Mobilization of Lipids Underpins Honey Bee and Colony Health During Limited Supplementary Feeding

Clara Castaños
The University of Western Australia
Mary Boyce
Edith Cowan University
Harvey Millar
University of Western Australia
Nathan Lawler
Edith Cowan University
Tiffane Bates
The University of Western Australia
Julia Grassl (julia.grassl@uwa.edu.au)
The University of Western Australia https://orcid.org/0000-0001-8653-4983

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Abstract

Honey bee nutritional health depends on nectar and pollen, which provide the main source of carbohydrate, protein, and lipids to individual bees. During malnutrition the insect metabolism accesses fat body reserves, this process however, is poorly understood, as well as its repercussions at a colony level. Using untargeted lipidomic analysis we examined the effects of different nutritional conditions during larval development followed by recovery and colony productivity. Nutritional stress led to an increase of unsaturation in triacylglycerols and diacylglycerols, as well as a decrease in free fatty acids. Suggesting the bees' metabolism triggers a process that makes lipid reserves rapidly accessible by changing their structure and allowing their mobilization. We also identified the specific lipid desaturase genes in honey bees that are induced in response to starvation. Even though nutritional stress was evident in starving and sugar-fed bees at a colony and physiological level, we found that only starved hives presented long-term effects as honey production.

Introduction

The Western honey bee *Apis mellifera* is amongst the most beneficial insects, providing pollination services to ecosystems and managed crops. The economic value of *Apis mellifera* as a honey producer and agricultural pollinator is recognized worldwide, especially as the main pollinator of large-scale crop monocultures[1], such as almond, avocado, canola and coffee[2,3]. However, honey bees and their essential services are at risk because of a decrease in health and survival, attributed to a variety of stressors interacting in the colony[4,5]. Among them, diseases and parasites, such as bacteria, viruses, fungi, and mites such as *Varroa destructor* [6,7]. Pesticides and insecticides can reduce the immune system responses and alter the nervous system, affecting navigation, foraging, development and increasing mortality of bees[8,9]. Hence, the decline in honey bee health is complex and its analysis requires a multifactorial approach.

The health status of a honey bee colony can be monitored through the measurement of different hive variables[5]. For example, those involved in maintaining adequate internal hive conditions, such as temperature and humidity, conditions that weak and unhealthy colonies are unable to maintain[10]. Temperature and humidity deregulation negatively affect the development of the larvae and pupae, decreasing their survival[11,12], promoting the development of diseases such as *Ascosphaera apis*[13], and influencing the adult bees behaviour when developing under suboptimal temperature conditions[14]. The colony nutritional condition is closely related to the health of honey bee colonies and its monitoring is critical to avoid colonies losses. Land fragmentation and alteration, loss of food resources and poor beekeeping management practices are highly responsible for nutritional stress[4,15].

Honey bees' nutritional health depends on the availability and collection of nectar and pollen in quantity and quality to meet the main nutritional requirements of carbohydrates, proteins, lipids, vitamins and minerals[15]. The weight of the whole hive is an indicator of colony growth and productivity, but mainly of

food reserves (especially honey)[16,17]. Nectar is the main component of honey and it is the principal source of carbohydrates for bees, especially important in providing energy for flight[18]. A deficiency of nectar in the colony leads to a reduction in the foraging activity and hygienic behaviour[19], which can make the colony susceptible to different diseases and decrease honey productivity[20].

Pollen is the main source of protein and amino acids; however, it also provides carbohydrates, lipids (including essential fatty acids and sterols), minerals and vitamins[15]. Pollen provides the nutrients for honey bee growth and development, for the rearing of the brood and stimulates the queen to lay eggs[21,22]. Therefore, pollen is indispensable for reproduction and the maintenance of a high number of bees, the quantity of brood being a common indicator of the reproductive output and size of the colony[23]. Honey production decreases when pollen is scarce because of the reduction in forager bee numbers, causing the colony not to take full advantage of nectar flows[24,25].

Among the nutrients that bees obtain from pollen, lipids are the long-term energy reserves and the major components of the fat body. The fat body is the insects' organ of energy storage with a great biosynthetic and metabolic activity[26]. During times of high energy demand, the insect's metabolism accesses triacylglycerols storage in lipid droplets of the fat body via lipolysis. Lipases catalyse the hydrolysis of the triacylglycerols making the fatty acids accessible for mobilization out of the fat body as diacylglycerols[26]. Diacylglycerols are hydrolysed further and free fatty acids are used in the generation of energy through β-oxidation[27,28]. Desaturases enzymes are necessary during this mobilization of lipids, by catalysing a double bond addition to fatty acid chains[29]. The presence of double bonds allow lipases activity[30,31] which has been reported during the lipolysis process of diapausing and overwintering insects[28]. Nutritional stress might generate a similar process of fat body lipolysis in insects[26]; where the role of desaturases might be necessary for the lipases to hydrolyse lipids. However, evidence that this process occurs and is important for bee nutrition is lacking.

Lipids also play an important role in the synthesis of pheromones and cuticular hydrocarbons[32,33] and are indispensable in the production of wax in the hive[34]. Furthermore, lipids are involved in the bees' behavioural maturation, where the transition from nurse to forager encompasses a decrease in fat body's lipid storage[35]. Thus, lipid depletion due to malnutrition in the colony, causes an early transition, with nurses becoming foragers at a younger age[35]. At the colony level, it has been demonstrated that by restricting pollen supply, the weight of worker bees decreased, but pollen collection and the number of foragers in the colony increased[25], most likely due to an early transition to foraging. Hence, lipid restrictions may affect the structure, health, and productivity of the whole colony, but direct evidence of this connection is limited.

To obtain a more comprehensive picture of malnutrition and its implications, organism physiology and colony structure should be studied as coordinated factors. The molecular events involved in changing the accessibility and mobilization of lipid contents during periods of starvation is poorly understood in honey bees, as well as the consequences of these changes on colony health. A high lipid concentrated diet

increases brood production[36], but a high-fat diet relative to optimal protein intake increases the risk of brood dying[37]. Furthermore, an increase in omega-6:3 ratio negatively affects brood rearing, cognitive performance, and survival[36,38], indicating bees appear to regulate their fat intake by an unknown mechanism[37,39]. Characterizing the molecular and colony implications of changes in lipid quality and quantity in bees' diet, especially during malnutrition periods, provide opportunities to improve the development of supplementary foods and beekeeping nutritional practices in the industry.

Material And Methods

Diet restriction field experiment

Construction and build-up of beehives

The experiment consisted of 18 full-size hives with 8 frames each: 2 drawn combs; 5 foundation combs (templates for the construction of the drawn combs) and a honeycomb (frame full of honey). Each hive was made up of one queen bee (genetically different from each other) and 2 kg of worker bees. A raiser and a mat were placed under the lid. Bees and equipment for this experiment were provided by the apiary of The University of Western Australia in Perth.

After the construction of the hives, we immediately placed them in a Red Gum (*Corymbia calophylla*) forest at Gidgegannup, WA (31°47'59.6"S 116°10'13.8"E), allowing free foraging for 4 weeks. Red Gum eucalypt was chosen since it is an excellent source of nectar and pollen for honey bees[40]. We placed 10 colonies on January 31st, 2019 and a further 8 colonies on February 5th, 2019.

Experimental set-up

After 4 weeks of freely foraging, the hives were shifted to a burnt unproductive site at Lancelin WA (30°47'11.8" S, 115°16'48.7"E), to stop foraging activity and to control the feeding. We randomly assigned the colonies to three different nutritional treatments, with six replicate colonies each: 1) Starving - no food provided, 2) Sugar - colonies fed each week with 1 L of sugar syrup 150% (w/v), and 3) Sugar + pollen - colonies fed each week with a pollen patty of 250 g of Red Gum pollen mixed with 50 mL of 150% (w/v) sugar syrup and 1 L of sugar syrup 150% (w/v). The pollen patties were placed under the mat of each hive, and the sugar syrup was placed on a 1.10 L bucket container under the lid of the hives and over the mat. Each week 27 newly hatched worker bees were collected from each hive, and immediately placed on dry ice and stored at -80°C for biochemical analyses. As health indicators of the hives, we monitored the weight, temperature, humidity, and quantity of brood.

