Lipidomic change in *Aedes aegypti* female following blood feeding reveals similar changes in functionally related lipids

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Abstract

We conducted lipidomic analyses of the whole body of female *Aedes aegypti* mosquitoes, before and at multiple time points after blood feeding, to assess how lipidome changed with feeding and subsequent reproduction. There was an increase in the levels of almost all lipids at the time of feeding, which mostly reflected the intake of lipids from human blood; this increase was followed by a sharp decrease, often to a level below that detected before blood feeding. Between 16 to 30 hours after feeding, several lipid classes increased in abundance again to levels observed immediately after feeding, followed by a further decrease when approaching 120 hours after feeding. The concerted temporal pattern was particularly strong in signalling lipids, many of which increased more than ten folds. Patterns for membrane lipids suggest the possibility that lyso-glycerophospholipids do not participate in the development of oocytes. Our study on the anautogenous insect *Ae. aegypti* provides an opportunity to compare the abundance of specific lipids between mosquitoes and humans, and the results point to specific lipids likely to be important in the reproductive process with a role in oocytes development.

Introduction

The yellow fever mosquito, *Aedes aegypti*, is one of the major vectors that transmits arbovirus diseases in tropical and subtropical areas, such as dengue and Zika (Gould & Higgs 2009; Bhatt *et al*. 2013; Brown *et al*. 2014). *Ae*. *aegypti* females are anautogenous: they must ingest human blood to obtain unique nutrients to produce their eggs, and diseases are transmitted through the blood ingestion process (Scott *et al*. 1997; Gulia-Nuss *et al*. 2015).

Reproduction in mosquito females is generally regulated through the provision of nutrition and the modulation of hormones. In *Ae*. *aegypti*, nutrients used to support egg development come from sugars and human blood (Ziegler & Ibrahim 2001; Attardo, Hansen & Raikhel 2005). Ovarian development can be separated into the pre-vitellogenic (before blood meal, BBM) and vitellogenic (post blood meal, PBM) stages depending on the timing of the blood meal. In the pre-vitellogenic stages, lipids are reserved in the fat body (Ziegler & Ibrahim 2001), while during the vitellogenic stages, consumption of blood meal triggers insulin-like peptide 3 (ILP3) from the brain to stimulate egg formation (Brown *et al*. 2008). The secretion of ecdysteroids 20-hydroxyecdysone then increases the expression of vitellogenin genes in the fat body to support yolk protein accumulation and oocytes development (Hansen *et al*. 2014; Belles & Piulachs 2015), which is stimulated by gonadotropin (Klowden 1997), and lipids are transferred from the fat body to oocytes (Ziegler & Ibrahim 2001). In the meantime, *Ae*. *aegypti* females also digest and absorb essential amino acids from human blood, where the concentration of amino acids increasing in haemolymph acts as a signal to activate ovarian development (Uchida *et al*. 1990) through the TOR pathway (Hansen *et al*. 2004).

Lipid reservation is essential to support *Ae*. *aegypti* reproduction (Zhou, Pennington & Wells 2004), particularly when a sugar source is compromised. Starving female mosquitoes before or after their blood feeding will not reduce their fecundity but will significantly reduce the amount of lipids in mothers after laying eggs (Zhou *et al*. 2004), suggesting a trade-off between reproduction and female survival. The source and the quality of blood that females are fed can directly impact their fertility (Phasomkusolsil *et al*. 2013; Ross, Lau & Hoffmann 2019), reflecting variation in the essential nutrients obtained from human blood. Variation in nutritional quality cannot be fully explained based on our current understanding of amino acids and cholesterol (Caragata *et al*. 2013; Caragata *et al*. 2014), though several artificial

blood substitute diets have been developed that rely on bovine proteins or blood (Talyuli *et al*. 2015; Dutra *et al*. 2017).

Nutrient reservation and mosquito reproduction are being increasingly studied within the context of *Wolbachia* bacteria which have now been successfully introduced into *Ae*. *aegypti* (Xi, Khoo & Dobson 2005; McMeniman *et al*. 2009; Ant *et al*. 2018; Ross, Turelli & Hoffmann 2019), and used in a population replacement strategy to control arboviral diseases (Moreira *et al*. 2009; Hoffmann *et al*. 2011; Nazni *et al*. 2019; Indriani *et al*. 2020). The success of this strategy requires *Wolbachia* to sustainably infect a population (Hoffmann *et al*. 2014), whereas *Wolbachia* dynamics and disease blocking may be affected by competition of nutrients between host *Ae*. *aegypti*, *Wolbachia*, and viruses (Heaton *et al*. 2010; Walker *et al*. 2011; Molloy *et al*. 2016; Rainey *et al*. 2016; Terradas & McGraw 2017; Koh *et al*. 2020), including competition for amino acids (Caragata *et al*. 2014) and cholesterol (Caragata *et al*. 2013; Geoghegan *et al*. 2017). However, current understanding of such competitive effects cannot fully explain the reduced fertility that can occur when *Wolbachia*-infected *Ae*. *aegypti* feed on different blood source (McMeniman, Hughes & O'Neill 2011), or when they are exposed to some environmental stresses (Lau, Ross & Hoffmann 2021).

Lipids are the main nutrients for supporting the reproduction process of many insects including *Ae*. *aegypti*. After female are engorged, ovarian development can be observed within 30 minutes (Clements & Boocock 1984); by the 2nd or 3rd day PBM, the dry weight of ovaries has increased substantially, and consist of 35% lipid, required for eggs to mature and be laid at the 4th or 5th day PBM (Anderson & Spielman 1971; Troy, Anderson & Spielman 1975). Over the course of the vitellogenic cycle, lipids in the fat body also undergo significant changes in general (Price *et al*. 2011; Pinch *et al*. 2021), although many aspects about the mechanisms of lipid digestion and recycling during egg development remain unknown.

In this study, we used liquid chromatography coupled with mass spectrometry (LCMS)-based lipidomic method and a comprehensive lipid standard mixture covering near all major lipid classes to identify and analyse lipid components of whole female individuals BBM and PBM. Our study aims to understand lipid metabolism in *Ae*. *aegypti* during reproductive process,

and to provide support for future studies about nutritional competition between *Ae. aegypti* and its parasites.

