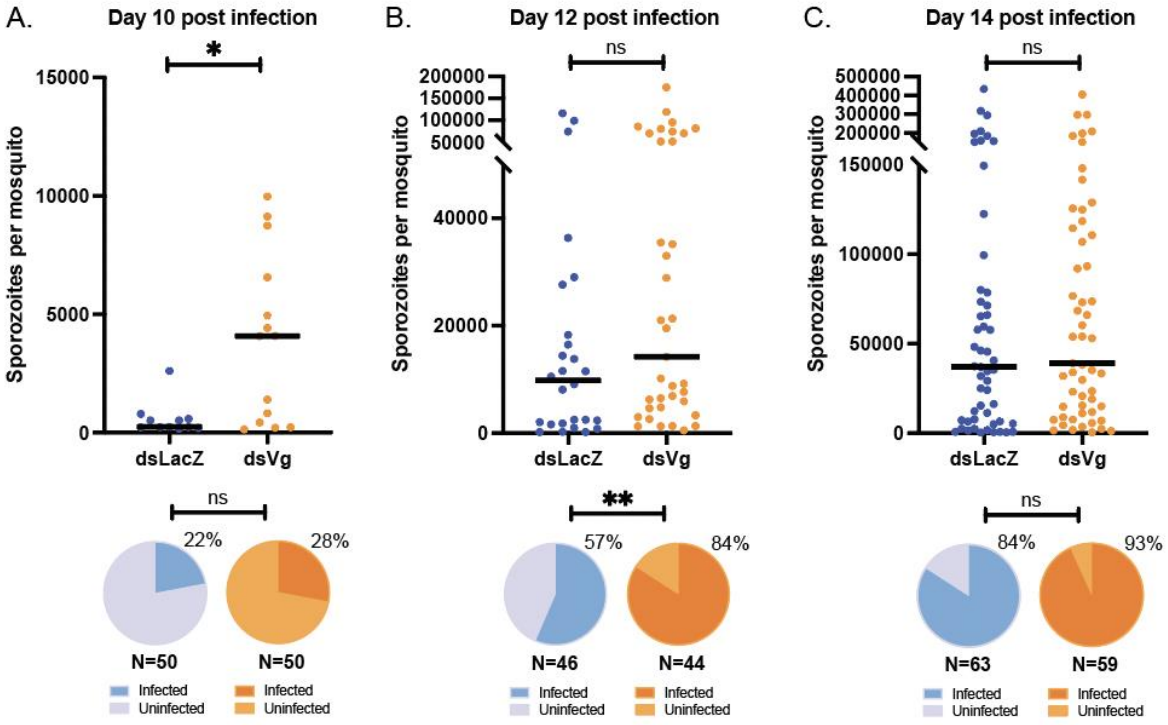

Figure 3.2. *Vg* knockdown increases the speed of *P. falciparum* sporozoites reaching the salivary glands of *An. gambiae* mosquitoes. (A, B and C) Number of sporozoites in the salivary glands of ds*LacZ* and *dsVg* females at 10, 12 and 14d pIBM; each dot is representative of the total sporozoite count in a single female; the pie charts represent the percent of infected and uninfected females; N = number of females, pooled from three biological replicates (Mann-Whitney for intensity and Fisher's exact test for prevalence: ns = not significant, * = $p < 0.05$, ** = $p < 0.01$).

Effects of *Vg* silencing on egg and parasite development are affected by Lp

Since previous work showed that Lp upregulation results in larger oocyst size (Werling et al. 2019), we asked whether the increase in *Lp* expression due to Vg depletion could be driving the difference in parasite development. To this end, we confirmed that *Lp* upregulation first displayed in our previous work (Stryapunina et al. 2023) persists during infection (Figure 3.3A). We then performed a double knockdown of *Vg* and *Lp*, which was successful as shown by mRNA quantification at 24h pIBM, and compared this group to controls as well as individual *Vg* and *Lp* knockdowns (Figure 3.3B and C).

Females with ds*Vg* and ds*Lp* double knockdown developed no eggs (Figure 3.3D), a phenotype that was more striking than the reduction in eggs induced by the two transporters when silenced individually, further underscoring the importance of these two nutrient carriers for fecundity. Moreover, they had significantly fewer oocysts per midgut compared to all other groups, including the ds*Lp* group which was previously demonstrated to cause a reduction in oocyst numbers (Figure 3.3E) (Werling et al. 2019). Oocyst size results were more puzzling. While we saw the expected increase upon *Vg* knockdown, we found a surprizing increase in ds*Lp* females, not previously observed in published work (Figure 3.3F) (Costa et al. 2018; Werling et al. 2019). Double knockdown resulted in an intermediate phenotype and oocyst size in ds*Vg*-ds*Lp* females was not statistically different from any other group.

We also assessed sporozoites in the salivary glands at day 12 pIBM, which is an intermediate timepoint for mosquito infectivity with the largest effect upon Vg depletion (Figure 3.2B). A total of 80% of ds*Vg* females had sporozoites in their salivary glands at this time point, while prevalence was less than 50% in the ds*LacZ*, ds*Lp*, and ds*Vg*/ds*Lp* groups (Figure 3.3G). Sporozoite prevalence for the ds*Lp* group is surprisingly not different from control although ds*Lp*

oocysts were larger. This could be due to the slightly lower number of oocysts in the midgut, or could signify that although oocysts in the ds*Lp* group were larger they did not actually develop faster. Intensity of infection also appeared to be higher upon *Vg* knockdown although it did not reach statistical significance, while ds*Lp* and ds*Vg*-ds*Lp* females had significantly lower sporozoite intensities than the ds*Vg* group. Together, these results point toward Lp indeed being involved in increasing the speed of parasite development upon Vg depletion since double knockdown of ds*Vg*/ds*Lp* results in an intermediate phenotype for oocyst size, and slows down the speed of sporozoite development to control levels, although the lower numbers of oocytes upon double knockdown could also impact the sporozoite results.

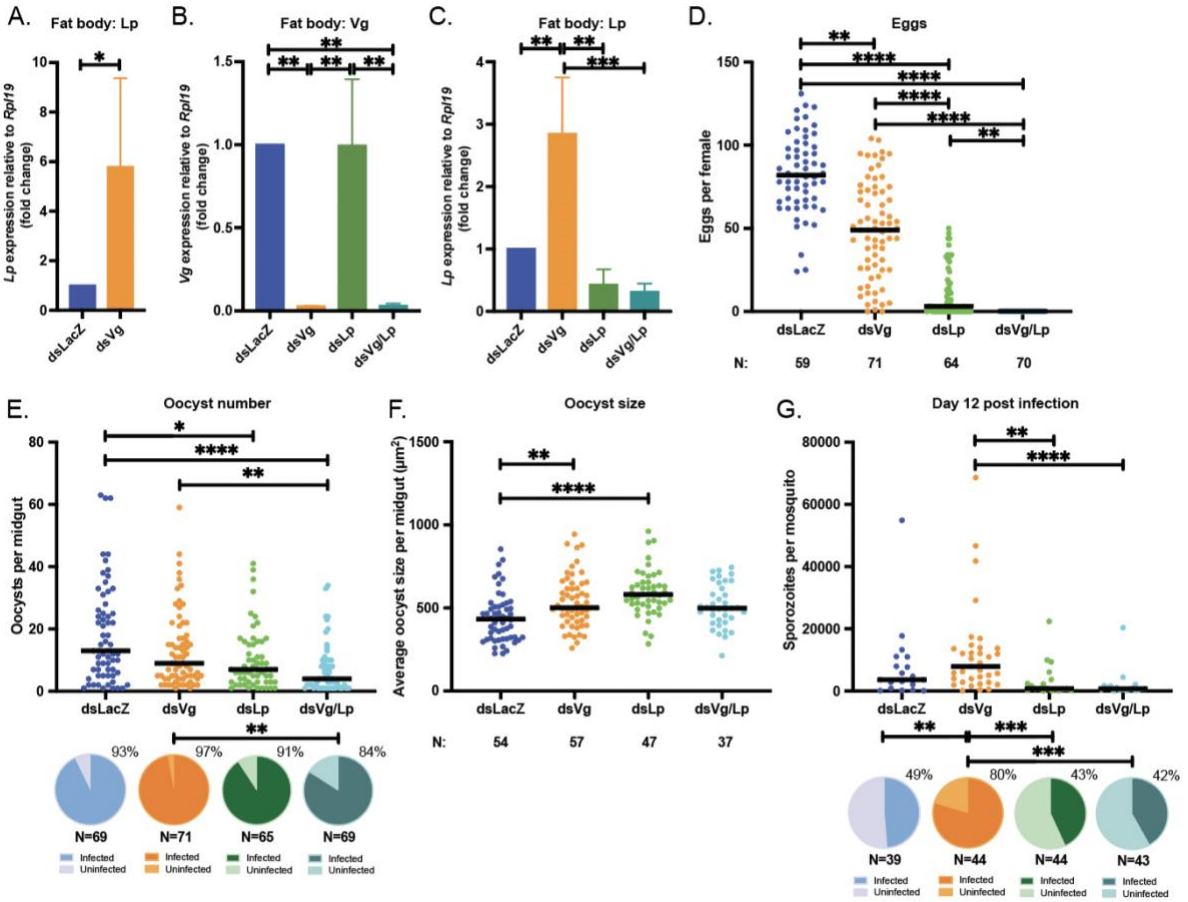

Figure 3.3. Effect of *dsVg* and *dsLp* double knockdown on egg and parasite development. (A) *Lp* mRNA levels, as measured by RT-qPCR in the fat body 24h post infection, are upregulated upon Vg depletion; all values normalized to ds*LacZ* (Unpaired t-test: * = $p < 0.05$; four biological replicates). (B) *Vg* mRNA is successfully depleted as measured by RT-qPCR in the fat body 24h post infection; all values normalized to ds*LacZ* (ANOVA: ** = $p < 0.01$; three biological replicates). (C) *Lp* mRNA is successfully depleted as measured by RT-qPCR in the fat body 24h post infection; all values normalized to ds*LacZ* (ANOVA: ** = $p < 0.01$, *** = $p < 0.001$; three biological replicates). (D) Females with depleted Vg, Lp or both develop fewer eggs; each dot represents eggs per female; N = number of females, pooled from three biological replicates (Kruskal-Wallis with Dunn's multiple comparisons: ** = $p < 0.01$, **** = $p < 0.0001$). (E) The number of oocysts developed in the four experimental groups at 7 days post infection; each dot represents oocysts per female; the pie charts show the percent of infected females; N = number of females, pooled from three biological replicates (Kruskal-Wallis with Dunn's multiple comparisons for intensity and Fisher's exact test for prevalence: * = $p < 0.05$, ** = $p < 0.01$, **** = $p < 0.0001$). (F) Oocyst size as measured at 7 days post infection; each dot is the average size of all oocysts in a midgut; midguts with fewer than 3 oocysts are excluded; N = number of females, pooled from three biological replicates (Kruskal-Wallis with Dunn's multiple comparisons: ** = $p < 0.01$, **** = $p < 0.0001$). (G) Number of sporozoites in the salivary glands of ds*LacZ*, ds*Vg*, ds*Lp* and *dsVg/Lp* females at 12 days post infection; each dot is representative of total sporozoites from a single female; the pie charts represent the percent of infected and uninfected females; N = number of females, pooled from three biological replicates (Kruskal-Wallis with Dunn's multiple comparisons: ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$).