Data and sampling collection lasted for 4 weeks at the unproductive site when we detected that the health condition of the hives from the starving diet treatment was deficient. The hives were moved to a honey flow in a White Gum (*Eucalyptus wandoo*) forest (excellent source for honey production according to Coleman 1962[40]) in Calingiri, WA (31°09'05.0" S, 116°18'05.1" E) to allow the free foraging of all the colonies. The recovery or maintenance of health was monitored with the same variables for a further 4

more weeks, and the sampling collection continued. From week 8 to 25, we shifted the hives into areas of good nectar sources, and we tracked their productivity by weighing the hives once a month (at weeks 12, 16, 21 and 25), and assessing the capped brood quantity once at week 21. Biochemical analyses were carried out for the bees collected from the diet treatment period and the recovery period (weeks 1 to 8, Fig. 1).

Nutritional disorders in the colony affect first-generation, but also the subsequent ones [41]. Nurse bees feed the worker larvae from the first day of hatching from the egg until before the cell is sealed. This is from day 4 to 9 of the worker bees' development in the cell, which hatch 12 days after being capped [12]. Because newly hatches bees were used for the study, they might represent the nutritional condition of the nurses that fed them on their larval stage, showing the nutritional condition of the bees 12 days ahead of the hive health indicators. Hence, the lipidomic analysis baseline was 12 days after week 0 of the experiment, which is between weeks 1 and 2 (Fig. 1). Subsequently, we used week 1 as the baseline for the lipidomic analyses, which represented bees that were still fed at the end of the build-up period, but not fed during the diet treatment period yet (Fig. 1).

Hive health monitoring

Weight, temperature, humidity, and quantity of the brood were measured each week. The hives were weighed using a digital weighing floor scale and hive weight was used as a measure of general hive strength and honey storage. We measured temperature and humidity by placing a digital monitor (remote relative humidity/temperature monitor-800027, Sper Scientific) in each hive. To measure the quantity of capped brood in each hive we took photos of both sides of each frame (Canon PowerShot A3300 IS). The photos were processed in the software “Beestly” (https://cyency.com/products/beestly/index.html) which allows the easy identification and quantification of capped brood cells. Finally, at the end of each sampling day the hives were fed according to the diet treatments.

Samples preparation for mass spectrometry analysis

The bees’ fat bodies from weeks 1 to 8 were dissected by removing the gut and the rest of the organs enclosed in the abdomen and conserving only the abdominal segment with the fat body attached at the dorsal region.

Each sample consisted of 20 abdomens (approx. 180 mg). The samples were weighed and processed for analysis in two separate batches that were created by randomization of hives. A solvent of 80% aqueous methanol (MeOH) was added at a concentration of 4:1 aqueous MeOH/bee material. The solvent contained a labelled internal standard at approximately 0.8 ppm of sphingosine-d9, tryptophan-d5, and taurodeoxycholic acid-d4. Samples were ground in 2 mL tubes containing ceramic beads (Precellys CKMix for tissue homogenizing), using a tissue homogenizer (Precellys evolution, Bertin instruments) at 6,000 g, 2x20 sec, at 0°C, with 15 sec of rest between cycles.

Untargeted lipidomic analysis by liquid chromatography high resolution mass spectrometry (LC-HR-MS)

For lipidomic analysis 100% chloroform ($CHCl_3$) was added to the extracts, reaching a final concentration of 1:1 aqueous MeOH/$CHCl_3$. Samples were vortexed for 10 sec and subsequently incubated for 30 min at room temperature and 750 rpm in a Thermomixer. The samples were dried under nitrogen and resuspended in 100% isopropanol (IPA) according to weight, obtaining a concentration of 4:1 IPA/bee material. The samples were centrifuged at 9,000 g, 4°C, for 10 min and the supernatant was split into 2 separate tubes, where 200 µL were destined for lipids analysis and 200 µL for fatty acid analysis. All the samples were dried under nitrogen and the samples destined for lipid analysis using liquid chromatography high resolution mass spectrometry (LC-HR-MS) were resuspended in 150 µL of IPA containing an internal standard of 1 ppm of deuterated cholesterol. Samples were vortexed and the supernatant isolated and transferred to a glass vial ready for analysis. Quality control (QC) samples were prepared by pooling 90 µL of each sample from batch 1.

Samples were analysed by high-pressure liquid chromatography (Dionex UltiMate 3000 RS) coupled to an Orbitrap Q-Exactive mass spectrometer (Thermo Fisher Scientific) with a heated electrospray ionisation probe (HESI) as the interface. Separation was achieved on a reversed phase Supelco Analytical Titan C18 column (2.1 × 75 mm, 1.9 µm particle size). The mobile phase was (Solvent A) 60:40 acetonitrile:water containing 10 mM ammonium formate and 0.1% formic acid and (Solvent B) 85.5:9.5:5 2-propanol (IPA):acetonitrile:water also containing 10 mM ammonium formate + 0.1% formic acid. The elution gradient was as follows: isocratic with 20% solvent B for 0.5 min, followed by an increase to 100% solvent B (0.5-8.5 min) and maintained at 100% B for 1 min. Then the system reverted to initial conditions (20% B) over 2 minutes and was equilibrated for 2.5 min before the next injection. The flow rate was 0.4 mL/min, the injection volume was 0.1 µL and column oven temperature was 55°C. Full scans with data-dependent tandem mass spectrometry were acquired on the Orbitrap mass analyzer. Full scans were acquired at a resolution of 70,000 at mass-to-charge ratio (*m/z*) 200 over the *m/z* range 150–2000 with the ESI conditions as follows: capillary temperature = 350°C, sheath gas = 48 (arbitrary units), auxiliary gas = 15 (arbitrary units), ion spray voltage = + 3.2 kV S-lens 60%. Tandem mass spectrometry analyses were performed at a resolution of 17,500 at *m/z* 200 on each sample with the collisional dissociation energy set at 40 eV. Data acquisition was carried out using Xcalibur software (Thermo Fisher Scientific).

Lipids data was analysed in MS-DIAL (version 4.18), where peak detection, identification and alignment were done using the LipidBlast adjusted Fiehn lipidomics dataset for the peak identification[42]. Data normalization and exportation was implemented as part of the MS-Dial software as recommended by Perez de Souza et al.[43]. To decrease the possibility of duplicated features and to increase the confidence of the identified compounds in the lipid analyses, only analytes that generated a MS fragmentation pattern were considered. We detected repeated diacylglycerols and triacylglycerols identities by sodium and ammonium adducts presenting the same results independently of the adduct. Both adducts display a structurally informative fragmentation pattern[44]. Hence, to avoid duplications, we conserved the ammonium adducts, which consistently presented a higher area abundance.

Fatty acids methyl ester (FAME) analysis

Previously dried samples for fatty acid analysis were resuspended in 305 µL of extraction buffer [MeOH containing 2% H_2SO_4 (v/v)]. The tubes were sealed with microtube cap locks, then mixed (using a vortex) for 10 sec and incubated for 2h at 80°C and 750 rpm in the Thermomixer. Samples were cooled to room temperature and 300 µL of NaCl [at 0.9%] and 300 µL of hexane were added. Samples were vortexed for 10 s and centrifuged at 15,700 g, for 3 min at room temperature. Next, 100 µL of the upper layer were transferred into a glass vial and used for FAME analysis by GC-MS. A QC sample was pooled from 90 µL of each sample from batch 1. The analysis was performed on a Gas Chromatograph (Agilent 6890N), equipped with a Gas Mass Selective Detector, 5975 series (Agilent Technologies). Separation was completed on a Agilent VF-5MS column (30m x 0.25mm, 0.25µm film thickness) + 10 m EZ-Guard column. The sample was injected at an initial temperature of 280°C and held for 5 min with a solvent delay of 5 min. Helium was used as a carrier gas with a flow rate of 1 mL/min. The purge flow was set to start at 2 min at 20 mL/min. The oven temperature was programmed as follows: initial temperature 70°C, hold for 2 min, then increase to 350°C at a rate of 7°C/min, and a post-run hold time of 5 min at 280°C. The transfer line temperature was kept at 250°C, and the ion source temperature was 230°C. The detector operated in scan mode from 40 to 600 Da. The MS Single Quad operated at 150°C and the collisional dissociation energy was set to 70 eV.

Fatty acids' data was first analysed with Agilent MassHunter Qualitative Analysis Navigator (version B.08.00) to discard background peaks from blank samples. The fatty acids were identified by deconvoluting peaks with the Automatic Mass Spectral Deconvolution Identification System (AMDIS) (version 2.64, 2006) and accessing the Mass Spectral Search Program (NIST/EPA/NIH/Mass Spectral Library, Version 2.0.d, 2005) to identify each compound. After the main compound features were identified and named, we used the program Agilent MassHunter Quantitative Analysis (version B.08.00) to align and quantify peak intensities.