Methods

Sample preparation

Aedes aegypti mosquitoes in this experiment were collected from *Wolbachia*-uninfected populations from Cairns, QLD, Australia and maintained in laboratory conditions (Lau, Ross & Hoffmann 2021). Samples were prepared by hatching *Ae. aegypti* eggs stored in an incubator (PG50 Plant Growth Chambers, Labec Laboratory Equipment, Marrickville, NSW, Australia) at 22-30 ± 1 °C, with a 12h : 12h photoperiod (low temperatures during the dark period and high temperatures during the light period). Larvae were reared in reverse osmosis (RO) with yeast and fed with Hikari Sinking Wafers fish food tablets until adult. All female mosquitoes were blood fed by the same volunteer's forearm. The first blood meal was provided to mosquito females at 4 ± 1 days post-emergence. Females were isolated individually and observed for seven days to ensure all have similar body size, are able to take blood and to lay enough eggs (>50). At the seventh day, a second blood meal was provided. Non- engorged individuals were removed from the experiment. Samples were then stored as different groups in their second gonotrophic cycle BBM and at different time PBM: 0 h PBM (just after blood meal), 4 h PBM, 16 h PBM, 30 h PBM, 72 h PBM, and 120 h PBM. Sugar and oviposition cups were provided during the entire period of the experiment.

Female mosquito measurement

Apart from analysis of the lipidome, we also measured the weight of different female individuals (4-7) at each time point from the additional collected samples using a Sartorius Analytical balance BP 210 D (Sartorius, Gottigen, Germany). After weighing, eight mosquitoes were randomly selected from all time points and their wing lengths were measured to indicate female body size (Briegel 1990). To further understand the development of female ovaries, we hatched batches of mosquito eggs and took photos of ovaries using an NIS Elements BR imaging microscope (Nikon Instruments, Tokyo, Japan) every day PBM.

Lipid extraction

For each group, we extracted the whole body of six mosquito females, keeping individuals separate. We added 10 μL of Mouse SPLASH® LIPIDOMIX® Mass Spec Standard (cat.

330707, Avanti) after 1 in 4 dilution to each sample prior the extraction and then homogenised samples in 200 µL ice-cold 60% methanol (MeOH) containing 0.01% (w/v) butylated hydroxytoluene (BHT) by using TissueLyserII (Qiagen, Hilden, Germany) at 25 Hertz for one minute and incubating for 20 minutes in an ultrasonic bath (Soniclean Pty Ltd., Thebarton, Australia). Following the method previously reported by Lydic *et al*. (2015), 120 µL of MilliQ water, 420 µl of MeOH with 0.01% (w/v) BHT, and 260 µL of chloroform ($CHCl_3$) were added to each tube and vortexed thoroughly before incubating in a Bioshake iQ (Quantifoil Instrument GmbH, Germany) setting at 1,400 rpm for 30 minutes and centrifuged at 14,000 rpm for 15 minutes. Supernatant containing lipids was transferred to a new 2 mL tube. The remaining sediments were re-extracted with 100 µL of MilliQ water and 400 µL of $CHCl_3$: MeOH (1:2, v:v) containing 0.01% (w/v) BHT as described above. Supernatants from the first and second extraction were pooled in the same 2 mL tube and then dried by evaporation under vacuum using a GeneVac miVac sample concentrator (SP Scientific, Warminster, PA, USA). Before HPLC, dried lipid extracts were resuspended in 50 µL of $CHCl_3$: MeOH (1:9, v:v, containing 0.01% BHT).

Mass spectrometry analyses

Samples were analysed by ultrahigh performance liquid chromatography (UHPLC) coupled to tandem mass spectrometry (MS/MS) employing a Vanquish UHPLC linked to an Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher Scientific), with separate runs in positive and negative ion polarity. Solvent A was 6/4 (v/v) acetonitrile/water with 10 mM ammonium acetate and 5 μM medronic acid and solvent B was 9/1 (v/v) isopropanol/acetonitrile with 10 mM ammonium acetate. Each sample was injected into an RRHD Eclipse Plus C18 column (2.1 × 100 mm, 1.8 μm, Agilent Technologies) at 50°C at a flow rate of 350 µl/min for 3 min using 3% solvent B. During separation, the percentage of solvent B was increased from 3% to 70% in 5 min, from 70% to 99% in 16 min, from 99% to 99% in 3 min, from 99% to 3% in 0.1 min and maintained at 3% for 3.9 min.

All experiments were performed using a Heated Electrospray Ionization source. The spray voltages were 3.5 kV in positive ionisation mode and 3.0 kV in negative ionisation mode. In both polarities, the flow rates of sheath, auxiliary and sweep gases were 20 and 6 and 1 'arbitrary' unit(s), respectively. The ion transfer tube and vaporizer temperatures were maintained at 350 °C and 400 °C, respectively, and the ion funnel RF level was set at 50%. In

both positive and negative ionisation modes from 3 to 24 min, top speed data-dependent scan with a cycle time of 1 s was used. Within each cycle, a full-scan MS spectrum was acquired firstly in the Orbitrap at a mass resolving power of 120,000 (at m/z 200) across a m/z range of 300–2000 using quadrupole isolation, an automatic gain control (AGC) target of 4e5 and a maximum injection time of 50 milliseconds, followed by higher-energy collisional dissociation (HCD)-MS/MS at a mass resolving power of 15,000 (at m/z 200), a normalised collision energy (NCE) of 27% at positive mode and 30% at negative mode, an m/z isolation window of 1, a maximum injection time of 22 milliseconds and an AGC target of 5e4. For the improved structural characterisation of glycerophosphocholine (PC) in positive mode, a data-dependent product ion (m/z 184.0733)-triggered collision-induced dissociation (CID)-MS/MS scan was performed in the cycle using a q-value of 0.25 and a NCE of 30%, with other settings being the same as that for HCD-MS/MS. For the improved structural characterisation of triacylglycerol (TG) lipid in positive mode, the fatty acid + NH3 neutral loss product ions (Supplementary table S1) observed by HCD-MS/MS were used to trigger the acquisition of the top-3 data-dependent CID-MS3 scans in the cycle using a q-value of 0.25 and a NCE of 30%, with other settings being the same as that for HCD-MS/MS.