Lipidomic analysis of midguts, hemolymph and fat bodies shows phospholipid accumulation upon Vg depletion

Since parasites require lipids for growth (van Schaijk et al. 2014; Nyasembe et al. 2023; Costa et al. 2018), and our finding of Lp upregulation upon *Vg* silencing, we performed lipidomic analysis of mosquito tissues in ds*Vg* females. We selected midguts, hemolymph and fat body as the tissues for analysis since oocysts are in direct contact with these mosquito tissues and can in theory exchange nutrients with all of them. We selected 0h, 24h, 48h and 72h PBM as timepoints since they represent the points of most Vg production (24h PBM) and early timepoints of oocyst development post establishment under the basal lamina of the midgut (48h and 72h pIBM), respectively. Of note, experiments were performed with uninfected females.

Our results show that total lipids, as well as the lipid classes of neutral lipids were not affected upon Vg depletion (Figure 3.4). However, phospholipids significantly increased at 24h PBM in the hemolymph, and at 48h and 72h PBM in the fat body, which are critical early timepoints in oocyst growth. There was also a significant increase in glycerophospholipids in the fat body at 48h PBM. Increase in phospholipids is of especial interest since they are carried by Lp, which has been shown to enter the oocysts, and since phospholipids may represent an important nutrient for parasite development since they are imperative for membrane formation, a key process during sporogony. No lipid class was affected in the midgut.

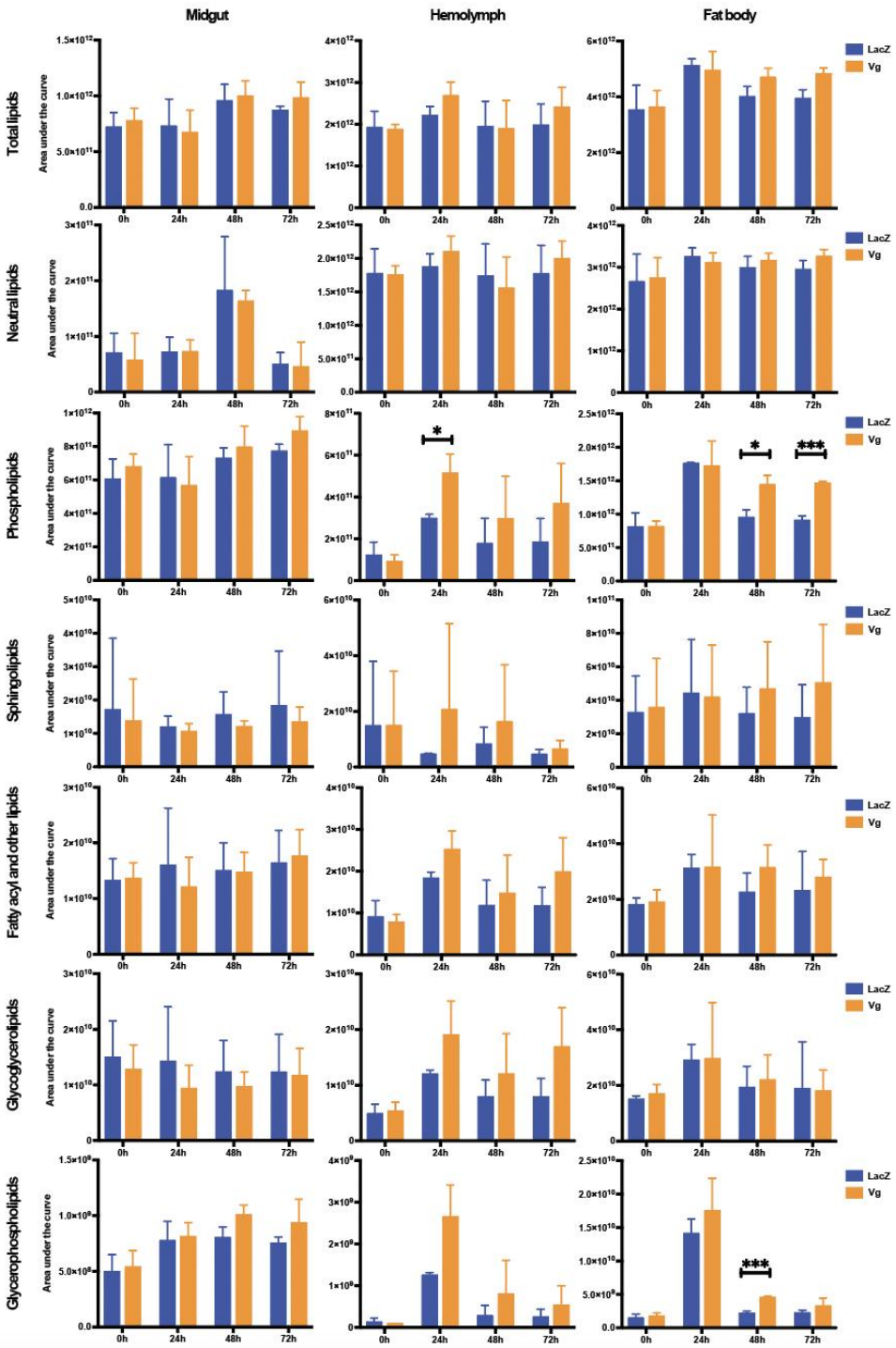

Figure 3.4. Lipidomic analysis of ds*Vg* midguts, hemolymph and fat bodies.
Midguts, hemolymph and fat bodies were collected from ds*LacZ* and ds*Vg* mosquitoes at 0, 24, 48 and 72 hours PBM. Lipidomic analyses were carried out using liquid chromatography and mass spectrometry. Areas under the curve are shown for the total lipids as well as the lipid classes of neutral lipids, phospholipids, sphingolipids, fatty acyl and other lipids, glycoglycerolipids and glycerophospholipids (Multiple unpaired t tests with FDR correction: * = $p<0.05$, *** = $p<0.001$; four biological replicates).

Amino acid levels are increased in the hemolymph and fat body upon Vg depletion

In addition to lipidomics, we also performed metabolomic analyses of midgut, hemolymph and fat body tissues in uninfected females upon Vg depletion at 0h, 24h, 48h, and 72h PBM (Supplemental Figure 5). All three tissues displayed major regulation of compounds upon blood feeding. We focused on amino acids since Vg is an amino acid transporter, and since we have previously shown that its knockdown results in an upregulation of total amino acids in the fat body (Stryapunina et al. 2023). In all three tissues amino acids are majorly upregulated upon blood feeding, and there does not appear to be much of a difference between control and Vg-depleted groups at 0h, 24h, or 72 PBM (Figure 3.5A). However, upon 48h PBM all three tissues showed an increase in many amino acids upon *Vg* knockdown. 48h pIBM is a critical timepoint for the parasite at which it has established itself as an oocyst and is starting to grow. It seems that upon *Vg* knockdown it receives a valuable boost in amino acids, potentially fueling its faster growth. Indeed, previous work has shown that providing an additional proteinaceous meal containing BSA following an infectious blood meal results in faster parasite growth (Kwon et al. 2021). We tested whether this effect is due to amino acids by supplementing them to the sugar solution of previously infected females starting at 3 days post infection, which also resulted in larger oocyst size (Figure 3.5B).

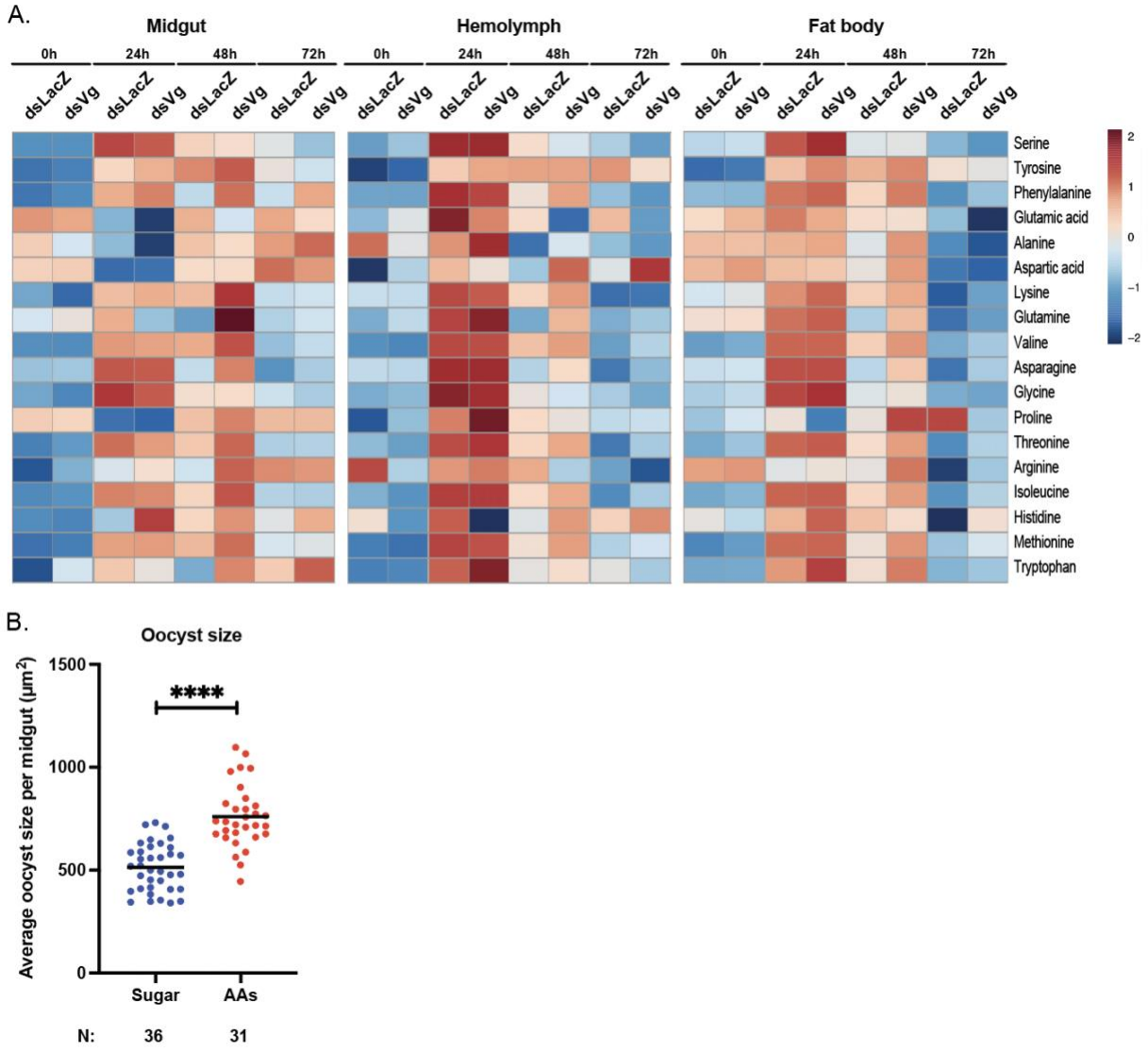

Figure 3.5. Amino acids are increased upon Vg depletion. (A) A total of 18 amino acids (leucine and cysteine were not detected), as identified by metabolomic mass spectrometry analysis, were measured in the midgut, hemolymph and fat body of control and Vg-depleted females at 0h, 24h, 48h and 72h PBM; area under the curve is visualized using Metaboanalyst (four biological replicates for midgut and hemolymph; three biological replicates for fat body). (B) Oocyst size is increased when amino acids are supplemented in the sugar starting at three days pIBM; mean oocyst size per midgut at 7 days pIBM is shown; midguts with fewer than 3 oocysts were excluded. N = number of females, pooled from two biological replicates (Unpaired t test: **** = $p < 0.0001$; two biological replicates).