Desaturase genes primers, RNA isolation and Quantitative Real-Time PCR

Desaturase genes of honey bee were found in the National Center for Biotechnology Information (NCBI). We used primers previously reported for five acyl-CoA Delta (11) desaturases from Falcon et al.[33] and one more designed from Vernier et al.[45] Using the software SnapGene 5.2 (www.snapgene.com) we designed primers for three more acyl-CoA Delta (11) desaturase or desaturase-like, and one sphingolipid Delta (4)-desaturase (Supplementary Table 1). AmACT was used as housekeeping gene since has been reported as a stable housekeeping gene for honey bees[46]. Primers sequences used for each gene and specific information is listed on Supplementary data Table 1.

Bees from weeks one, three and four were dissected as previous and ground in liquid nitrogen using mortar and pistil (using 7 bees per sample and samples consisting of 3 to 4 hives per treatment). RNA isolation was done with 15 mg of ground tissue from each sample using SV Total RNA Isolation System (Promega, Madison, WI), according to the manufacturer's specifications. 1.2 µg of RNA were used for cDNA synthesis using iScript cDNA synthesis kit (Bio-rad, 1708890). cDNA was further diluted and used for quantitative real-time PCR using QuantiNova SYBR green PCR kit (Qiagen, 208056). Values were

normalized against the housekeeping gene and the relative abundance of each gene by sample was calculated by the comparative Ct method[47].

Statistics and reproducibility

For hive health data, we fitted generalized linear models with quasi-poisson distribution for capped brood cell counts and gamma distribution for the rest predicted variables (hive weight, temperature, and humidity). We considered treatment (6 hives per diet treatment), time (sampling week) and the second order interaction of both as response variables. Akaike information criterion (AIC) value was used to find better-fitted model and distribution. To determine significant differences between specific time points we did post-hoc comparisons, adjusting the *P*-value with the false discovery rate (FDR).

Precision post-analysis tests were done to clean the MS data for both FAME and lipidomic results, determining the relative standard deviation (RSD) and the dispersion ratio (D-ratio) for untargeted analysis, discarding the compounds that were over the acceptance criterion (RSD > 20% and D-ratio > 50%), (for more details refer to Broadhurst et al.[48]. Lipidomics data was analysed with generalized linear mixed effect models with gamma distribution, where the logarithm of each compound peak area was the predicted variable. The response variables were treatment, time, and their interaction. The batch of laboratory analyses (two levels) was included as a random effect variable since we detected an effect in the raw data of the samples (Supplementary Fig. 1). Significance was tested with Least Squares Mean (LS mean) comparisons using FDR for adjusting the *P*-value. Fatty acid results did not present a batch effect; however, the machine presented a performance decrease during the running of the samples. Subsequently, we performed a QC based random forest signal correction (QC-RFSC) using statTarget package in R language[49,50]. The values were normalized to time 1 by treatment and Mann-Whitney-Wilcoxon test was used to evaluate significant differences. In a closer examination of the lipidomics' data the software Perseus 1.6.15.0 was used to generate hierarchical clusters with the estimates of the models considering only the LS mean significant compounds in each treatment ($P < 0.05$).

The relative expression of desaturase genes was analysed using generalized linear models with gamma distribution or generalized linear mixed effect models when considering hive as random effect resulted in a better-fitted model (according to the AIC value). We ran LS mean comparisons using FDR to test significance. In every analysis assumptions of homogeneity of variance and dispersion were checked with Levene test and residuals distribution plots. Apart from Perseus software for the clustering analysis, the main statistical analyses and plots were performed in R[50].

Results

Different types of supplemental feeding affect malnutrition and honey bee colony health

Comparing colony health between hives treated with different supplementary treatments, we found differences between the diet treatments and time. The generalized linear model showed differences in the hive weight of the different diet treatments over time and between treatments (deviance= 6.143, $df = 24$, P

< 0.001), (Fig. 1A and Supplementary Table 2). Hives of the starving treatment decreased in weight at week 3 (post-hoc FDR, $P = 0.004$) and week 4 (post-hoc FDR, $P = 0.002$) relative to week 0. Conversely, hives fed with supplementary diets (sugar and sugar + pollen) maintained their weight during the diet treatment period (Fig. 2A and Supplementary Table 3). All hives in all 3 treatments recovered when placed on a honey flow, however, the starvation-treated hives never reached the same weight as the supplementary fed hives (Fig. 2A and Supplementary Table 3).

The differential diets caused a difference in the quantity of capped brood in the 3 diet treatments over time (deviance= 413.57, $df = 18$, $P < 0.001$) (Fig. 2B and Supplementary Table 2), where the 3 treatments showed a significant decrease (Fig. 2B and Supplementary Table 4). However, the starving group presented a larger decrease of capped brood cells (Fig. 2B and Supplementary Table 4). Moreover, no capped brood was found for 3 of the 6 hives at weeks 4 and 5 in the starving treatment hives. Due to this brood gap, we could not find recently hatched bees in 3 of the 6 hives during week 4 of sampling, and in 5 hives at weeks 5 and 6. Consequently, samples from weeks 5 and 6 are absent from the lipidomic results for the starving treatment and week 4 consisted of sampled from 3 hives rather than 6 for this treatment. The absence of newly hatched bees was also observed in the sugar treatment hives, where newly hatched bees were absent in 1 of 6 hives at week 4 and 2 hives at weeks 5 and 6. Consequently, in the lipidomic statistical analysis the sugar-fed treatment did not contain bees from hives in these time points.

The hive temperature was consistent in the 3 treatments (Fig. 2C and Supplementary Table 2), until week 4 when the starvation hive temperatures decreased relatively to week 0 (post-hoc FDR, $P < 0.018$) and became more deregulated than the feed treatments for the rest of the experiment (Fig. 2C, and Supplementary Table 5). Hive humidity did not present differences between treatments or time.

Honey bee lipid stores change in quantity and quality during supplementary feeding and malnutrition

To detect the global impact of supplemental feeding on fatty acid contents, Fatty Acid Methyl Ester (FAME) analysis was performed on extracts from honey bee fat bodies. In total 13 fatty acids were quantitated and identified with high confidence. A heatmap of the abundance, normalized to time 1, shows the fatty acid abundances within and between each of the treatments, over 8 weeks, covering the diet treatment and recovery period (Fig. 3). The main changes were present in the sugar treatment, presenting a decrease of most of the measured fatty acids during weeks 6, 7, and 8, (Wilcoxon test $P <$ 0.05 indicated by asterisks in Figure 3A (Supplementary Table 6). No significant differences were found in the fatty acid abundances for the sugar + pollen treatment across the entire experiment period (Fig. 3A, B and Supplementary Table 6).

To identify specific lipid molecules that might contribute to or facilitate these differences, untargeted lipidomic analysis identified a total of 158 lipids belonging to 4 different lipid classes: 96 triacylglycerols (TGs), 11 diacylglycerols (DGs), 9 phosphatidylcholines (PCs), 10 lysophosphatidylcholines (LPCs), 13 phosphatidylethanolamines (PEs), 13 lysophosphatidylethanolamines (LPEs), 3 sphingomyelins (SMs), 1 steryl ester (SE), 1 sterol (ST), and 6 unknowns identified from the RIKEN P-VS1 database. The estimated abundance values generated from the generalized linear mixed effect models that evaluated treatment

interacting with time were plotted in a heatmap (Fig. 4). Weeks 3 and 4 of the starving treatment clustered together, identifying a group of lipids with increased abundance and another group with decreased abundance. The lipids of these two groups consisted mainly of triacylglycerols (Fig. 4). However, a linear model considering the interaction of treatment and time showed no differences in the abundance of these 158 lipids together ($F = 0.623$, $df = 12$, $P = 0.824$) (Supplementary Fig. 2).

When examining the molecular structure annotation in the names of the main lipids that changed in abundance. They appeared to be clustered accordingly to the number of double bonds in their fatty acyl chains, leading to degrees of unsaturation. To further examine these lipids changing in abundance relative to their level of unsaturation, and generated hierarchical clusters (in Perseus software) of the significantly changing compounds per treatment over time (LS mean FDR, $P < 0.05$). Using a Pearson correlation with a distance threshold of 0.78, four hierarchical clusters of lipids were produced (Supplementary Fig. 3A) from 91 significant lipids in the starving treatment (LS mean FDR, $P < 0.05$) (Supplementary Table 7). Two of these clusters contained most of the lipids, constituting 85% of the lipids changing in abundance (Fig. 5A). On closer inspection, the first cluster consisted of 52% of the total lipids significantly changing in abundance during starvation. The abundance of lipids in this group was principally decreasing at weeks 3 and 4 into starvation and mainly consisted of TGs with a low number of unsaturations (Fig. 5A). For graphical representation, we considered lowly unsaturated lipids as those with 3 or less double bonds in their fatty acid chains. The second cluster of the starving group consisted of 33% of the lipids. This cluster had lipids with highly increased abundances at week 4 and contained mainly highly unsaturated TGs (with more than 3 double bonds) (Fig. 5B).