Lipid identification and quantification

LC-MS/MS data was searched through MS Dial 4.80 (Tsugawa *et al*. 2015; Tsugawa *et al*. 2020). The mass accuracy settings are 0.005 Da and 0.025 Da for MS1 and MS2. The minimum peak height is 50000 and mass slice width is 0.05 Da. The identification score cut off is 80%. Post identification was done with a text file containing name and m/z of each standard in Mouse SPLASH® LIPIDOMIX® Mass Spec Standard. In positive mode, $[M+H]^+$ and $[M+NH4]^+$ were selected as ion forms. In negative mode, $[M-H]^-$ were selected as ion forms. All lipid classes available were selected for the search. The retention time tolerance for alignment is 0.1 min. Lipids with maximum intensity less than 5-fold of average intensity in blank were removed. All other settings were default. All lipid LC-MS features were manually inspected and re-integrated when needed. We also removed four other types of lipids: (1) lipids with only sum composition except PC and SM; (2) lipids identified due to peak tailing; (3) retention time outliers within each lipid class; and (4) LPA and PA artifacts generated by in-source fragmentation of LPS and PS. The shorthand notation used for lipid classification and structural representation follows the nomenclature proposed previously (Liebisch *et al*. 2020).

Relative quantification of lipid species was achieved by comparison of the LC peak areas of identified lipids against peak areas and quantities of the corresponding internal lipid standards in the same or similar lipid class (Table 1), and the concentrations of all lipids were reported at nmol/individual. Given that the commercially available stable isotope-labelled lipid standards are limited, some of the identified lipids were normalised against a standard from a different class or sub-class, and no attempts were made to quantitatively correct for different ESI responses of individual lipids due to concentration, acyl chain length, degree of unsaturation, or matrix effects caused by differences in chromatographic retention times compared with the relevant standards. The results reported here are for relative quantification and should not be considered to reflect the absolute concentrations of each lipid or lipid sub-class.

Table 1. List of lipid standards, adduct type and number of lipids that were identified in each class.

Name (Abbreviation)	Standard	Adduct type	Number of lipids identified
Diacylglycerol (DG)	15:0-18:1(d7) DAG	$[M+NH_4]^+$	14
Triglycerides (TG)	15:0-18:1(d7)-15:0 TAG	$[M+NH_4]^+$	41
Phosphatidylethanolamines (PE)	15:0-18:1(d7) PE	$[M-H]^-$	43
Ether-linked PE (EtherPE)	15:0-18:1(d7) PE	$[M-H]^-$	18
Oxidized PE (OxPE)	15:0-18:1(d7) PE	$[M-H]^-$	8
Phosphatidylinositol (PI)	15:0-18:1(d7) PI	$[M-H]^-$	33
Ether-linked PI (EtherPI)	15:0-18:1(d7) PI	$[M-H]^-$	3
Phosphatidylserine (PS)	15:0-18:1(d7) PS	$[M-H]^-$	10
Phosphatidic acids (PA)	15:0_18:1(d7) PA	$[M-H]^-$	5
Phosphatidylglycerol (PG)	15:0_18:1(d7) PG	$[M-H]^-$	24
Phosphatidylcholines (PC)	15:0_18:1(d7) PC	$[M+H]^+$	52
Ether-linked PC (EtherPC)	15:0_18:1(d7) PC	$[M+H]^+$	7
Lyso-PE (LPE)	18:1(d7) Lyso PE	$[M-H]^-$	13
Ether-linked lyso-PE (EtherLPE)	18:1(d7) Lyso PE	$[M-H]^-$	2
Lyso-PI (LPI)	15:0-18:1(d7) PI	$[M-H]^-$	7
Lyso-PS (LPS)	15:0-18:1(d7) PS	$[M-H]^-$	6
Lyso-PA (LPA)	15:0_18:1(d7) PA	$[M-H]^-$	4
Lyso-PG (LPG)	15:0_18:1(d7) PG	$[M-H]^-$	7
Lyso-PC (LPC)	18:1(d7) Lyso PC	$[M+H]^+$	9
Cardiolipin (CL)	15:0_18:1(d7) PG	$[M-H]^-$	66
Carnitine (CAR)	15:0_18:1(d7) PC	$[M+H]^+$	15
Sphingomyelin (SM)	d18:1-18:1(d9) SM	$[M+H]^+$	26

Ceramides (Cer)	d18:1-18:1(d9) SM	$[M-H]^-$	23
Hexosylceramides (HexCer)	d18:1-18:1(d9) SM	$[M-H]^-$	4
Sulfatides (SHexCer)	d18:1-18:1(d9) SM	$[M-H]^-$	16

212

213 **Data analysis**

214 All data were analysed through R v. 3.6.0. Female weight was analysed by ANOVA across
215 time followed by posthoc Tukey HSB tests. The concentrations of different lipid classes
216 (nmol/individual) were used as variables for principal component analysis (PCA). For
217 specific comparisons between treatments for particular lipids, concentration was natural log-
218 transformed before undertaking paired t-tests, and p values were corrected for lipid number
219 considered in a comparison by the Benjamin-Hochberg method.

220

221 **Ethics statement**

222 Female mosquitoes were blood fed on a volunteer in this experiment, with ethics approval
223 from the University of Melbourne Human Ethics committee (approval 0723847).

224

225 **Results:**

226 **Female mosquito measurement**

227 We first weighed female mosquitoes from samples collected at each time point (Figure 1),
228 with the difference between BBM and 0 h PBM representing the amount of blood a female
229 engorged. Sampling time had a significant effect on female weight (ANOVA, $F_{7,41} = 13.06$, P
230 < 0.001). The weights of engorged female mosquitoes were approximately double their
231 weights BBM. The females then started to digest blood, and their weight dropped
232 significantly from 0 h PBM to 4 h PBM, thereafter stabilising until 30 h PBM, and then
233 dropping again further to the BBM level at 72 h PBM; this change in weight reflects the
234 process of blood digestion, which takes around 32 hours after females are engorged
235 (Mukabana *et al*. 2002; Oshaghi *et al*. 2006). The appearance of female ovaries was captured
236 every day under a microscope (Sup Figure 1). We also calculated the coefficient of variation
237 (CV, CV = (Standard Deviation/Mean) × 100) for female wing length for the eight samples
238 we measured (mean ± se = 2.07 ± 0.06 mm, CV = 8.71) to indicate the spread of mosquito
239 body sizes in this study.