Discussion:

P. falciparum parasite development is intimately linked with mosquito reproductive processes. The parasite has co-evolved with its arthropod host for 13-64 million years, ensuring that it does not reduce her reproductive output (Shaw, Marcenac, and Catteruccia 2022). Firstly, it has to ensure that the female's immune system does not get activated during infection and thus divert resources from reproduction. Secondly, the parasite needs to thread a delicate balance of utilizing mosquito nutrients for its own growth without jeopardizing the female's developing eggs.

This study further explores the relationship between mosquito reproduction and *P. falciparum* development by assessing the function of the yolk protein Vg, which is essential for fertility, during the parasitic mosquito stages. Although Vg has immune functions in *An. gambiae* when infected with *P. berghei* by shrouding the parasite from TEP1 identification, it does not appear to protect *P. falciparum* in a similar manner. Vg depletion did not influence oocyst numbers, which is consistent with *P. falciparum*'s expression of Pfs47 safeguarding it against TEP1-mediated lysis, thus making Vg dispensable in this protective role.

However, Vg affected the speed of parasite development. Vg depletion increased oocyst size and reduced the time it takes sporozoites to invade the salivary glands. This increase in the speed of development could be partially attributed to an increase in Lp levels upon *Vg* knockdown. Indeed, fewer sporozoites reached the salivary glands at 12 days pIBM in the double knockdown of *Vg/Lp* compared to *Vg* alone. However, the double knockdown also resulted in lower oocyst numbers, which could have also led to a decrease in sporozoite prevalence on day 12, making the data difficult to interpret in an unequivocal manner. Similar experiments have been carried out showing that Lp upregulation resulted in faster parasite growth upon *EcR* knockdown. Considering

that *Vg* transcription is under EcR control, the same mechanism of increase in the speed of parasite growth is likely.

Lipids are an essential nutritional resource that malaria parasites can synthesize or scavenge from their hosts at multiple stages of the life cycle (O'Neal et al. 2020). Liver stage parasites scavenge host phospholipids and cholesterol, as well as undertake their own lipid biosynthesis (Itoe et al. 2014; Labaied et al. 2011; Kluck et al. 2019), while blood stage parasites acquire lipid building blocks such as ethanolamine, choline and fatty acids from serum (Mikkelsen et al. 1988; Wein et al. 2018). Lipid scavenging during mosquito stages has not been studied as thoroughly, but *P. falciparum* has been shown to upregulate the mosquito adipokinetic hormones which mobilize lipid reserves (Nyasembe et al. 2023). A study in *Plasmodium gallinaceum* has shown that Lp is internalized by oocysts (Atella et al. 2009), and other studies have shown that upon Lp depletion *P. berghei* oocysts fail to accumulate neutral lipids, and both *P. falciparum* and *P. berghei* oocysts exhibit slower growth (Rono et al. 2010; Costa et al. 2018). The consequence of this impaired development upon *Lp* knockdown for *P. berghei* was that the sporozoites were less infectious when exposed to liver cells (Costa et al. 2018). However, other studies were not able to confirm that Lp depletion results in smaller oocysts under otherwise normal reproductive conditions (Werling et al. 2019).

Here we show that phospholipids and glycerophospholipids are significantly upregulated in the mosquito fat body during early time points of oocyst growth (48h and 72h pIBM). Lipid classes do not seem to be affected in the midgut upon *Vg* knockdown. A caveat of these experiments is that they were performed in uninfected females; thus, if a parasite sequesters nutrients in the midgut these analyses would not be able to identify those changes. These changes in phospholipids are of interest to oocyst growth since Lp travels between tissues, has been shown

to transport phospholipids and be internalized into oocysts. Phospholipids are also of especial importance to the growing oocysts due to their need to build membranes of developing sporozoites. Thus, an increase in phospholipids may increase the speed of parasite development resulting in earlier sporozoite production. Future work can assess which phospholipids are necessary for sporozoite development, and whether they are indeed internalized by the oocysts.

Another explanation for an increase in the speed of oocyst development could be that Vg depletion leads to an increase in amino acid concentrations in mosquito midgut, hemolymph and fat body, thus resulting in more protein building blocks for the parasite. Indeed, supplementation with amino acids post infection results in larger oocysts (Figure 5A). To prove that increased amino acid levels lead to faster growth upon ds*Vg* knockdown we require parasite amino acid transporter knock out (KO) lines. If we see no increase in parasite size upon *Vg* knockdown in the KO line, this would show that amino acids are involved in expediting growth upon Vg depletion.

An influx of free amino acids leads to activation of TOR signaling, which is a master regulator of cell growth and division in response to nutritional stimuli. As shown in our previous work, Vg depletion also leads to an increase in TOR signaling in the fat body at 24h PBM (Stryapunina et al. 2023), which is instrumental in regulating cell size in many species including insects (Jacinto and Hall 2003). Although TOR signaling is highly conserved in eukaryotes, apicomplexans have lost the majority of the pathway's components including the TOR kinase (Serfontein et al. 2010; van Dam et al. 2011). Future work could assess the impact of inhibiting mosquito TOR signaling by applying rapamycin, which is a TOR inhibitor, to the mosquito, or carrying out *TOR* dsRNA knockdown experiments, since these manipulations would not directly affect the parasite. Similar experiments in *Anopheles stephensi* with *P. berghei* parasites have shown a decrease in oocyst numbers but the authors did not provide oocyst size results (Feng et

al. 2021). *TOR* knockdown in the mosquito would most likely increase available amino acid levels since TOR is a global translation activator. The results should thus be similar to feeding mosquitoes amino acids, as was done in Figure 3.5B. Although it has lost TOR machinery, *P. falciparum* has retained the ability to respond to the amino acid concentrations in its environment utilizing Maf1, which is repressor of RNA Pol III (Babbitt et al. 2012; McLean and Jacobs-Lorena 2017). Thus, one could utilize an Maf1 KO *P. falciparum* line to assess nutrient sensing upon *Vg* knockdown.

Overall, the results of this study characterize Vg as an essential metabolic molecule that is important for proper levels of lipids and amino acids throughout the female's body, in addition to its function in embryogenesis. Upon Vg depletion, there was an increase in phospholipids in the hemolymph and fat body, and an increase in glycerophospholipids in the fat body. Furthermore, Vg depletion resulted in higher amino acid levels in the midgut, hemolymph and fat body. *P. falciparum* can hijack this higher nutrient availability and as well as nutrient transporters, such as Lp, from its mosquito host to its own benefit, resulting in faster growth. Specifically, the hemolymph is vital to the parasite, as it is bathed in it, while it is separated from the midgut and the fat body by other membranes. Furthermore, there is a possible additive effect of both Lp-shuttled lipids and amino acids resulting in increased parasite growth upon Vg depletion. These findings bring support to the existence of a metabolic link between mosquito reproduction and parasite development, even though these processes are happening in different tissues.

Finally, our data are important for vector control measures that aim to affect reproduction since they add to a growing body of evidence showing that impairing egg development results in faster growing parasites (Werling et al. 2019). If a vector control intervention that imparts sterility is utilized it could actually hamper malaria control efforts by making females infectious sooner. Studying mosquito reproduction not only helps us better understand their basic biology in hopes

of controlling vector populations but also provides insight into how parasites can hijack these processes to ensure their own transmission.

Materials and Methods:

Mosquito lines and rearing:

Anopheles gambiae G3 colony was reared at 27°C at 70-80% humidity. The colony was fed with 10% glucose solution and purchased human blood (Research Blood Components, Boston, MA). Females and males were separated as pupae for all experiments.

***P. falciparum* culture and infections:**

P. falciparum NF54 strain was cultured at 37°C in RPMI medium 1640 supplemented with 25mM HEPES, 10mg/l hypoxanthine, 0.2% sodium bicarbonate, and 10% heat-inactivated human serum (Interstate Blood Bank) at 5% O_2, 5% CO_2 and balanced N_2 between 0.2 and 2% parasitemia for up to 8 weeks (Trager and Jensen 1976; Ifediba and Vanderberg 1981). To induce gametocytogenesis, parasitemia was raised to 4%, and cultures were incubated for 14-20 days with daily media changes. This strain is used with permission by a material transfer agreement from Carolina Barillas-Mury.

For infections, 4 day old mosquitoes (3 days post injection) were blood fed on ~300 μL of parasite culture using a membrane feeder. Any unfed mosquitoes were removed post blood feeding. Mosquitoes were given free access to cotton balls soaked in 10% glucose for the duration of the experiments.

Oocyst counts and measurements:

7 days post infection mosquitoes were aspirated into 80% ethanol and then transferred to PBS. Midguts were dissected and stained with 2 mM mercurochrome (mercury dibromofluorescein

disodium salt from Sigma-Aldrich) in diH_2O. Midguts were mounted using 0.2 mM mercurochrome and imaged at 100X on the Olympus Inverted CKX41. Images from two planes were stitched together using Duo Peng's code deposited on GitHub (https://github.com/duopeng/image_merge-z-stack_and_stitch). Oocysts were counted and their size was measured using FIJI (Schindelin et al. 2012). Midguts with fewer than 3 oocysts were excluded from measurements. Ovaries from the same mosquitoes were dissected in a paired fashion and stored in 70% ethanol for later counts.

Sporozoite counts:

At 10, 12 or 14 days post infection mosquitoes were aspirated into ice-cold PBS and their heads were cleaved from the rest of the body. Salivary glands were dissected into a small volume of PBS (about 30-40 µL). Salivary glands were homogenized using a hand-held pestle, which was then rinsed with 100 µL of PBS to collect any sporozoites clinging to it. Samples were centrifuged at 8,000g for 10 minutes at 4°C, and 100 µL of supernatant was removed leaving about 30-40 µL, which was then re-suspended and volume was measured. Sporozoites in 0.1 µL were counted using a disposable hemocytometer (C-Chip from INCYTO) at 200X magnification using the Olympus Inverted CKX41 with phase-contrast. The number of sporozoites per mosquito was calculated using the following formula:

$$(\text{Sporozoites counted}) \times 10^4 = \#\text{ of sporozoites in } 1000\ \mu L$$

$$(\#\text{ of sporozoites in } 1000\ \mu L)/1000\ \mu L \times (\text{volume measured}) = \#\text{ of sporozoites per mosquito}$$

dsRNA generation, dsRNA injection, RNA extraction, cDNA synthesis and RT-qPCR:

These techniques were performed as described in the previous chapter.