From 75 lipids significantly changing in abundance over time in the sugar treatment (LS mean FDR, $P < 0.05$) (Supplementary Table 8), 5 clusters were identified with a distance threshold of 0.73 (Supplementary Fig. 3B), where 2 clusters contained 93% of these 75 lipids. The first major cluster consisted of 79% of the lipids and mainly decreased in abundance at week 6 of the sugar treatment and contained principally TGs with few unsaturations (Fig. 5C). The second cluster contained 15% of the lipids that increased in abundance at week 4 of the sugar treatment and contained mainly highly unsaturated TGs (Fig. 5D).

From the 73 lipids significantly changing in abundance over time in the sugar + pollen treatment (LS mean FDR, $P < 0.05$) (Supplementary Table 9), three clusters were generated with a distance threshold of 0.94 (Supplementary Fig. 3C), with the main two clusters containing 99% of these lipids. The first cluster of 69% was mainly lipids not changing very much in abundance over time and consisted of mainly saturated TGs (Fig. 5E). The second cluster of 30% presented a small number of lipids increasing over time in the sugar + pollen treatment but not containing any unsaturated TGs (Fig. 5F).

Lipid unsaturation increases at starvation

More in-depth interrogation of the data revealed several lipids that changed in abundance with the same number of carbons and fatty acid side chains but with differing numbers of unsaturations. Our analysis could not identify the exact position of the double bond, however, the number of unsaturations on each

fatty acid side chain was determined from MS fragmentation pattern and database searching. We grouped identified lipids with the same structure but a different number of double bonds in their fatty acyl chains with each other (Fig. 6). We hypothesized they consisted of the same lipid but with modifications to the number of double bonds over time. Based on this approach, we provide evidence for 11 triacylglycerols and 2 diacylglycerols which during the starvation period steadily increased the abundance of lipid molecules with a greater number of unsaturations (LS mean FDR, $P < 0.05$). In contrast, the same lipid with a fewer number of unsaturations decreased in abundance at week 4 (Fig. 6 and Supplementary Table 10).

The expression of specific desaturase genes is induced by malnutrition

To understand the molecular mechanism involved in the unsaturation increase of lipids during the starvation period in honey bees, as well as the effect of different dietary treatments over time, the expression levels of the 12 annotated lipid desaturase genes from the honey bee genome were measured at weeks 1 (baseline), 3, and 4 in each treatment. LS mean comparisons analyses of the generalized linear models showed that three of these evaluated genes (GB194, GB218, and GB217) increased expression during malnutrition and starvation (Fig. 7 and Supplementary Table 11). Gene GB194 expression increased significantly at week 3 (LS mean FDR, $P < 0.039$) and marginally significantly at week 4 (LS mean FDR, $P < 0.057$) in the starving bees relative to week 1 (Fig. 7A and Supplementary Table 11). However, bees fed with supplementary diets maintained low expression over time. The expression of gene GB218 increased significantly at week 3 (LS mean FDR, $P < 0.029$) and week 4 (LS mean FDR, $P < 0.029$) relative to week 1 in starving bees. Furthermore, the increased expression of this gene was marginally significant in sugar feed bees at weeks 3 (LS mean FDR, $P < 0.082$) and 4 (LS mean FDR, $P < 0.089$) compared to week 1, but did not change expression in the sugar + pollen feed bees (Fig. 7B, and Supplementary Table 11). Gene GB217 expression was also increased in starvation, marginally significant at week 3 relative to week 1 in starving bees (LS mean FDR, $P < 0.081$), (Fig. 7C and Supplementary Table 11). The mean expression level of gene GB217 appeared to increase in sugar feed bees as well but was not statistically significantly different to control due to sample variations. No gene expression differences were detected for the sugar + pollen treatment for any of the desaturase genes (Fig. 7 and Supplementary Table 11).

Discussion

Changes in the environment and beekeeping practices are putting honey bees increasingly under nutritional stress. Due to the loss of habitat and hive sites as well as intensive beekeeping, beekeepers are forced to rely more and more on supplementary feeding to keep their bees alive and productive through periods of low availability of natural resources of pollen and nectar[4,51]. Many factors such as feed availability, accessibility of the hives as well as economic investment influence the decisions when and how to feed as well as if to supplementary feed[51,52]. Here we investigated the molecular and physiological effects of decisions to use three different diet treatments on wider nutritional health and performance of honey bee colonies. The effects of starvation, sugar syrup, and sugar + pollen feeding

were assessed measuring colony health as well as lipidomic changes in the access and mobilization of stored resources in individual honey bees.

Hive health and performance indicators of malnutrition

Honey bee colonies under nutritional stress demonstrate several rescue behaviours for colony survival. One of the first signs of severe nutritional stress in colonies is the reduction of brood cells, where a decrease in rearing allows the colony to conserve resources[53]. In this process bees cannibalize young larvae, obtaining protein which they use to feed and maintain the older larvae until no more brood is produced when the queen stops laying eggs[53,54]. In our experiment, hives of the starving diet treatment suffered a quick decrease in the quantity of capped brood cells during the starvation period (evident from weeks 3 to 5), and cannibalism would have started before week 3 when the capped brood decreased and no capped brood was found in the following 2 weeks. The absence of newly hatched bees during and after the diet treatment in the starving hives and some of the sugar treatment hives, demonstrates that colonies of both treatments were nutritionally affected, although the starving treatment more severely.

Colony productivity is measured by hive weight which represents the numbers of workers, developing brood, and stored food reserves[16,17]. The reduced hive weight observed in the starved hives throughout the treatment as well as during and after the recovery period showed the long-term adverse consequences of an earlier starvation event, reducing the honey productivity of these hives until the end of the experiment. Interestingly, even though hives fed with sugar presented early signs of malnutrition such as the absence of newly hatched bees, honey productivity after the recovery period was not decreased. Furthermore, the high fluctuations of temperature in the colonies, especially in the starving and sugar diets, starting during the treatment period is a clear sign of malnutrition[10,12–14]. This incapacity of maintaining proper thermal conditions is a consequence of a reduction in the number of worker bees and/or a coordinated behavior in the colony that further affects the development of the brood[10]. This agrees with the decrease in brood quantity and lack of newly hatched bees present in both starved and sugar-fed colonies.

Differentially abundant lipids and their mobilization during malnutrition

Apart from colony effects, nutrition can affect the individual bee, for instance, carbohydrates are the bees' strict metabolic fuel for flight affecting foraging when sugar is not available[55,56]. A lack of protein affects the proper development of the brood and the queen laying eggs[21,22]. In an environment rich in nutrients, carbohydrates are acquired mainly in the form of glucose that is stored as glycogen or converted into fats [57]. Insect metabolism can rapidly degrade glycogen, accessing glucose and meeting its energy needs, such as movement. For this reason, carbohydrates are considered as the immediate energy source[26]. Conversely, protein as an energy source is accumulated during the larval stage and used in metamorphosis but also during periods of starvations[57].

Among the nutrients that bees obtain from pollen, lipids, which include neutral lipids as triglycerides, sterols, sterol esters, and free fatty acids, are an important nutritional source[15]. Lipids are accumulated in lipid droplets in the fat body for long-term energy reserves in the form of TGs, which constitute the major components of the fat body[26,58]. During periods of adequate nutrition, triglycerides are synthesized from dietary carbohydrates, proteins, and free fatty acids[26]. During times of energy demand such as starvation, the insect organism accesses these accumulated TGs in the fat body via lipolysis. Lipases catalyse the hydrolysis of the triglycerides making the fatty acids accessible for mobilization [26]. Fatty acids are then translocated to the mitochondria of different cells where they are substrates in the production of ATP[27,28]. During bees' nutritional stress, we did not detect changes in the total abundance of lipids but lipolysis signs were pronounced, such as the specifically increased abundance of highly unsaturated lipids that were mainly triacylglycerols. This abundance was present in the starving and sugar feed treatments but not in the sugar + pollen feed bees. Conversely, lipids with saturated and lowly unsaturated fatty acyl chains decreased in concentration in the starving and the sugar treatments, but again no changes were observed in the sugar + pollen feed bees. The accessibility and mobilization of lipids lead to increases and decreases of different lipids altering their concentrations, such as we observed in malnourished bees and agreeing with previous fasting studies in different animals[59–62]. Here we show evidence of a structural change in the stored TGs, as a greater increase of unsaturations in the starved bees, compared to a less pronounced increase in the sugar feed bees and no increase of unsaturations in sugar + pollen feed bees. These unsaturation changes lead to the mobilization of TGs, by the addition of double bonds in the fatty acid chains, as a high number of double bonds is known to increase lipases affinity and activity[30,31].