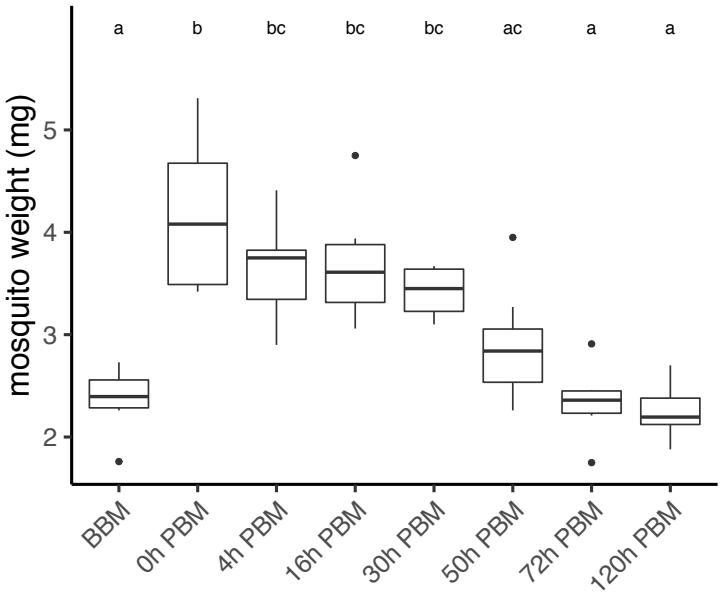

Figure 1. Weight changes across time. Female mosquitoes were weighed using samples from different time points over the course of feeding and reproduction. The same letter at the top of the boxplots represents groups that are not significantly different following Tukey HSD post-hoc tests.

Lipidomic change for uninfected mosquito

We measured lipidomic changes in individual females BBM and at different time points PBM, covering the entire vitellogenic cycle. In total, we identified 456 lipids (Table 1). We divided lipids into six functionally relevant groups: 1) glycerolipids, which include TG and DG; 2) diacyl-glycerophospholipids, which include PE, EtherPE, OxPE, PI, EtherPI, PS, PA, PG, PC and EtherPC; 3) lyso-glycerophospholipids, which include LPE, EtherLPE, LPI, LPS, LPA, LPG and LPC; 4) CL; 5) CAR; and 6) sphingolipids, which include SM, Cer, HexCer and SHexCer. The most abundant lipid group in female mosquitoes was the neutral glycerolipid, with 55 molecules making up approximately 80% of the total lipid amount. Glycerophospholipids accounted for 55% of the molecules identified, with 193 diacyl-glycerophospholipids and 48 lyso-glycerophospholipids. We also identified 66 CL, 15 CAR and 69 sphingolipids.

In order to obtain an overview of lipidomic changes during the reproductive process, we first undertook a principal component analysis (PCA) (Figure 2). This indicated that lipid components of female mosquitoes went through two main changes: the first occurred immediately after a blood meal (0 h PBM), and the second change occurred between 16 - 30

263 h PBM. Between these periods, the amounts of lipid in most classes also changed, except for
264 lyso-glycerophospholipids, in which case the total amount remained relatively stable during
265 the reproduction process (Figure 3).
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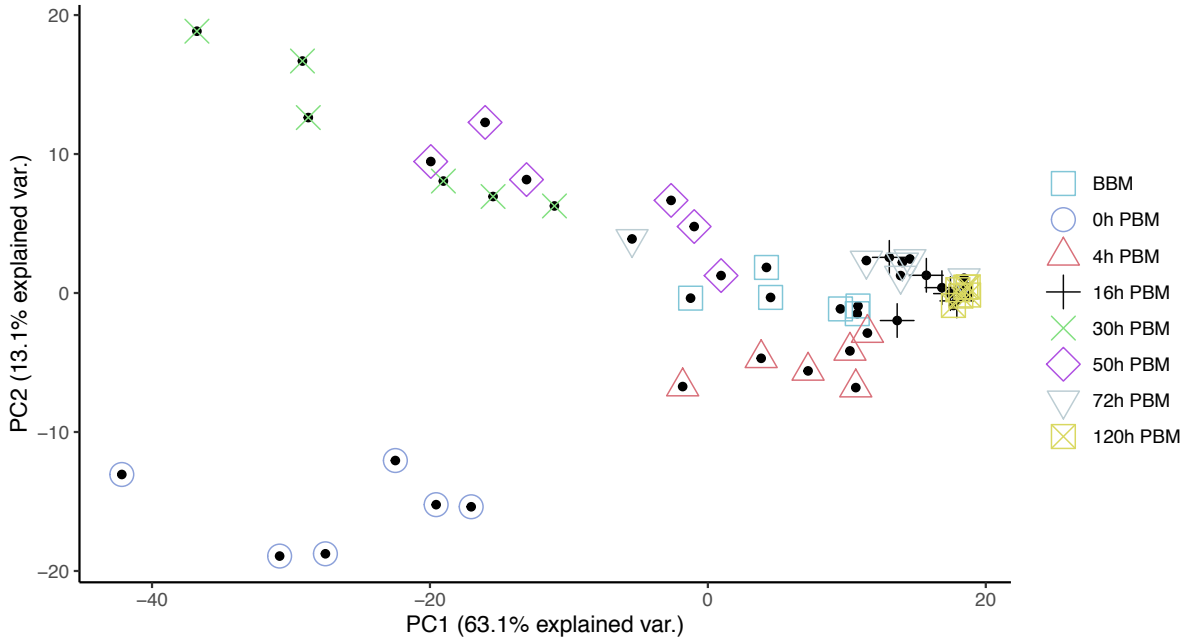


267
268 Figure 2. PCA plots of lipidome of *Aedes aegypti* females at different time points over the
269 course of feeding and reproduction.