Amino acid supplementation:

Three days post infection 10% glucose solution containing amino acids at physiological concentrations was supplied to the mosquitoes (Chung et al. 2017). Control mosquitoes were provided with 10% glucose solution.

Lipidomics and metabolomics sample collection:

10 midguts and fat bodies were collected into 1 mL methanol and stored at -80°C until processing. Blood bolus was removed from midguts at 24h and 48h. Midguts with no blood bolus were washed in the same manner as those with a blood bolus to ensure that processing did not affect the analysis. Hemolymph from 10 mosquitoes was collected by making a small incision on the seventh ventral abdominal segment and injecting diH_2O into the thorax. The first drop for every mosquito was collected into a microcentrifuge tube. The volume was measured and additional diH_2O was added to equal 200 µL. 800 µL of methanol was added to make 1 mL of solution and stored at -80°C until processing. Samples were processed as described in the previous chapter.

Lipidomics and metabolomics mass spectroscopy:

These techniques were performed as described in the previous chapter.

Statistical analyses:

All statistical tests were performed in GraphPad Prism 9.0. The number of replicates and statistical tests performed are mentioned in the figure legend.

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4
Discussion

The role of Vg in egg development

The findings outlined in this thesis shed light on the processes regulating egg development in *An. gambiae*. Specifically, these findings reveal a previously underappreciated interplay between the two major nutrient transporters, Vg and Lp. During egg development, the first phase of Lp-mediated lipid accumulation is halted or slowed down by the expression of Vg, whose incorporation into the oocytes prevents excessive accumulation of lipids by Lp. Thus, this work adds to our understanding of egg development by showing that the lipid deposition phase is regulated by the protein deposition phase. Furthermore, the findings show that Vg has a feedback loop on TOR signaling. It is known from other species that TOR signaling turns on *Vg* expression (Hansen et al. 2004), which we also confirmed for *An. gambiae*. To our knowledge, this is the first work showing that Vg expression, in turn, affects TOR signaling. Indeed, *Vg* knockdown resulted in upregulation of phosphorylated S6K, which is downstream of TOR kinase. This upregulation of TOR signaling also led to increased Lp levels.

These observations help us reconstruct the series of events triggered by blood feeding. First, lipids mobilized from the midgut and fat body are transported by Lp into the ovaries. This is paralleled by ecdysone synthesis and release by the ovaries, and 20E synthesis in the fat body. When amino acids derived from the blood meal are incorporated into the Vg polypeptide following the 20E peak, TOR signaling is decreased, which in turn decreases *Lp* levels, resulting in a switch from lipid to protein deposition into the eggs. Together with previously published work, this study adds to our understanding of *Lp* regulation. Specifically, this work shows that Vg and TOR regulate *Lp* expression at the early (12h and 24h) timepoints after blood feeding, while 20E signaling via EcR regulates *Lp* downregulation at 36h and 48h PBF (Werling et al. 2019). Future experiments could further solidify the link between Vg, TOR signaling and Lp by performing a double

knockdown of *TOR* and *Vg* rather than using an inhibitor such as rapamycin, as well as injecting amino acids into the hemolymph and measuring *Lp* levels to identify whether it is indeed amino acid signaling that is responsible for regulation of *Lp* expression.

In other insect species 8-15% of Vg is composed of lipids, specifically phospholipids, hydrocarbons, diglycerides and triglycerides carried in a lipid-binding cavity (Maruta et al. 2002; Swevers et al. 2005; Raikhel and Dhadialla 1992; Thompson and Banaszak 2002). To the best of our knowledge, the lipid content of mosquito Vg has not been assessed. The lipidomics results in this thesis show that significantly fewer phospholipids and glycerophospholipids were present in embryos 3-5h post oviposition, while significantly more phospholipids and glycerophospholipids were retained in the fat body. A hypothesis that results from these findings is that *An. gambiae* Vg transports phospholipids and glycerophospholipids from the fat body to the ovaries during egg development. A future study could characterize the lipids carried by Vg post blood feeding. Furthermore, Lp-carried lipids have only ever been characterized in unfed mosquitoes (Atella et al. 2006), and the lipid profile is likely vastly different upon blood feeding, yet, it remains to be characterized.

Vg is essential for *An. gambiae* fertility

Vg depletion results in a fully penetrant infertility phenotype. This phenotype was first observed by Rono et al., however, it was only briefly mentioned and data were not shown (Rono et al. 2010). Due to Vg's multiple functions (source of amino acids, ecdysteroid storage, and antioxidant functions), we wondered which one of these was most likely to be causing the infertility. DAPI staining of the embryos' nuclei allowed us to determine that embryonic development was stopped very early in embryogenesis, before the formation of a syncytial

blastoderm 4h post oviposition. Vg, a known antioxidant molecule implicated in longevity, is upregulated in the spermathecae upon mating (Seehuus et al. 2006; Rogers et al. 2008). Thus, we considered its potential function in sperm storage as a possible cause of infertility. However, if this were the case, oviposited eggs would not have undergone mitotic divisions, since poorly stored sperm would be defective and unable to undergo fertilization. Thus, a possible role of Vg in sperm maintenance is unlikely as a cause of infertility. In other insect species, ecdysteroids have been found bound to vitellin and released to regulate organ formation and cuticle deposition (Lagueux et al., 1981; Bownes et al., 1988; Yamada et al., 2005). However, Vg-depleted embryos do not reach the organ formation stage; thus, this hypothesis is unlikely as well. The developmental stage at which embryos are blocked most closely aligns with Vg's function as a source of amino acids. Indeed, when *D. melanogaster* embryos are treated with cycloheximide, which is a translation inhibitor, embryos do not make it past the blastoderm stage (Marcos et al. 1982). Vg-depleted embryos have significantly decreased amounts of 14 of 19 detected amino acids, which would prevent loading of tRNAs and effective translation potentially leading to apoptosis (Sikalidis 2013; Dong et al. 2000; Qin et al. 2017). Consistent with this hypothesis, nuclei of embryos derived from ds*Vg* mothers displayed blebbing, which is characteristic of apoptosis. Future experiments, such as ribosomal profiling and caspase cleavage assays, are needed to determine whether translation is stalled, and whether embryos are indeed undergoing apoptosis. TOR signalling in embryos is most likely also impacted due to a decrease in amino acids, and this signalling remains to be assessed. Finally, whether ecdysteroids are stored in yolk and their role during embryonic development remain to be fully studied *An. gambiae*.

Since Vg depletion results in complete infertility, this phenotype could potentially be translated into vector control methods. There are multiple potential ways of reducing Vg levels in

mosquitoes in a field settings. One could potentially encapsulate dsRNA into liposomes or nanoparticles and deliver those by attractive sugar baits or residual spraying (Coy et al. 2012; Airs and Bartholomay 2017). There are also multiple compounds that have been shown to decrease Vg levels. Fluvastatin and allatostatin inhibit Vg release from fat body in cockroaches due to an effect on glycosylation (Martín, Piulachs, and Bellés 1996). Pyriproxyfen, juvenile hormone analog, dramatically inhibited Vg synthesis in honeybees, and pyriproxyfen-treated nets reduced fertility in mosquitoes in Burkina Faso (Pinto, Bitondi, and Simões 2000; Grisales et al. 2021). Gene drives for knockout of *Vg* or *VgR* could be utilized. Indeed multiple CRISPR-Cas9 gene drives have been developed targeting reproductive proteins in *An. gambiae*, leading to sterility (Hammond et al. 2016; Kyrou et al. 2018). Since Vg is incorporated into the eggs by receptor-mediated endocytosis one could imagine blocking the interaction between Vg and VgR with peptides or with chemicals designed to target the binding region (Hershberger, Lee, and Chmielewski 2007; Arkin, Tang, and Wells 2014). This method would require better understanding of the specific residues involved in Vg binding to VgR. Although most of these strategies may be far-fetched at present, improvements of systems for delivering small molecules to mosquitoes could be instrumental for their future use in mosquito control.

The role of Vg as an immune molecule in *An. gambiae* and other mosquitoes remains to be determined

Although Vg depletion resulted in lower parasite numbers upon *P. berghei* infection due to TEP1-mediated lysis (Rono et al. 2010), these results could not be replicated in *P. falciparum*, in line with previous publications showing that *P. falciparum* does not activate the *An. gambiae* immune system (Molina-Cruz et al. 2013) and adding evidence to a line of thought arguing that

coevolution of *P. falciparum* with *An. gambiae* has resulted in mosquito immune tolerance to infection (Marcenac et al. 2020).

Vg is a known immune molecule in insects and fish (Tong et al. 2010; Singh et al. 2013; Salmela, Amdam, and Freitak 2015). Although Vg does not seem to have immune functions during *P. falciparum* infection since there was no difference in oocyst number, future experiments should assess Vg as an immune molecule in bacterial or viral infection in this species. In anophelines, Vg immune function against bacteria would be valuable to evaluate to better understand mosquito interactions with their microbiome, and since anophelines are vectors of viruses like O'nyong'nyong and Mayaro, the antiviral functions of this yolk protein could also be examined (Rezza, Chen, and Weaver 2017; Brustolin et al. 2018).

Studying Vg's immune functions in *Ae. aegypti* mosquitoes is even more imperative since they can transmit arboviruses vertically from mother to offspring, which is postulated to be how the virus persists during inter-epidemic periods (Danis-Lozano et al. 2019). Vertical transmission is poorly studied because it was believed to happen at low frequency. However, Sanches-Vargas *et al*., showed that this frequency increases from 10% during the first gonotrophic cycle to 55% during the second gonotrophic cycle, and the mechanism of vertical transmission has not yet been identified (Sánchez-Vargas et al. 2018). Honeybee Vg can bind bacterial pathogen-associated molecular patterns (PAMPs) and carry them into the developing oocytes (Salmela *et al*., 2015), and Vg has also been shown to bind a plant virus in the brown planthopper, facilitating viral transmission (Huo *et al*., 2018). Vg could thus bind dengue virions in the hemolymph and carry them into the *Ae. aegypti* ovary, which is usually an immune privileged site, favoring vertical transmission.

Vg depletion expedites parasite development

Although Vg depletion does not affect *P. falciparum* numbers, it speeds up parasite development, resulting in larger oocyst size and earlier sporozoites invasion of the salivary glands. These results are similar to those observed when silencing *EcR*, which showed that impairing 20E signaling also expedites oocyst growth (Werling et al. 2019). 20E is one of the signals for *Vg* transcription, and EcR depletion also results in reduced *Vg* levels. Since both of these modulations (EcR and Vg depletion) result in the same phenotype, and since Vg is downstream of EcR, it is possible that the *EcR*-knockdown mediated phenotype is actually a result of decreased Vg levels. EcR regulates many genes and follow-up experiments would need to be carried out to establish this relationship.

The mechanism mediating increased oocyst growth upon Vg depletion was also explored in this work. In previous studies, upregulated Lp levels and feeding of BSA have both been shown to accelerate oocyst growth (Werling et al. 2019; Kwon et al. 2021). In my work, I observed that Vg depletion results in increased Lp levels as well as increased amino acid levels, and that injection of amino acids also induced accelerated oocyst growth. These data are therefore in line with what observed in earlier studies. These findings highlight that the parasite interacts with the mosquito reproductive system, since both processes utilize the same molecules of lipids and amino acids for growth. The parasite can hijack any extra nutrients leftover from reproductive processes, such as any increased Lp-shuttled lipids or available amino acids.