Just as for phospholipids, where changes in saturation are associated with maintaining the cell membrane fluidity under cold temperatures[28,63,64], bees modify the fluidity of their lipids under greater energy demand. This agrees with Vukašinović et al.[65] refusing the idea that only cold increase unsaturation levels in the fat body, but also different metabolic stressors such as diapause and in this case malnutrition by the action of desaturases. In this study, we showed the likely involvement of three desaturases: GB194, GB218, and GB2017 in the mobilization of fatty acids from lipid stores during honey bees' malnutrition. These three desaturases have been reported to function as D9-desaturases in honey bees, which insert a double bond at the 9th position from the carboxyl group of fatty acid chains[33]. Previously, genes GB218 and GB217 have been reported to be involved in the biosynthesis of cuticular hydrocarbons of the exoskeleton of honey bees[33] and GB194 in the biosynthesis of unsaturated fatty acids in the pathway of honey bee capping pheromone components[32]. Here we add knowledge to their function as putative, inducible stored lipid mobilization enhancers/enablers in newly hatched worker honey bees. Genes GB194 was highly expressed in fat body tissue of starved newly hatched bees compared to bees with carbohydrate and protein sources. Genes GB218 and G217 increased expression in starved bees as well as in bees that had been fed sugar during their development. Therefore, GB218 and GB217 seem to have a more sensitive response to malnutrition, however, we think that the marginally statistically significant differences were due to the small number of samples and not due to their gene

expression. Gene expression of the three desaturases never changed across all the time points in the sugar + pollen fed bees and hence are inactive, meaning that under adequate nutrition the bees do not increase lipolysis.

Lipid catabolism of fasting has not been reported in detail for nutrition in honey bees, but our results match the lipolysis process on energy demand of other insects under high stress conditions such as flying, diapausing, and overwintering[27,28,66,67]. For instance, in different flying insects but not bees, the access of energy during the flight, involves the release of DAGs from TAGs in the fat body to the hemolymph (the insect body fluid). Associated with lipoproteins, DAGs are then mobilized to somatic cells and hydrolysed into free fatty acids chains (FFA), which are then transported into the mitochondria and finally used in β-oxidation for energy generation in the form of ATP[26,27]. Moreover, during high energy demand, this lipolysis process might be enhanced by the addition of double bonds in the TGs. This is because lipases activity is higher when TGs are rich in unsaturated fatty acids, restructuring the angle of the carbons chain and the geometry of the fatty acid[30,31,68], allowing the enzyme to hydrolyse the TG, which further cleave them into DGs and FFA[26,69]. A similar lipidomic response of lipolysis and unsaturation increase seems to be activated in newly hatched bees coming under nutritional stress during their development. In that way, this process would start with the increase of unsaturations in the fatty acid chains of TGs accumulated in the lipid droplets of the fat body. The desaturases involved in this process would be GB194, GB127, and GB128, with GB194 being exclusive for severe malnutrition. Secondarily, as previously described for flight insects[26,27], TGs lipolysis and further mobilization of DGs (associated with lipophorines and binding proteins) from fat body would provide FFA to somatic cells. Finally, by using these FFA in β-oxidation, energy would be supplied to other tissues in the absence of carbohydrate and protein resources (Fig. 8). As under optimal conditions or with a satisfactory amount of pollen, carbohydrates and proteins would be utilised for meeting energy demands[15].

Most of the fatty acids acquired from the diet are transported to the fat body primarily in form of diglycerides, and further integrated into triglycerides[57]. Fatty acids are also involved in regulating many physiological processes, as the development, reproduction, and biosynthesis of pheromones[26,32,33], but they are mainly used as an energy source from the TGs reserves[26]. A decrease of FFA was constantly present in the sugar diet treatment in the recovery period which matches with the decrease of lowly unsaturated lipids in this treatment at the same period. The amount of fatty acids incorporated in the fat body is in part influenced by the feeding status of the insect[26]. Our result shows the low energy acquisition in malnutrition, matching again the energy demand according to the nutritional stress level suffered as the sugar diet contained carbohydrates, which is the immediate source of energy but did not contain a lipid base, which is the long-term energy reserve[26]. Nurses need sufficient lipid reserves and a proper balance of essential free fatty acids obtained from the diet to produce sufficient jelly and optimal brood rearing[38,70], which explains the unbalance suffered by the sugar-fed and starved recently hatched bees. The high decrease of FFA present during the recovery period of the sugar treatment is not present in the starved groups and we attribute this to the action of the desaturases. The desaturase GB194 gene expression was induced during the starvation period only in starved bees, but not in sugar feed ones.

Hence, three desaturases are likely to be activating the further release of FFA from lipid reserves in starved bees, but only two of the desaturases (GB218 and GB2017) in sugar-fed bees, causing a depletion of FFA in sugar-fed bees at a later stage, not observable in starved bees. Therefore, we conclude that desaturase GB194 is the prime candidate for being responsible for the release of FFA from TGs during starvation.

Our study supports the theory of bees preferring pollen from a variety of sources[71,72], which provide benefits for the improvement of immune functions or longer survival under conditions of parasitism[73,74]. Even when we provided redgum pollen, which is a high-quality protein source[40] we detected a dysregulation in the abundance of a few lipids over time and a decrease of capped brood cells of those colonies. Additionally, it is unclear currently if the pollen patties provided were fed to the developing brood in the same way as beebread would be fed by nurse bees[75]. Our study suggests that supplementary feed in-hive cannot replace the free foraging of mixed pollen sources without impacting the bees in a colony also an individual physiological level.

Conclusions

According to our study, the lipidomic response of bees under starvation and malnutrition consists of the mobilization of triacylglycerols, in a process that involves the addition of double bonds in the fatty acid chains. Furthermore, three desaturases are involved in the early process of lipolysis, being potential biomarkers for honey bee nutritional stress. Recently a kit that tests honey bee stress induced by a variety of factors through the measurement of lipolysis activity has been developed[76]. Following this idea, we propose that the desaturases we reported could be used to develop an assay for assessing specifically malnutrition stress levels by measuring specific molecules involved. Moreover, earlier detection of malnutrition could be achieved due to the early action of desaturases enzymes in the lipolysis process. However, the expression of the reported desaturases will need to be tested with a larger sample size and under different conditions to confirm their specificity and measure their sensitivity to malnutrition.

Sugar nutrition without pollen was shown to be less optimal as the mix of both components at a molecular and colony level, but no long-term decrease in productivity was detected in sugar-fed colonies, as it was for starved ones. Moreover, providing supplementary food of pollen + sugar in-hive also leads to changes in the lipid landscape in individuals and altered colony health indicators. Future studies focused on colony behaviour, structure and productivity need to be done addressing the further disadvantages of feeding sugar without a protein source, which is a common practice in the honey bee industry, but also feeding sugar + pollen in-hive. Moreover, more molecular studies can provide valuable information to integrate an accurate assay for the early detection of malnutrition for the honey bee industry.

Declarations

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Author contributions

Main study design, J.G.; investigation, C.C. and J.G; field experiment, T.B. and C.C.; laboratory analyses, M.B., N.L. and C.C.; data analyses C.C.; results interpretations, J.G. H.M. and C.C.; main manuscript writing, C.C.; reviews and editions; J.G., M.B. and H.M.

Competing interests

The authors declare no competing interests.

Data availability

Lipidomics data to support the findings of this study will be deposited in a database for Metabolomics experiments and derived information MetaboLights (https://www.ebi.ac.uk/metabolights/). The authors declare that the other data supporting the findings of this study are available within the paper and its supplementary information files.