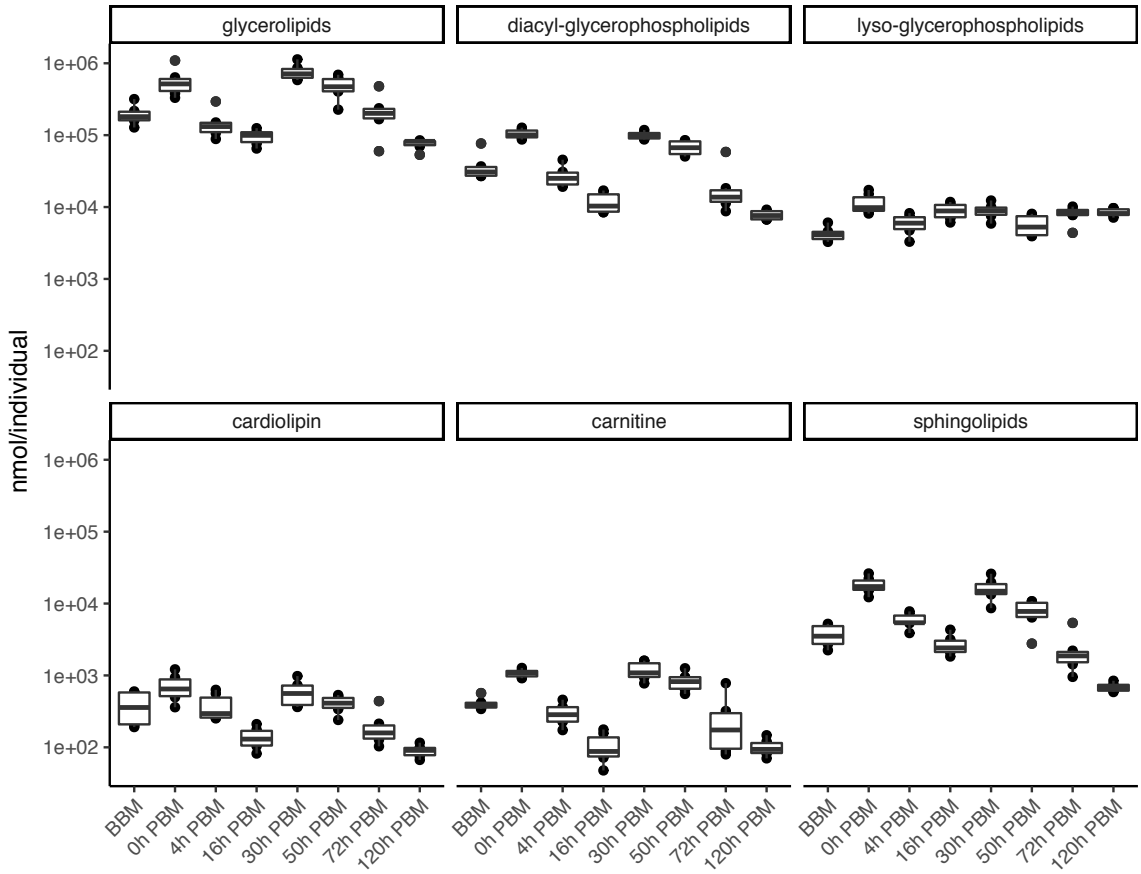


Figure 3. Total amount (nmol/individual) of glycerolipids, diacyl-glycerophospholipids, lyso-glycerophospholipids, cardiolipins, carnitines and sphingolipids in *Aedes aegypti* females at different time points over the course of feeding and reproduction.

We then also did PCA analyses on specific groups of lipids (Figure 4); for most groups the first PCs explained more than half of the variation, with the exception of the lyso-glycerophospholipids where it only explained 31.8% of variation and there was no obvious change directly after blood feeding but there was a shift at a later time point (Figure 4c), indicating that most changes in lyso-glycerophospholipid reflect mosquito metabolism rather than direct blood ingestion. However, all other lipid classes showed clear immediate changes in response to the blood meal as well as at a later time point (Figure 4a, b, d-f).

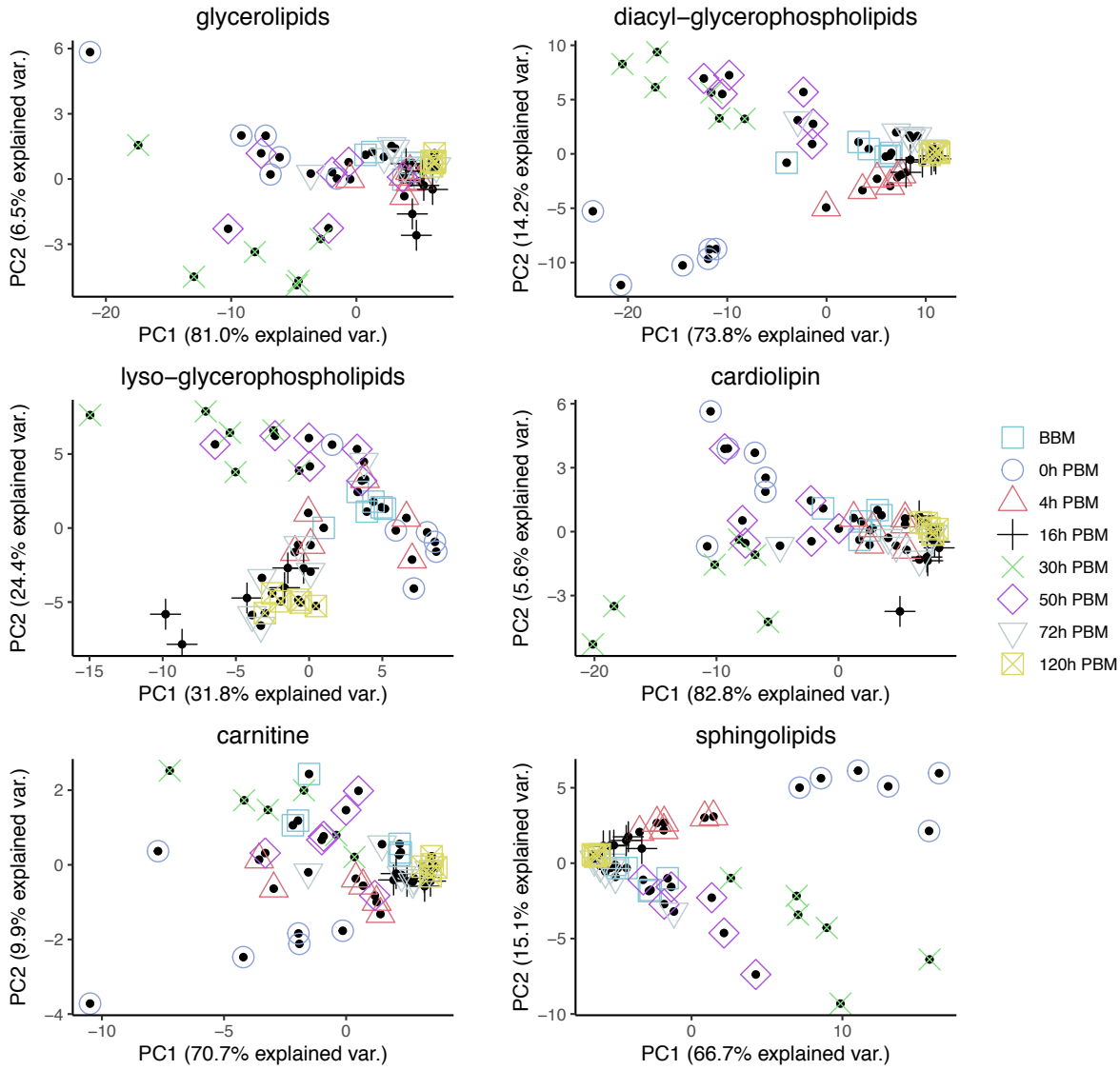


Figure 4. PCA plots of glycerolipids; (B) diacyl-glycerophospholipids; (C) lyso-glycerophospholipids; (D) cardiolipin; (E) carnitine; and (F) sphingolipids in *Aedes aegypti* females at different time points over the course of feeding and reproduction.