There remain many unanswered questions about *P. falciparum* growth in mosquito stages and future experiments are needed to further characterize the effects of increased amino acids and lipids. Firstly, the parasite does not express TOR, so what directs its amino acid sensing? Which amino acids are of most importance to the parasite during mosquito stages? How does the

mosquito's TOR signaling impact *P. falciparum* development? Which lipids are important for oocyst growth and what growth process do they contribute to (energy, membrane formation, etc)? Ideally, a future study would characterize the resources that the parasite can synthesize itself and which molecules it must scavenge from its host for optimal development during its mosquito stages.

The findings that targeting Vg favors parasite growth have significant implications for vector control. If vector control methods impairing Vg production or internalization into the oocytes were to be developed, then careful experimentation combined with mathematical modeling would need to be applied to ensure that resulting mosquitoes would not be infectious earlier. Considering that in the wild few mosquitoes survive past the 14 days post blood feeding that are necessary for sporogonic development and for the female to become infectious to humans (Gillies and Wilkes 1965), increased rates of parasite development could be a major barrier to utilizing sterile Vg-depleted mosquitoes. These results therefore highlight the importance of thoroughly assessing any vector control method to avoid to inadvertently favor pathogen transmission.

Final remarks

Overall, Vg is a molecule of many functions, some of which are still to be explored in the mosquito. During mosquito egg development, Vg regulates TOR signaling and Lp expression, and it is essential for successful reproduction resulting in 100% infertility when depleted. It is a significant nutritional investment by the female, and its depletion majorly disturbs nutrient deposition across mosquito tissues, of which the malaria parasite can take advantage. *Vg* knockdown results in faster parasite development, making mosquitoes infectious sooner, which

has repercussions for vector control methods targeting reproduction. Elucidating the mechanisms of mosquito reproduction and malaria parasite development leads to a better understanding of host-pathogen interactions and could result in better control approaches.

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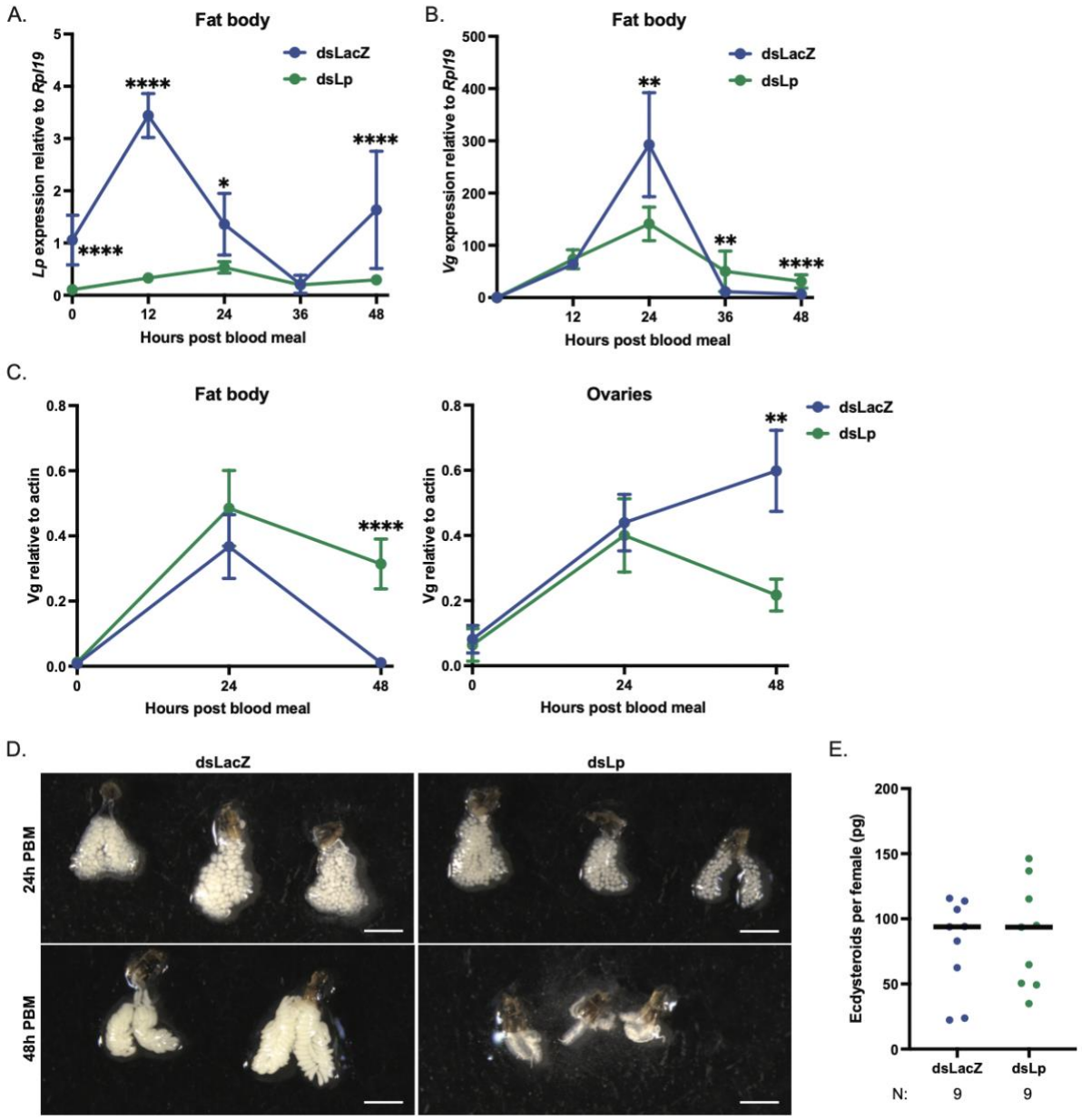
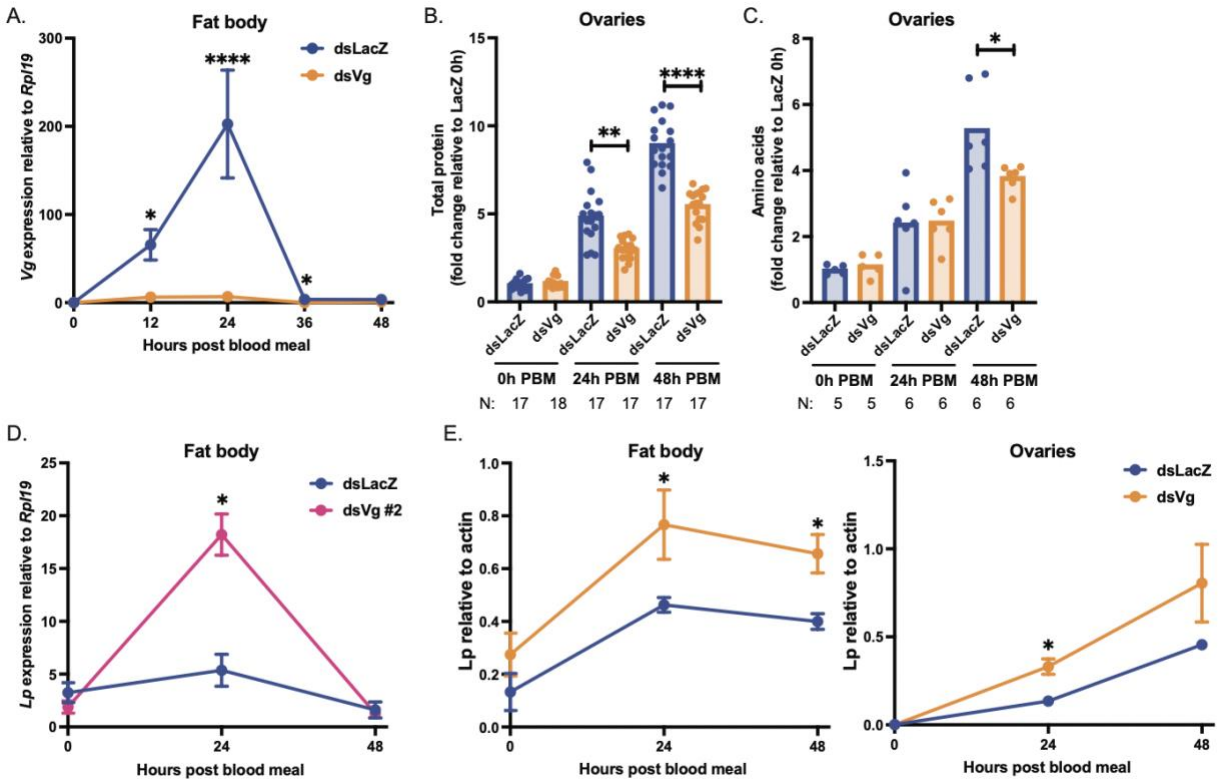
Appendix A