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Figures

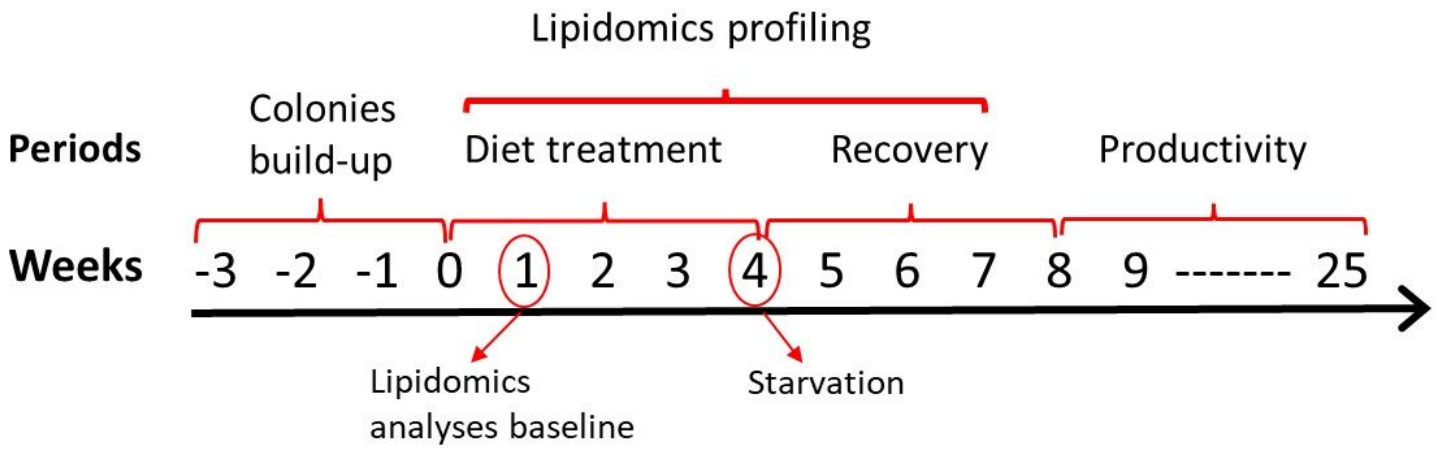

Figure 1

Schematic timeline of the differential nutrition protocol of honey bee hives by weeks. Colonies build up (-3 to 0 weeks); the diet treatment period (0 to 4 weeks); recovery period (4 to 8 weeks) and a productivity period (8 to 25 weeks). The first red oval represents the approximate theoretical moment of the lipidomics baseline (considering the nourishing periods of the larva development), and the second red oval indicates the starvation moment of the hives.

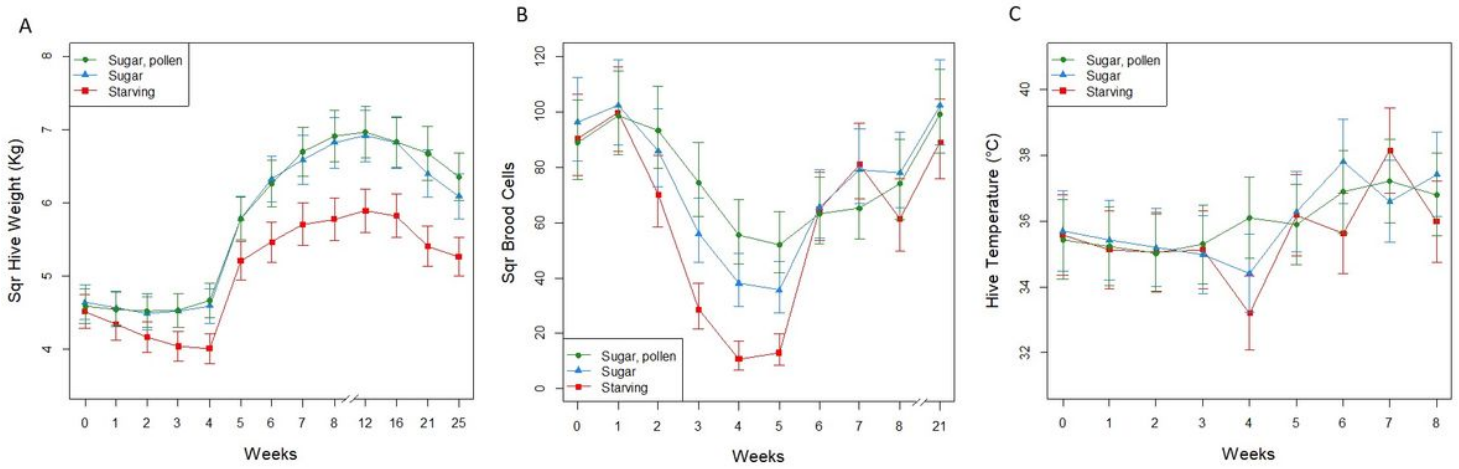

Figure 2

Hive health and performance indicators of colonies exposed to 3 different diet treatments over time. A) Hive weight over 25 weeks, B) Capped brood cells over 21 weeks and D) Hive temperature over 8 weeks. Statistically significant differences given by ANOVA (LS mean FDR, $P \leq 0.05$). Error bars represent the standard errors, 95 % confidence interval.

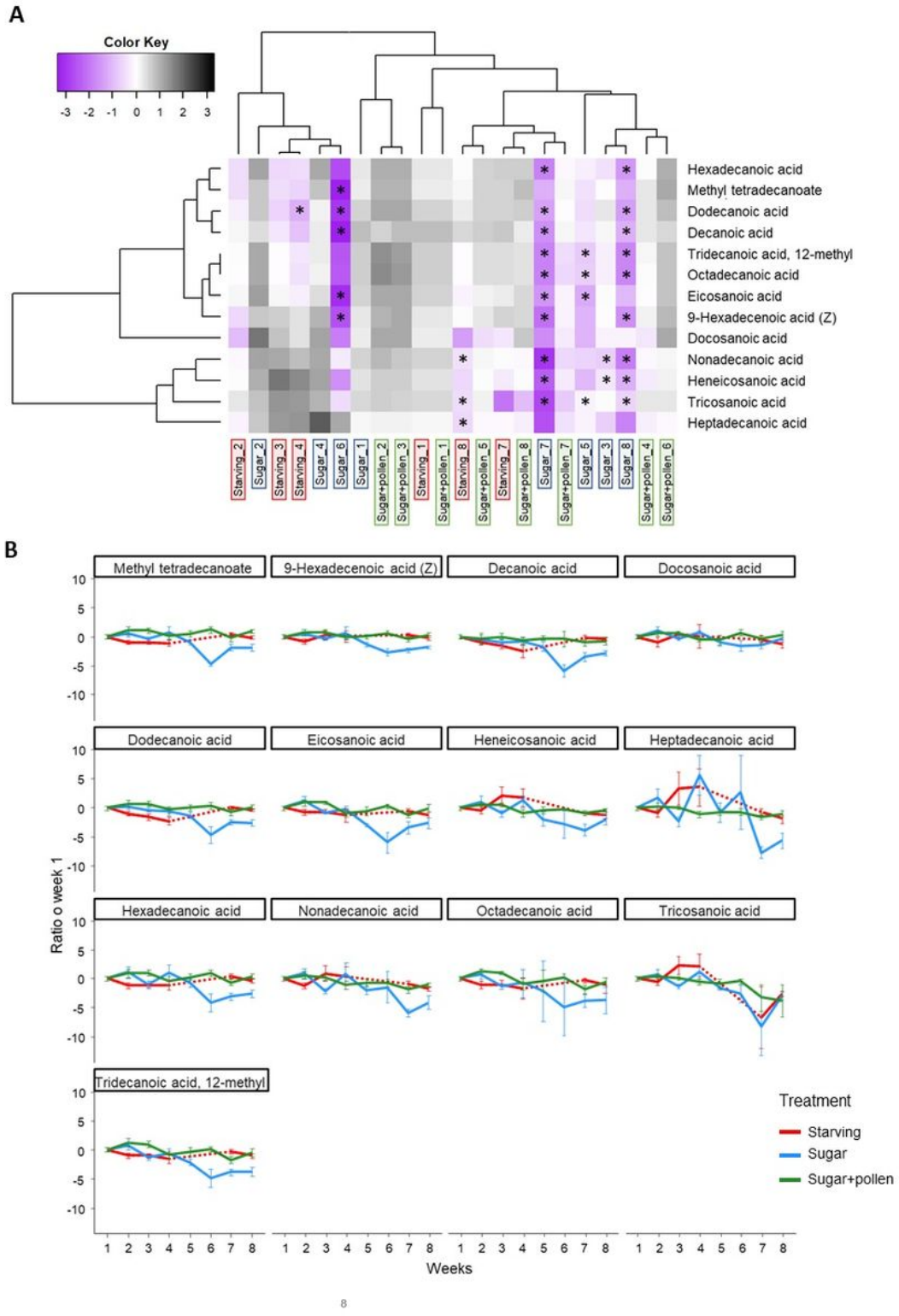

Figure 3

Identified fatty acids (as FAME) changing over time and treatment. A) Heatmap of all identified fatty acids across all samples, where purple indicates decreased abundance and black increased abundance compared to week 1 of each treatment. Asterisks (*) indicate significant differences according to Wilcoxon test ($P < 0.05$). Diet treatment is indicated at the bottom, represented by a colour code, and

followed by treatment week indicated after the underscore (_). B) Line plots of each identified fatty acid changing over time relative to week 1 in the tree treatments (± standard errors, 95% confidence interval).