Identifying patterns for specific lipids

The amount of blood a female mosquito takes in when fully engorged can be similar to the weight of the individual, or even nearly twice the individual's weight (Roy 1936; Harrington, Edman & Scott 2001). We compared the time points BBM and 0 h PBM for individual lipid to determine which lipids were taken in with human blood. 86% of all the lipids identified significantly increased at the PBM monitoring time, while 14 (3%) of them significantly decreased and 50 (11%) did not change significantly (Sup Table 1). Specifically with reference to each lipid class, we obtained the following findings. (1) 75% (48 molecules) of lipids that did not increase are glycerophospholipids, and 29 are lyso-glycerophospholipids.

The latter represent 60% of the lyso-glycerophospholipids identified, while the 19 diacyl-glycerophospholipids that did not increase only accounted for 10% of molecules in this class. (2) All ether-glycerophospholipids (EtherPE, EtherPI, EtherPC and EtherLPE) and PS increased significantly. (3) Tetradecasphingenine (C14) and hexadecasphingenine (C16) CAR did not change significantly, while most of the other carnitines (7 out of 11) increased only slightly (adjusted $0.05 > p > 0.01$); only one lipid (CAR 20:1) in this class increased highly significantly (adjusted $p < 0.001$). (4) Most glycerolipids, CL and sphingomyelins increased.

The second significant lipidomic changes happened during the period spanning from 16 to 50 h PBM, and we specifically considered the difference between 16 and 30 h PBM when most lipids increased (87.7%, 400 molecules). These changed lipids may be used by female mosquitoes for egg development. 12 lipid molecules (2.6%) decreased significantly, and 44 lipid molecules (9.6%) did not change significantly (Sup Table 2). Of those lipids that increased, 392 showed increases of at least twice from 16 to 30 h PBM. These lipids are expected to be particularly important in mosquito egg production. For each group, we noted the following. (1) 60.7% (34 molecules) of those that did not increase are lyso-glycerophospholipids (constituting 70.8% of lyso-glycerophospholipids molecules), including all LPC and LPE. Interestingly, all LPA identified increased more than ten-fold from 16 to 30 h PBM. (2) For diacyl-glycerophospholipids, all EtherPI, OxPE, PA, PE, PG, PI and PS increased; 30 out of 43 PE and 18 out of 33 PI identified increased more than ten-fold. (3) All CAR and most glycerolipids, CL and sphingolipids increased. Specially, 42 out of 66 CL increased more than ten-fold and the four HexCer identified increased about ten-fold.

We found lyso-glycerophospholipid, a group composed of various sub-groups of lipids with different functions, had a different pattern of change in the above analyses. Specifically, the two most abundant lyso-glycerophospholipids LPE and LPC, which play an important role in membrane composition, were relatively stable in terms of total amount during reproduction. LPA showed dramatic increase from 16 to 30 h PBM, which the pattern is similar to most lipids in other groups. And LPI, LPG and LPS only had one significant increase at time period from 0 to 16 h PBM, the period when most of lipids in other groups decreased.

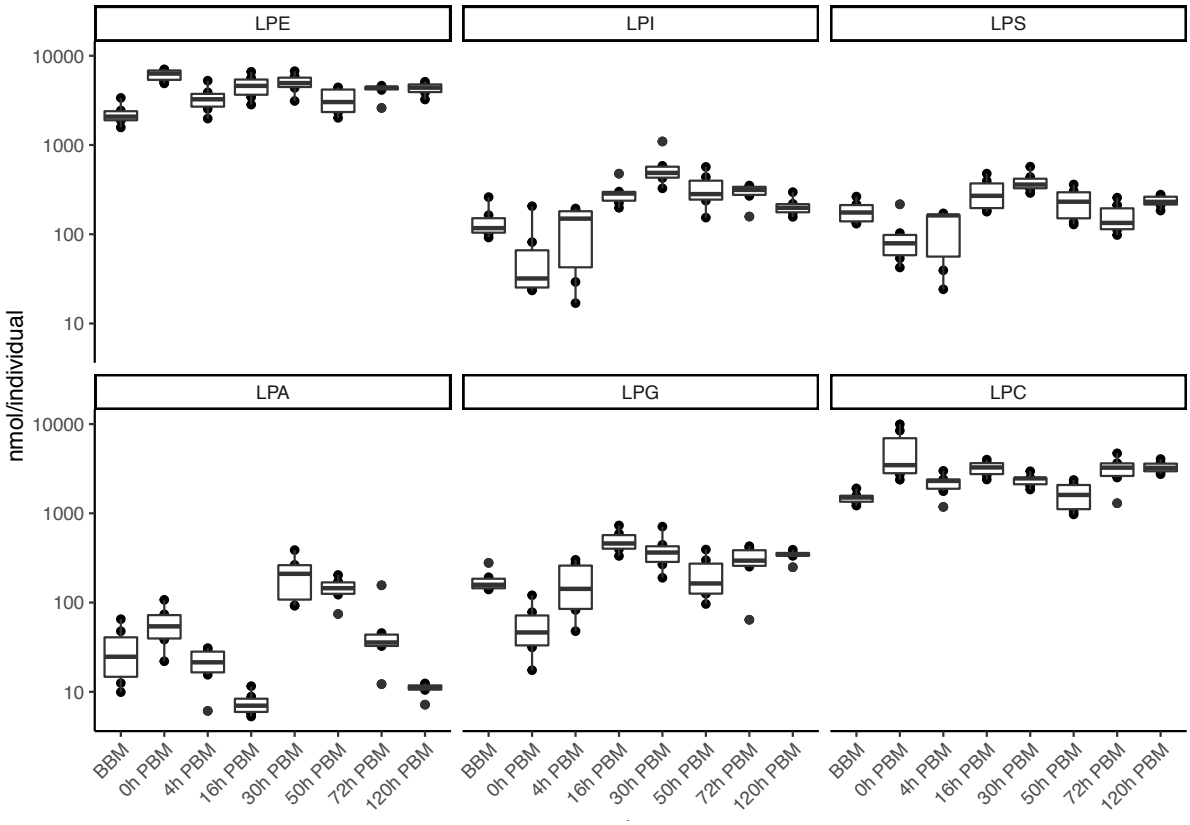


Figure 5. Total amount (nmol/individual) of LPE; LPI; LPS; LPA; LPG and LPC in the class of lyso-glycerophospholipids in *Aedes aegypti* females at different time points over the course of feeding and reproduction.