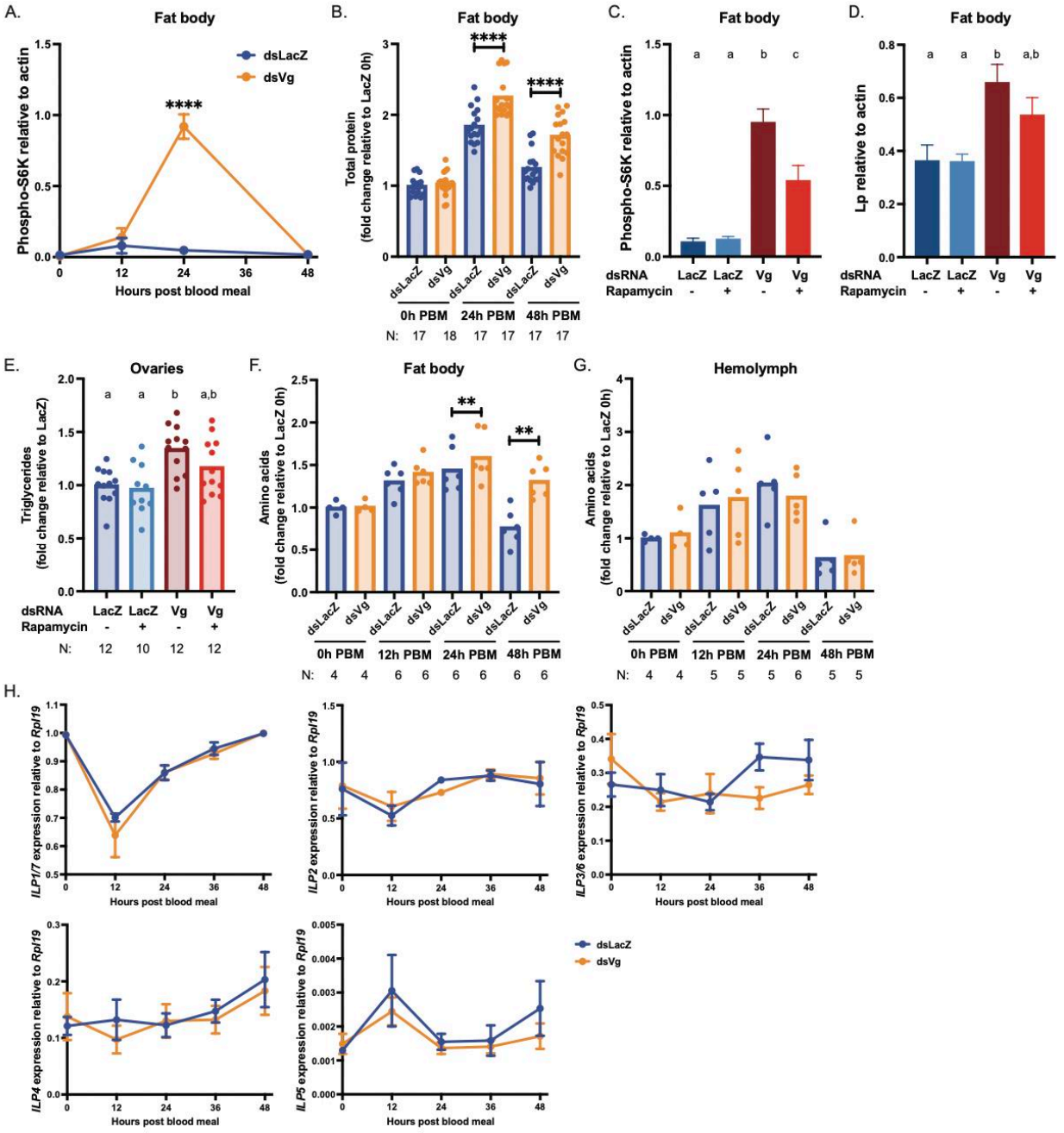
Supplementary material for Chapter 2



Supplemental Figure 1. *Lp* knockdown significantly impairs oogenesis and affects Vg expression and localization. (A) Successful *Lp* knockdown as determined by RT-qPCR of *Lp* expression levels relative to *Rpl19* in the fat body of ds*LacZ* and ds*Lp* females (REML variance component analysis: * = $p < 0.05$; **** = $p < 0.0001$; three biological replicates). (B) RT-qPCR of *Vg* expression levels relative to *Rpl19* in the fat body of ds*LacZ* and ds*Lp* females (REML variance component analysis: ** = $p < 0.01$; **** = $p < 0.0001$; four biological replicates). (C) Western blot quantification from Figure 1C showing an accumulation of Vg in the fat body and a decrease of Vg in the ovaries upon *Lp* knockdown (REML variance component analysis: ** = $p < 0.01$; **** = $p < 0.0001$; three biological replicates). (D) Images of ovaries at 24 and 48h post blood meal showing that Lp depleted ovaries develop normally at first before degenerating by 48h; scale bar = 2mm. (E) There is no difference in ecdysteroid levels of whole female bodies at 26h PBM upon *Lp* knockdown; each dot represents ecdysteroid level per female derived from a sample of 10 females (Unpaired t-test: not significant; three biological replicates of 3 samples each).



Supplemental Figure 2. *Vg* knockdown effects in fat body and ovaries. (A) Successful *Vg* knockdown as determined by RT-qPCR of *Vg* expression levels relative to *Rpl19* in the fat body (REML variance component analysis: * = $p < 0.05$; **** = $p < 0.0001$; three biological replicates). (B) Fold change in protein levels measured by Bradford assay in the ovaries of ds*LacZ* and ds*Vg* females before blood meal and at 24h and 48h post blood meal (PBM); each dot is representative of three ovaries; N=number of samples of three tissues, pooled from three biological replicates (REML variance component analysis by timepoint: ** = $p < 0.01$; **** = $p < 0.0001$). (C) Fold change in free amino acid levels in the ovaries of ds*LacZ* and ds*Vg* females before blood meal and at 24h and 48h PBM; each dot is representative of five ovaries; N=number of samples of five tissues, pooled from three biological replicates (REML variance component analysis by timepoint: * = $p < 0.05$). (D) *Vg* knockdown by a second *Vg* RNAi fragment also results in an increase in *Lp* levels as determined by RT-qPCR (REML variance component analysis: * = $p < 0.05$; three biological replicates). (E) Western blot quantification from Figure 2G showing an accumulation of Lp in the fat body and ovaries upon *Vg* knockdown (REML variance component analysis: fat body – dsRNA: $p < 0.01$; * = $p < 0.05$; ovaries – dsRNA: $p < 0.05$; * = $p < 0.05$; three biological replicates).

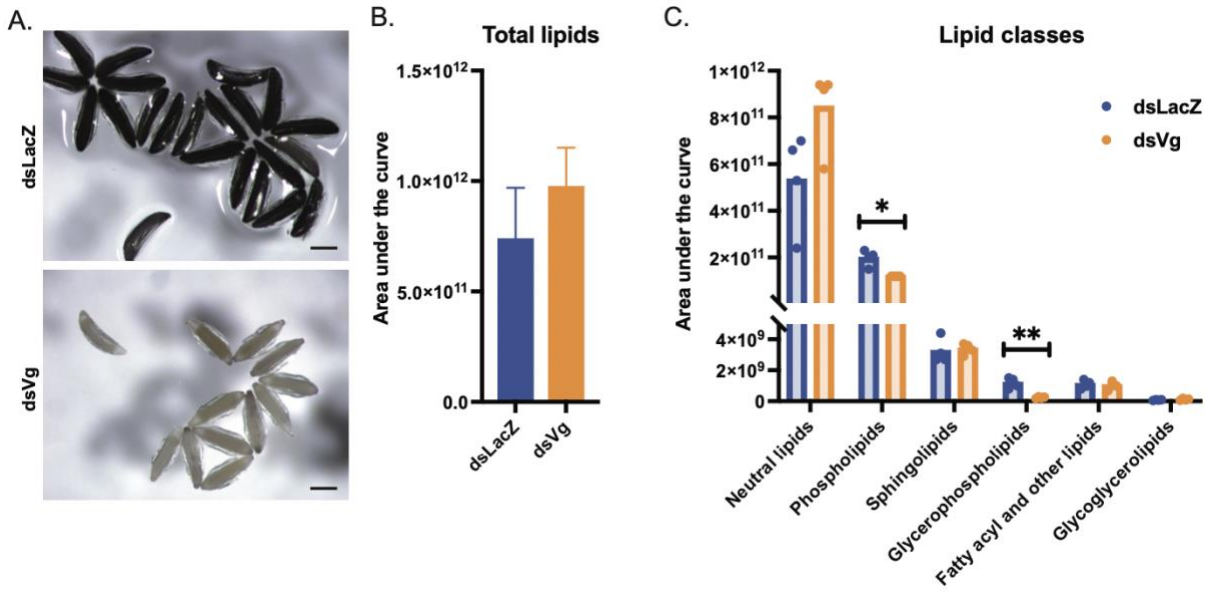


Supplemental Figure 3. *Vg* expression regulates Lp-mediated accumulation of lipids via TOR signaling. (A) Western blot quantification from Figure 3B showing an increase in phospho-S6K levels in the fat body upon *Vg* knockdown (REML variance component analysis: **** = $p < 0.0001$; three biological replicates). (B) Fold change in protein levels, measured by Bradford assay of the fat body are increased in ds*Vg* females; each dot is representative of three fat bodies; N=number of samples of three tissues, pooled from three biological replicates (REML variance component analysis by timepoint: **** = $p < 0.0001$). (C) Western blot quantification from Figure 3D showing a decrease in phospho-S6K levels in the fat body upon rapamycin treatment (ANOVA; three biological replicates). [Legend continues on next page]

Supplemental Figure 3 [Continuted]. (D) Western blot quantification from Figure 3E showing Lp protein levels upon *Vg* knockdown and rapamycin treatment (ANOVA; three biological replicates). (E) Triglyceride levels measured in ds*LacZ* and ds*Vg* ovaries upon 0.5 µl of 40µM rapamycin treatment at 72h post blood meal and normalized to mean ds*LacZ* levels in that replicate; each dot is representative of ovaries from three females; N=number of samples of three tissues, pooled from two biological replicates (ANOVA). (F) Fold change in free amino acid levels in the fat bodies of ds*LacZ* and ds*Vg* females before blood meal and at 12h, 24h and 48h post blood meal; each dot is representative of five ovaries; N=number of samples of five tissues, pooled from three biological replicates (REML variance component analysis by timepoint: ** = $p < 0.01$). (G) Fold change in free amino acid levels in the hemolymph of ds*LacZ* and ds*Vg* females before blood meal and at 12h, 24h and 48h post blood meal; each dot is representative of hemolymphs collected from five females; N=number of samples of five hemolymphs, pooled from three biological replicates (REML variance component analysis by timepoint). (H) RT-qPCR of *ILP* expression levels relative to *Rpl19* in the heads of ds*LacZ* and ds*Vg* females (REML variance component analysis; ILP1/7: two biological replicates; other ILPs: three biological replicates).

Supplemental Table 1. Vg amino acids and their decrease in embryos upon Vg depletion.

Amino acid	Percent content in Vg	Decrease upon Vg KD in mothers (fold change)	Significance	
Ser (S)	8.50%	2.2	0.09205	
Tyr (Y)	8.00%	52.6	0.00010	***
Phe (F)	7.70%	63.2	0.00000	****
Glu (E)	6.50%	1.2	0.33025	
Ala (A)	6.30%	2.0	0.02859	*
Asp (D)	6.30%	2.1	0.15012	
Lys (K)	6.20%	10.0	0.00000	****
Gln (Q)	6.10%	1.1	0.88614	
Val (V)	5.80%	3.1	0.00005	****
Asn (N)	5.60%	5.5	0.00006	****
Leu (L)	5.60%	3.7	0.00024	***
Gly (G)	4.40%	2.2	0.02060	*
Pro (P)	4.40%	1.4	0.01066	*
Thr (T)	4.40%	3.9	0.00015	***
Arg (R)	3.90%	2.9	0.00012	***
Ile (I)	3.20%	8.7	0.00001	****
His (H)	2.90%	1.0	0.69815	
Met (M)	2.20%	4.9	0.00010	***
Cys (C)	1.10%	Not detected		
Trp (W)	0.80%	13.2	0.00002	****



Supplemental Figure 4. *Vg* knockdown in females prevents embryo melanization and causes early embryonic arrest. (A) Light microscopy of embryos from ds*LacZ*- and ds*Vg*-derived females at 3-5h post oviposition; scale bar = 200 µm. (B-C) Total lipids (B) and lipid classes (C) in ds*LacZ*- and ds*Vg*-derived embryos 3-5h post oviposition as determined by mass spectrometry (unpaired t tests, followed by FDR correction: * = $p < 0.05$; ** = $p < 0.01$; four biological replicates).