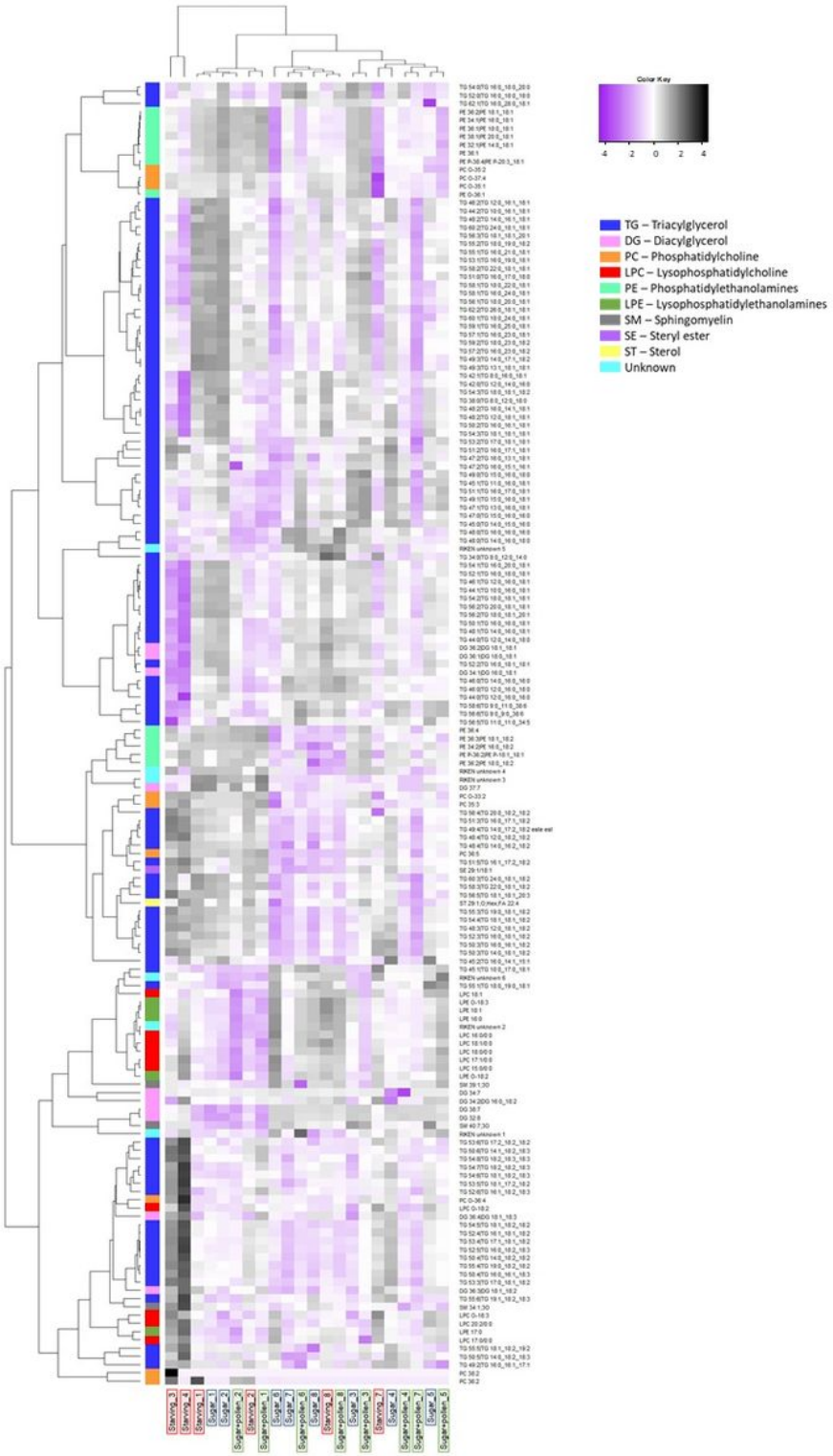

Figure 4

Heatmap of the abundance of different lipids identified by MS fragmentation pattern matches in the software MS-Dial. Unsupervised clustering was performed for both the treatments and the lipids. Lipid

classes are represented by a colour code in the left column and lipid abbreviations are listed at the top right. Diet treatment is indicated at the bottom, represented by a colour code, and followed by week indicated after the underscore (_).

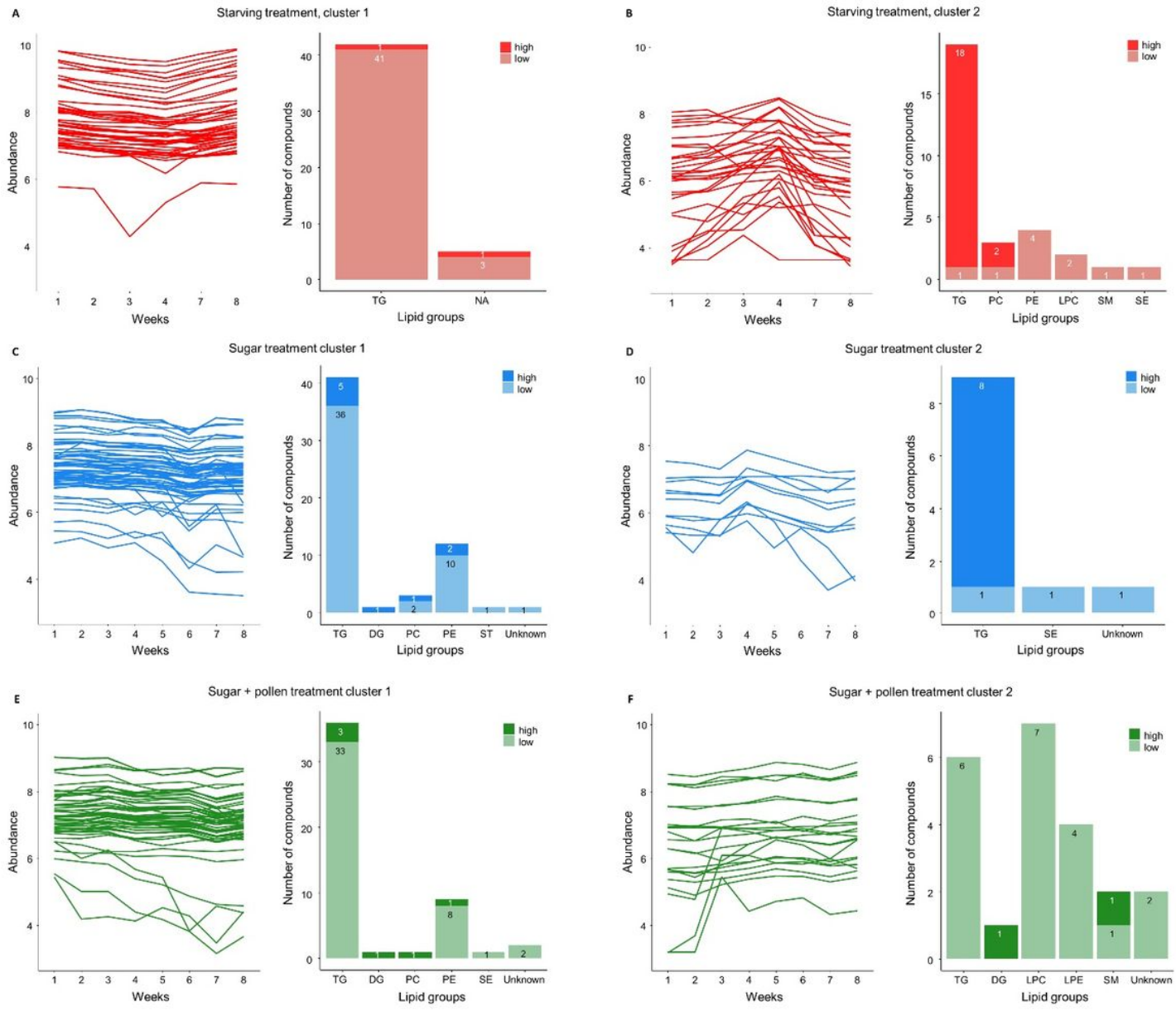

Figure 5

Main clusters of the lipids significantly changing in abundance by treatment, with the number of lipids by lipid class and levels of unsaturation indicated in each cluster. A) and B) Starving treatment clusters, where dark red on the bar plots represents highly unsaturated lipids (double bonds > 3) and light red lowly unsaturated lipids (double bonds ≤ 3). C) and D) Sugar treatment clusters with highly unsaturated lipids as dark blue and lowly unsaturated lipids as light blue in the bar plots. E) and F) Sugar + pollen treatment clusters with highly unsaturated lipids as dark green and lowly unsaturated lipids as light green in the bar plots. The lipid class is indicated as the abbreviated name at the bottom of each bar plot. TG – triacylglycerols, DG – diacylglycerols, PC – phosphatidylcholines, LPC – lysophosphatidylcholines, PE –