By comparing the differences between the two periods when most increases occurred, we found that most lipids we identified were present in both human blood and mosquitoes as well as being affected by mosquito egg development (detected or increased in both changes). Nevertheless, some lipids seemed to be present at a stable level in mosquitoes and were not significantly increased after blood feeding, such as tetradecasphingenine (C14) and hexadecasphingenine (C16) CAR, most of the LPG; LPI and LPS (Figure 5); and some seemed to exist typically in human blood, which we define here as those that increased more than 100-fold after blood feeding but did not increase significantly (adjust $p > 0.05$) at the later period. These are PE O-36:5 (16:1_20:4), PE O-38:6 (16:0_22:6), PE O-38:6 (18:2_20:4), PE O-38:7 (16:1_22:6), PE O-40:5 (18:0_22:5), PE O-40:6(18:1_22:5), PE O-40:7 (18:2_22:5), PC 40:6 (18:0_22:6), SM 33:1;2O (17:1;2O/16:0), SM 34:2;2O (18:2;2O/16:0), SM 36:2;2O (18:2;2O/18:0) and SM 42:3;2O (18:1;2O/24:2).

Discussion

The reproductive process of *Aedes aegypti* is tightly linked to regulation of its nutritional status (Attardo, Hansen & Raikhel 2005). Essential nutrients mainly come from human blood such as amino acids and cholesterol (Hansen *et al*. 2004; Geoghegan *et al*. 2017); or via the fat body (Arrese & Soulages 2010), which supports gene products involved in yolk development (Price *et al*. 2011). Apart from these changes of amino acids, cholesterol and yolk protein, the vittellogenic cycle is also expected to involve lipid generates, with lipids attributing to 35% of ovarian dry weight (Troy, Anderson & Spielman 1975; Briegel, Hefti & DiMarco 2002).

In the current study, we focused on lipidomic changes of whole female mosquitoes during the reproductive process. Whole-body investigations provided an overall appraisal which detected changes that might not be evident when using organs or cells. We found that there are biphasic increases in most lipids at 0 h PBM and 16 to 30 h PBM. The first increase mainly reflected the intake of lipids from human blood which the increase is also consistent with lipidome composition of human plasma (Burla *et al*. 2018), and the second most likely reflected the biosynthesis of lipids to support egg formation. The amount of lipids dropped between these increases, which is similar to the change of lipids observed in the fat body (Pinch *et al*. 2021), and suggesting the digestion of lipids from blood. The amount of lipid dropped again after the second increase until the end of the experiment when females were finished laying eggs (120 h PBM); this change is expected to reflect the process of lipids being used in supporting egg formation and deposition. Among lipid groups, lyso-glycerophospholipids as a whole showed a different change pattern and a relatively static abundance. Although Pinch *et al*. (2021) observed a small decrease from 12 h to 24 h PBM, they specifically focused on the fat body and with different time points and output units, which makes it difficult to directly compare our results with theirs.

The weight of mosquitoes did not show a corresponding decrease at the first time of lipids decreased, indicating that lipids were transformed into different metabolites, such as LPI, LPG and LPS that were observed to increase in this period. However, lyso-glycerophospholipids only constituted a very small component of total lipids in *Ae. aegypti* females, so perhaps it is more likely that lipase activity produced free fatty acids for eventual consumption in the process of beta-oxidation to provide energy for reproduction (Toprak *et al*. 2020).

Pinch *et al*. (2021) observed a relatively lower level of TG in the *Ae*. *aegypti* female fat body at around 30 h PBM, while our study of whole body lipidome showed highest level of TG at 30 h PBM. The discrepancy may reflect the transportation of lipids from the fat body or midgut into oocytes during the insect reproductive process through lipophorin, a major lipid-carrying protein (Kawooya & Law 1988). Glycerolipids, in particular TG, account for a high percentage of the lipids present in insects. TG that are stored in the fat body can be transformed into DG and fatty acids and then exported to the hemolymph for transportation (Beenakkers, Van der Horst & Van Marrewijk 1985; Arrese *et al*. 2001; Canavoso *et al*. 2001). However, we did not find a substantial increase for DGs at the time when overall glycerolipids declined between 4 and 16 h PBM. This may not be important, because there is evidence that *Ae*. *aegypti* and some other Diptera predominantly transport TG via lipophorin, not DG as is the case of most other insects (Ford & Van Heusden 1994; Pennington, Nussenzveig & Van Heusden 1996; Arrese *et al*. 2001). Moreover, a general observation for insects is that the midgut can convert DG to TG in the presence of high concentrations of potentially toxic fatty acids; this conversion could maintain low intracellular levels of DG and fatty acids to prevent lipotoxicity (Canavoso & Wells 2000; Canavoso *et al*. 2001; Toprak *et al*. 2020). Additionally, there are other possibilities for lipid movement not based on DG transport including evolutionarily conserved mechanisms for movement of fatty acids (Black & DiRusso 2003).

Lyso-glycerophospholipids were highlighted as a lipid group of interest by the present study due to their unusual temporal pattern in abundance. Lyso-glycerophospholipids have diverse functions and they are also found in cell membranes (Rietschel *et al*. 1994); as precursor of phospholipid biosynthesis, they may act as signal molecules involved in a broad range of biological processes (Ishii *et al*. 2004; Tan *et al*. 2020). However, they might also reflect a highly regulated reservoir of lipids with accessible utility and concentration-dependent lipid toxicity liability (Arouri & Mouritsen 2013), which can result from lipid overload generated by dietary excess (Levental *et al*. 2020) as might be the case when mosquitoes blood feed. LPE and LPC incorporate into membranes and change membrane permeability and stability. The change in permeability and stability can result in membrane toxicity unless concentrations of lyso-glycerophospholipids are kept below a cytotoxic limit (Arouri & Mouritsen 2013), which may explain the stability of these two lipid groups. Also, we found