Supplemental Table 2. Primers used for RT-qPCR

Gene	Primer	Citation, if previously published
Rpl19	F CCAACTCGCGACAAAACATTC R ACCGGCTTCTTGATGATCAGA	Werling, Shaw, Itoe et al., 2019
Lp	F CAGCCAGGATGGTGAGCTTAA R CACCAGCACCTTGGCGTT	Werling, Shaw, Itoe et al., 2019
Vg	F CCGACTACGACCAGGACTTC R CTTCCGGCGTAGTAGACGAA	Werling, Shaw, Itoe et al., 2019
ILP1/7	F GCAAAAAGTCCGAGAATCTACTGATGA R CGAACGATCGTTCAATGTGTGGA	
ILP2	F CTACCTCTACGCCCAACAGC R CGTGTACATAATCTGTGCGATAGTG	
ILP3/6	F GGTAAAGGTACTGTCCTTCCTG R AGTATCTGCTGCGTGTTGTC	Arsic and Guerin, 2008
ILP4	F TCTCCGAAAGAACACAGTTGA R GGTTTCTGCCTGAACCACAT	Arsic and Guerin, 2008
ILP5	F GTGGCACCAGGAGAGTCATT R GCCCAGTACAGATGGCGTAT	

Supplemental Table 3. Details of statistical tests and outputs are summarized for each figure. For qRT-PCR, at least three independent biological replicates of a gene expression timecourse were analyzed, except for ILP1, where one replicate was excluded as an outlier. Effect test outputs are reported here. Multiple comparisons were calculated using pairwise Student's t tests at each timepoint followed by FDR correction (see **Table S4**). KD = knock down; rand = random effect; FDR = false discovery rate.

Fig	Comparison	Statistical test	Effect Test Outputs
2.1A	Egg number after *Lp* KD	Mann-Whitney	p<0.0001
2.1B	TAG levels after *Lp* KD (Ovaries)	Ln(x+1.1) transformation; Linear Mixed Model at each timepoint	(0h) dsRNA p=0.7964 replicate p=0.3755 (24h) dsRNA p<0.0001 replicate p=0.3400 (48h) dsRNA p<0.0001 replicate p=0.0109
2.1B	TAG levels after *Lp* KD (Midgut)	Ln(x+1.1) transformation; Linear Mixed Model at each timepoint	(0h) dsRNA p=0.2984 replicate p=0.3317 (24h) dsRNA p<0.0001 replicate p=0.3573 (48h) dsRNA p<0.0001 replicate p=0.3228
2.2A	Egg number after *Vg* KD	Mann-Whitney test	p<0.0001
2.2B	Fertility after *Vg* KD	Kruskal-Wallis test	dsLacZ vs dsVg: p<0.0001 dsLacZ vs dsVg#2: p<0.0001 dsVg vs dsVg#2: p>0.05
2.2E	Triglycerides after *Vg* KD (ovaries 48h)	Unpaired t test on transformed data	p=0.0222
2.2F	*Lp* expression after *Vg* KD	4^{th} root transformation; Linear Mixed Model followed by 5 post-hoc t-tests (**Table S6**)	timepoint p<0.0001 dsRNA p<0.0001 dsRNA x timepoint p=0.0032 replicate[rand] p=0.3090
2.3A	*Vg* upon rapamycin treatment	Unpaired t test	p<0.0001
2.3C	*Lp* mRNA levels upon rapamycin treatment	ANOVA	LacZ Control vs. LacZ Rapamycin p=0.9956 LacZ Control vs. Vg Control p=0.0002 LacZ Control vs. Vg Rapamycin p=0.2500

			LacZ Rapamycin vs. Vg Control p=0.0003 LacZ Rapamycin vs. Vg Rapamycin p=0.3469 Vg Control vs. Vg Rapamycin p=0.0226
2.4D	Embryo triglycerides	Unpaired t test	p=0.0496
S1A	*Lp* expression after *Lp* KD	4th root transformation; Linear Mixed Model followed by 5 post-hoc t-tests (**Table S6**)	timepoint p<0.0001 dsRNA p<0.0001 dsRNA x timepoint p=0.0004 replicate[rand] p=0.478
S1B	*Vg* expression after *Lp* KD	4th root transformation; Linear Mixed Model followed by 5 post-hoc t-tests (**Table S6**)	timepoint p<0.0001 dsRNA p=0.044 dsRNA x timepoint p=0.0001 replicate[rand] p=0.536 replicate x timepoint[rand] p=0.033
S1C	Vg protein expression after *Lp* KD (Ovaries)	Ln(x+1) transformation; Linear Mixed Model followed by 3 post-hoc t-tests (**Table S6**)	timepoint p<0.0001 dsRNA p=0.0103 dsRNA x timepoint p=0.0192 replicate p=0.3836
S1C	Vg protein expression after *Lp* KD (Fat body)	No transformation; Generalized Linear Model followed by 3 post-hoc t-tests (**Table S6**)	timepoint p<0.0001 dsRNA p=0.0148 dsRNA x timepoint p<0.0001 replicate p=0.0012
S2A	*Vg* expression after *Vg* KD	8th root transformation; Linear Mixed Model followed by 5 post-hoc t-tests (**Table S6**)	timepoint p<0.0001 dsRNA p<0.0001 dsRNA x timepoint p=0.0068 replicate[rand] p=0.6864
S2B	Protein levels after *Vg* KD (Ovaries)	No suitable transformation; Generalized Linear Mixed Model at each timepoint	(0h) dsRNA p=0.8072 replicate p=0.4399 (24h) dsRNA p=0.0044 replicate p=0.0002 (48h) dsRNA p<0.0001 replicate p=0.0092
S2C	Amino acid levels after *Vg* KD (Ovaries)	Square root transformation; Linear Mixed Model at each timepoint	(0h) dsRNA p=0.5067 replicate p=0.4232 (24h) dsRNA p=0.8833 replicate p=0.4843 (48h) dsRNA p=0.0163 replicate p=0.3562
S2D	*Lp* expression after *Vg* KD#2	4th root transformation; Linear Mixed Model	timepoint p=0.0004 dsRNA p=0.1804

		followed by 3 post-hoc t-tests (**Table S6**)	dsRNA x timepoint p=0.0409 replicate[rand] p=0.9751
S2E	Lp protein expression after *Vg* KD (Ovaries)	Square root (x+0.03) transformation; Linear Mixed Model followed by 3 post-hoc t-tests (**Table S6**)	timepoint p<0.0001 dsRNA p=0.0100 dsRNA x timepoint p=0.1130 replicate p=0.8073
S2E	Lp protein expression after *Vg* KD (Fat body)	No transformation; Linear Mixed Model followed by 3 post-hoc t-tests (**Table S6**)	timepoint p=0.0004 dsRNA p=0.0025 dsRNA x timepoint p=0.5277 replicate p=0.6241
S3A	Protein levels after *Vg* KD (Fat body)	Cube root transformation; Linear Mixed Model at each timepoint	(0h) dsRNA p=0.4585 replicate p=0.3221 (24h) dsRNA p<0.0001 replicate p=0.3232 (48h) dsRNA p<0.0001 replicate p=0.3260
S3B	pS6K expression after *Vg* KD (Fat body)	No transformation; Generalized Linear Model followed by 4 post-hoc t-tests (**Table S6**)	timepoint p<0.0001 dsRNA p=0.1251 dsRNA x timepoint p<0.0001 replicate p=0.0084
S3C	Phospho S6K protein levels upon rapamycin treatment	ANOVA	LacZ Control vs. LacZ Rapamycin p=0.9976 LacZ Control vs. Vg Control p<0.0001 LacZ Control vs. Vg Rapamycin p=0.0072 LacZ Rapamycin vs. Vg Control p<0.0001 LacZ Rapamycin vs. Vg Rapamycin p=0.0100 Vg Control vs. Vg Rapamycin p=0.0102
S3D	Lp protein levels upon rapamycin treatment	ANOVA	LacZ Control vs. LacZ Rapamycin p>0.9999 LacZ Control vs. Vg Control p=0.0185 LacZ Control vs. Vg Rapamycin p=0.2184 LacZ Rapamycin vs. Vg Control p=0.0172

			LacZ Rapamycin vs. Vg Rapamycin p=0.2044 Vg Control vs. Vg Rapamycin p=0.4834
S3E	Triglycerides upon rapamycin treatment	ANOVA	LacZ Control vs. LacZ Rapamycin P=0.9860 LacZ Control vs. Vg Control p=0.0030 LacZ Control vs. Vg Rapamycin p=0.2565 LacZ Rapamycin vs. Vg Control p=0.0018 LacZ Rapamycin vs. Vg Rapamycin p=0.1618 Vg Control vs. Vg Rapamycin p=0.2566
S3F	Amino acid levels after *Vg* KD (Fat body)	No transformation Linear Mixed Model at each timepoint	(0h) dsRNA p=0.8672 replicate p=0.3832 (12h) dsRNA p=0.1087 replicate p=0.3257 (24h) dsRNA p=0.0064 replicate p=0.3201 (48h) dsRNA p=0.0027 replicate p=0.4067
S3G	Amino acid levels after *Vg* KD (Hemolymph)	No transformation Linear Mixed Model at each timepoint	(0h) dsRNA p=0.7177 replicate p=0.7615 (12h) dsRNA p=0.6672 replicate p=0.4125 (24h) dsRNA p=0.2457 replicate p=0.3536 (48h) dsRNA p=0.8222 replicate p=0.7415
S3H	*ILP1* expression after *Vg* KD (2 replicates)	arcsine transformation; Linear Mixed Model No post-hoc t-testing	timepoint p<0.0001 dsRNA p=0.426
	ILP2 expression after *Vg* KD	arcsine transformation; Linear Mixed Model No post-hoc t-testing	timepoint p=0.0089 dsRNA p=0.920
	ILP3 expression after *Vg* KD	No transformation; Linear Mixed Model No post-hoc t-testing	timepoint p=0.248 dsRNA p=0.367

	ILP4 expression after *Vg* KD	No transformation; Linear Mixed Model No post-hoc t-testing	timepoint p=0.002 dsRNA p=0.408
	ILP5 expression after *Vg* KD	5^{th} root transformation; Linear Mixed Model No post-hoc t-testing	timepoint p=0.002 dsRNA p=0.241
S4B	Embryo total lipids	Unpaired t test	p=0.1606

Supplemental Table 4. Post-hoc testing for significant differences using an FDR of 0.05. See **Supplemental Table 3**.

2.2F **Lp after Vg KD**	**p-value**	**FDR-adjusted p-value**	**Significant?**
dsLacZ – dsVg at 0h	0.6414	0.6414	No
dsLacZ – dsVg at 12h	0.0731	0.0914	No
dsLacZ – dsVg at 24h	4.13×10^{-6}	2.07×10^{-5}	Yes
dsLacZ – dsVg at 36h	0.0020	0.0050	Yes
dsLacZ – dsVg at 48h	0.0450	0.0750	No
S1B **Vg after Lp KD**	**p-value**	**FDR-adjusted p-value**	**Significant?**
dsLacZ – dsLp at 0h	0.9198	0.9198	No
dsLacZ – dsLp at 12h	0.7704	0.9198	No
dsLacZ – dsLp at 24h	0.0029	0.0048	Yes
dsLacZ – dsLp at 36h	0.0028	0.0048	Yes
dsLacZ – dsLp at 48h	7.55×10^{-5}	3.78×10^{-4}	Yes
S1A **Lp after Lp KD**	**p-value**	**FDR-adjusted p-value**	**Significant?**
dsLacZ – dsLp at 0h	2.64×10^{-5}	6.60×10^{-5}	Yes
dsLacZ – dsLp at 12h	4.36×10^{-7}	2.18×10^{-6}	Yes
dsLacZ – dsLp at 24h	0.0138	0.0173	Yes
dsLacZ – dsLp at 36h	0.8884	0.8884	No
dsLacZ – dsLp at 48h	2.67×10^{-4}	4.45×10^{-4}	Yes
S1C (Ovaries)	**p-value**	**FDR-adjusted p-value**	**Significant?**
dsLacZ – dsVg at 0h	0.7766	0.7766	No
dsLacZ – dsVg at 24h	0.6119	0.7766	No
dsLacZ – dsVg at 48h	0.0009	0.0027	Yes
S1C (Fat body)	**p-value**	**FDR-adjusted p-value**	**Significant?**
dsLacZ – dsVg at 0h	0.7447	0.7447	No
dsLacZ – dsVg at 24h	0.1780	0.2670	No
dsLacZ – dsVg at 48h	2.86×10^{-10}	8.56×10^{-10}	Yes
S2A **Vg after Vg KD**	**p-value**	**FDR-adjusted p-value**	**Significant?**
dsLacZ – dsVg at 0h	0.9000	0.9000	No
dsLacZ – dsVg at 12h	0.0057	0.0115	Yes
dsLacZ – dsVg at 24h	2.94×10^{-5}	1.47×10^{-4}	Yes
dsLacZ – dsVg at 36h	0.0069	0.0115	Yes
dsLacZ – dsVg at 48h	0.5501	0.6877	No
S2D **Lp after Vg KD#2**	**p-value**	**FDR-adjusted p-value**	**Significant?**
dsLacZ – dsVg at 0h	0.3785	0.5678	No

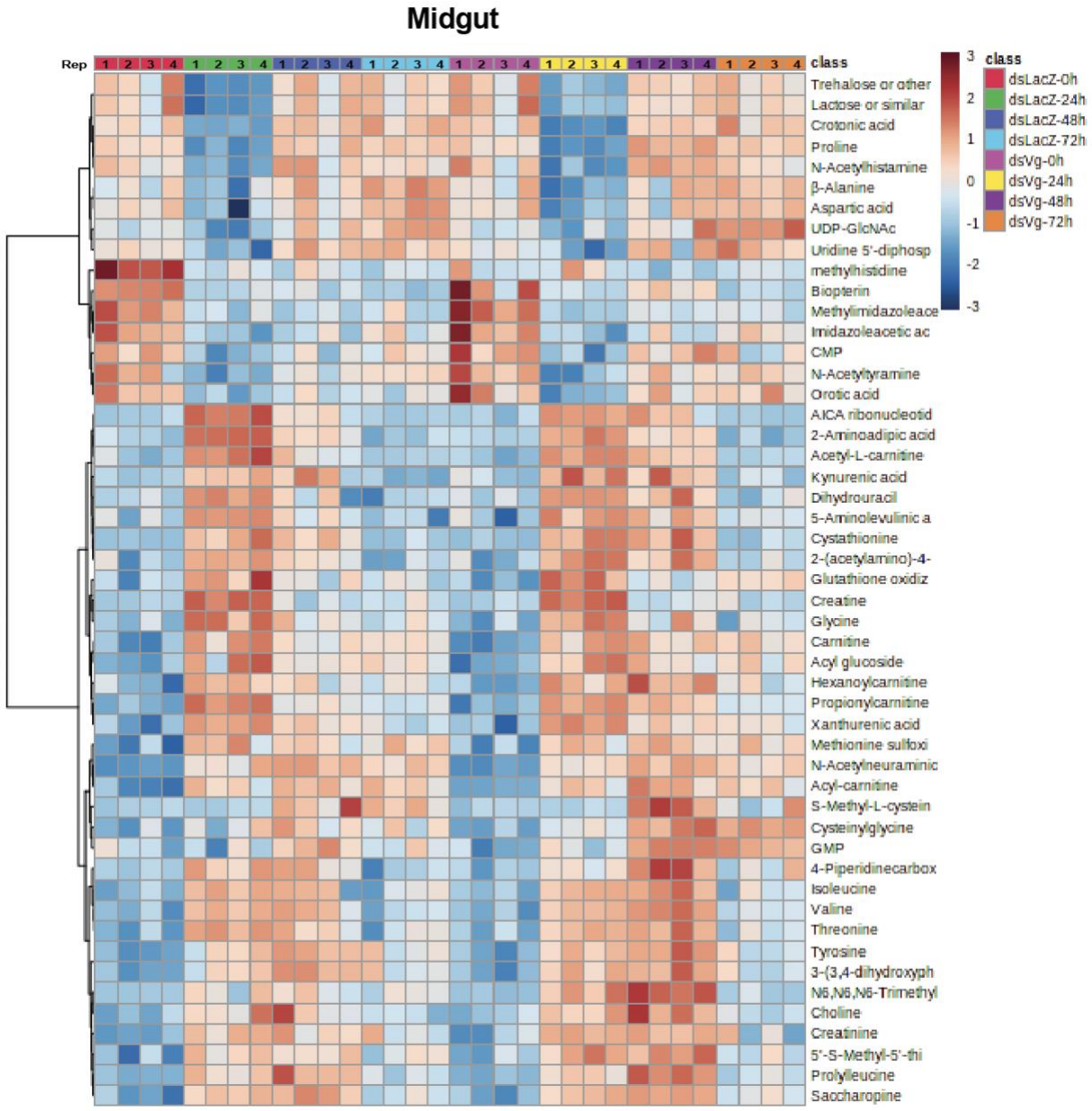
dsLacZ – dsVg at 24h	0.0098	0.0294	Yes
dsLacZ – dsVg at 48h	0.8191	0.8191	No
S2E (Ovaries)	**p-value**	**FDR-adjusted p-value**	**Significant?**
dsLacZ – dsVg at 0h	0.9392	0.9392	No
dsLacZ – dsVg at 24h	0.0210	0.0315	Yes
dsLacZ – dsVg at 48h	0.0177	0.0315	No
S2E (Fat body)	**p-value**	**FDR-adjusted p-value**	**Significant?**
dsLacZ – dsVg at 0h	0.1927	0.1927	No
dsLacZ – dsVg at 24h	0.0133	0.0399	Yes
dsLacZ – dsVg at 48h	0.0298	0.0447	Yes
S3A (Fat body)	**p-value**	**FDR-adjusted p-value**	**Significant?**
dsLacZ – dsVg at 0h	0.9058	0.9058	No
dsLacZ – dsVg at 12h	0.1829	0.3658	No
dsLacZ – dsVg at 24h	8.06×10^{-25}	3.22×10^{-24}	Yes
dsLacZ – dsVg at 48h	0.6257	0.8343	No

Appendix B

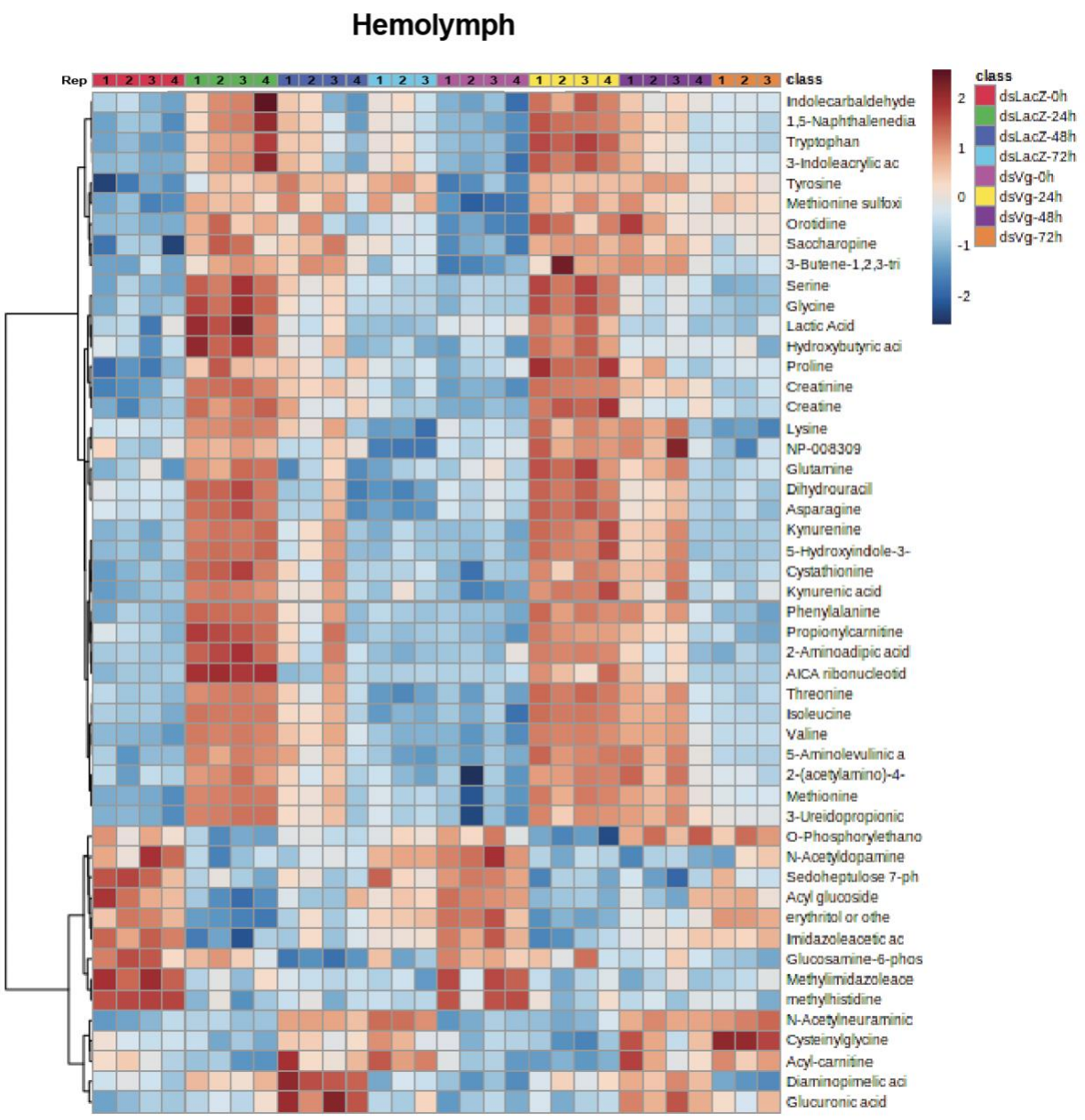
Supplementary material for Chapter 3

Supplemental Figure 5. Metabolomics of midgut, fat body and hemolymph upon *Vg* knockdown. Top 50 metabolites dysregulated metabolites (as determined by Metaboanalyst) identified by metabolomic mass spectrometry analysis. Metabolites were measured in the midgut, hemolymph and fat body at 0h, 24h, 48h and 72h PBM in control and Vg-depleted females; area under the curve is visualized using Metaboanalyst (four biological replicates for midgut and hemolymph; three biological replicates for fat body).

Supplemental Figure 5 (continued)



Supplemental Figure 5 (continued)



Supplemental Figure 5 (continued)