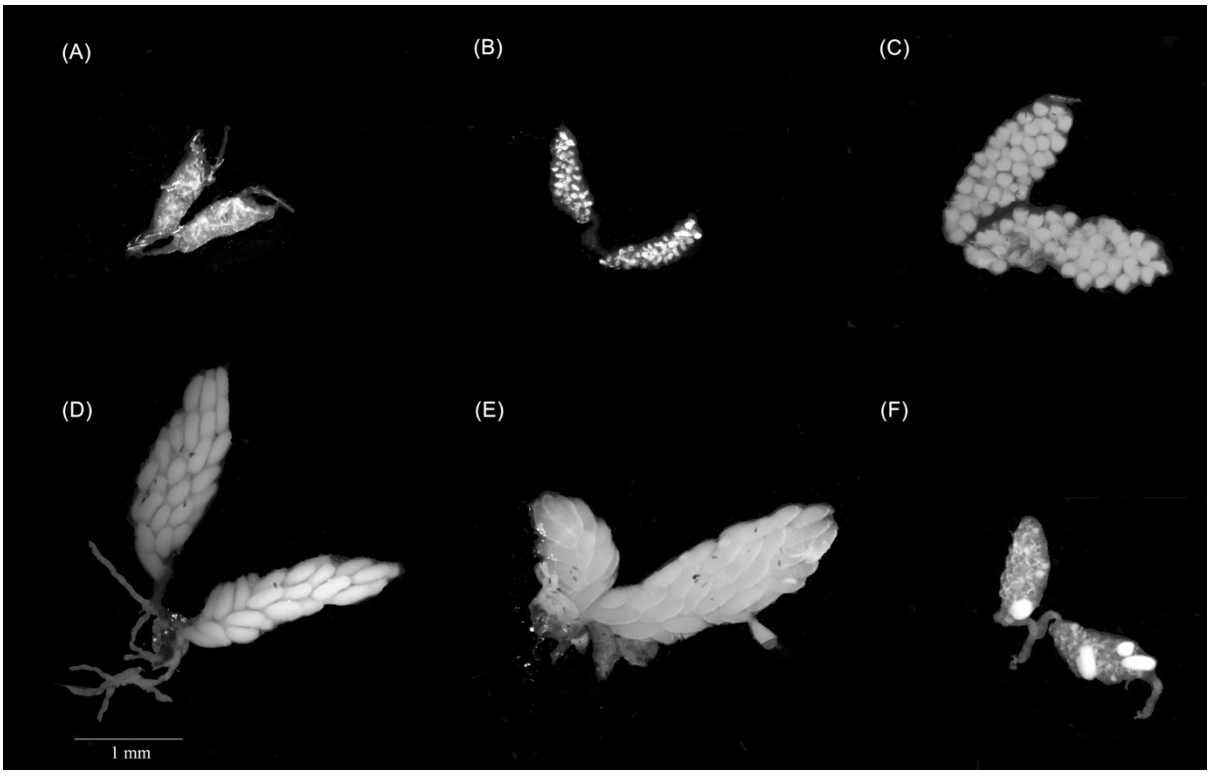
substantial increases in cell membrane lipids between 16 and 30 h PBM as in the cases of PE and PC, reflecting the large number of cells formed during the reproductive process. Moreover, lyso-glycerophospholipids could act as a collective reserve which provides acyl chains for beta-oxidation when needed (Black & DiRusso 2003), or they could be a source of partially assembled molecules for further biosynthesis, which may explain the unusual increase of LPI, LPG and LPS between 0 and 16 h PBM followed by increase of near all major lipid groups from 16 to 30 h PBM. It's worth noting that LPA, the only group from lyso-glycerophospholipids showing a bimodal pattern with increases from 16 to 30 h PBM, are essential signalling molecules (Luquain, Sciorra & Morris 2003).

We also found that many lipids from groups PI and CL increased dramatically from 16 to 30 h PBM. These lipids are closely connected with metabolic activity and signalling. For example, PIs are important in regulating phototransduction, cell growth and the developmental process of fruit flies (Milligan *et al*. 1997; Garcia-Murillas *et al*. 2006; Gupta *et al*. 2013). And CLs are integral to mitochondrial membranes, with important roles in ATP-synthase and phospholipid remodelling (Schlame & Ren 2009; Acehan *et al*. 2011). Moreover, SM play significant roles in mammal cells being involved in diverse signalling pathways (Hannun & Bell 1989; Hannun & Obeid 2008), whereas for dipteran insects such as *Drosophila*, there is a lack of SM and instead they synthesize the SM analogue phosphoethanolamine ceramide (Acharya & Acharya 2005). Though we did not look into phosphoethanolamine ceramide in the present study, we also note that most of SMs were either lacked or changed slightly between 16 h and 30 h PBM. Finally, it is worth noting that approximately a quarter of the lipids, including almost all SHexCer we identified, contained odd chains. Pinch *et al*. (2021) investigated the fat body of mosquitoes feeding on bovine blood and supposed that odd-chain lipids may come from ruminal microflora or microbes in larval rearing water or branched chain amino acids from hemoglobin. Our study showed that the last hypothesis is most likely, as we observed these odd-chain lipids from human blood and also increased in mosquitoes between 16 h and 30 h PBM, these odd chain fatty acid-containing lipids did not show any distinct change patterns comparable to even chain fatty acid-containing lipids from the same lipid group.

In summary, we assessed lipidomic changes in female *Ae. aegypti* mosquitoes at different time points over the course of feeding and reproduction. We observed temporal biphasic

increases of most lipids with peaks at the time of feeding (0h PBM) and 1-2 days after feeding (30 h PBM); the first peak mostly represented the intake of human blood and the second mostly represented the biosynthesis of mosquitoes to support the reproduction. The abundance of many lipids dropped to a lower level between the two peaks while the weight of mosquitoes remained stable indicating a large amount of intermediates were likely generated and transported. The generation of phospholipids at the second peak points indicating the development of oocytes and our study also highlighted lipids involved with metabolic activity and signalling that increased dramatically at the second peak. Our study provides a rich arena for further work to understand how lipid metabolism is involved in different components of the reproduction process of *Ae. aegypti*.

Supporting information



Sup Figure 1. Ovarian appearance of female mosquitoes dissected at (A) BBM, (B) 1 d PBM, (C) 2 d PBM, (D) 3 d PBM, (E) 4 d PBM and (F) 5 d PBM. There is some variation in the stages of development of the ovaries among individuals as captured by variation in weight (Fig. 1).

Sub Table 1. List of lipids that changed before and after female *Aedes aegypti* mosquitoes blood fed (between BBM and 0 h PBM).

Sub Table 2. List of lipids that changed between 16 and 30 h after female *Aedes aegypti* mosquitoes blood fed (between 16 and 30 h PBM).

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