phosphatidylethnolamine, LPE – lysophosphatidylethanolamines, SM – Sphingomyelin, SE – steryl esters, ST – sterols. Statistically significant lipids were determined by LS mean comparisons with FDR adjustment ($P < 0.05$). Clusters were obtained using Pearson correlation in Perseus software.

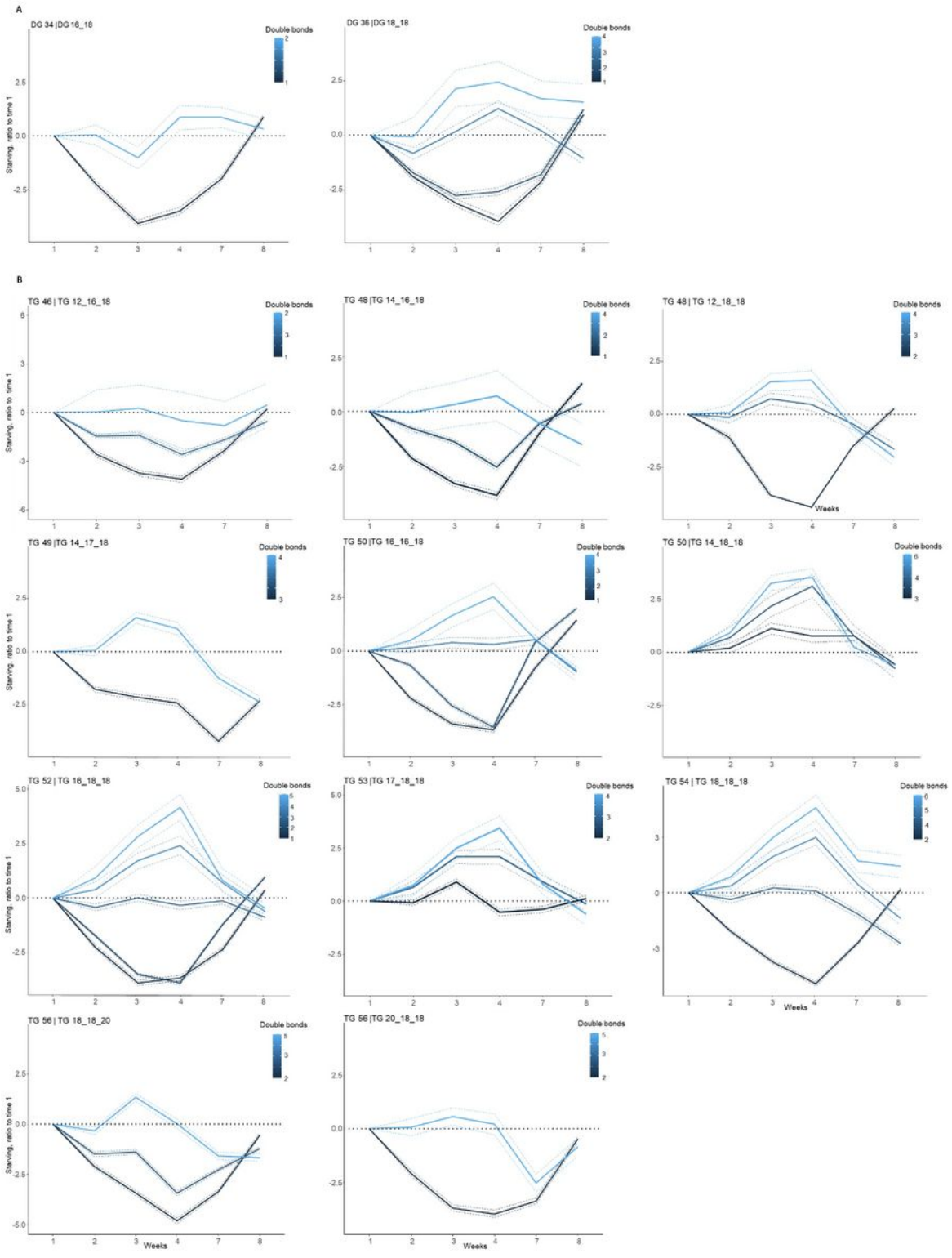

Figure 6

Abundance of different classes of storage lipids at starvation, relative to week 1 and their number of unsaturations. A) diacylglycerols (DGs), and B) triacylglycerols (TGs). In the legend, the number next the column presents the quantity of double bonds, from black to blue relative to the increase in double bonds. Ratio difference to week 1 determined by LS mean comparisons with FDR adjustment represented by solid lines, while dashed lines represent standard errors, 95 % confidence interval.

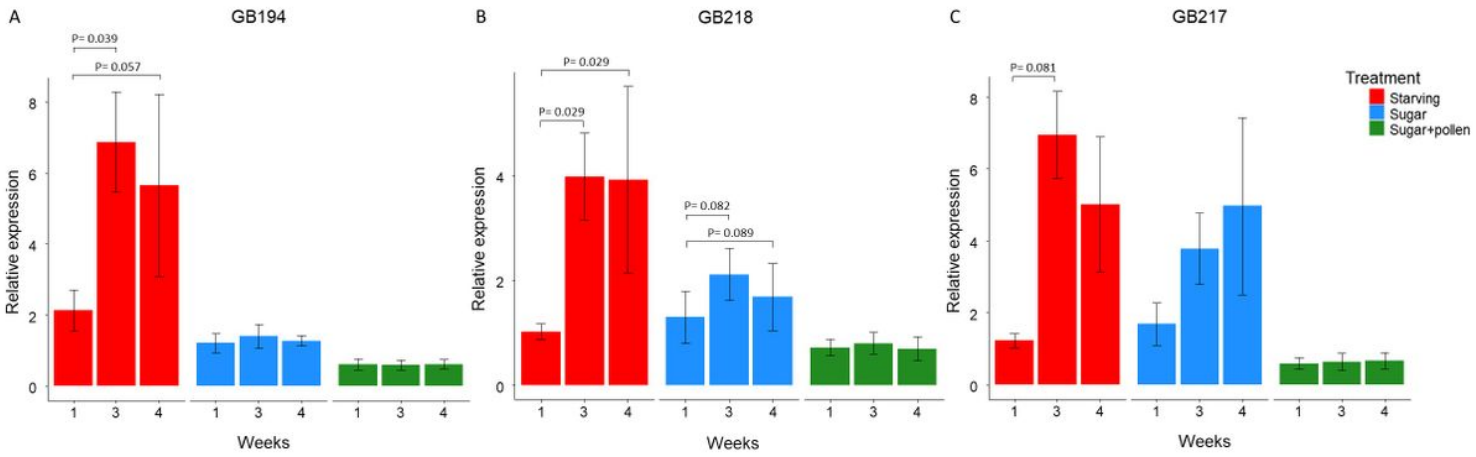

Figure 7

Relative gene expression of 3 desaturases over time in each diet treatment. A) GB194 desaturase B) GB218 desaturase and C) GB217 desaturase. Bars represents the mean expression levels for each gene at weeks 1, 3 and 4 (± standard errors, 95 % confidence interval) and statistical significance (*P*-values) from LS means with FDR adjustment denoted above bars. Expression of all desaturases was low in the sugar + pollen fed bees, whereas expression of GB194, GB218, and GB217 was elevated in the starving bees at 3 and 4 weeks.

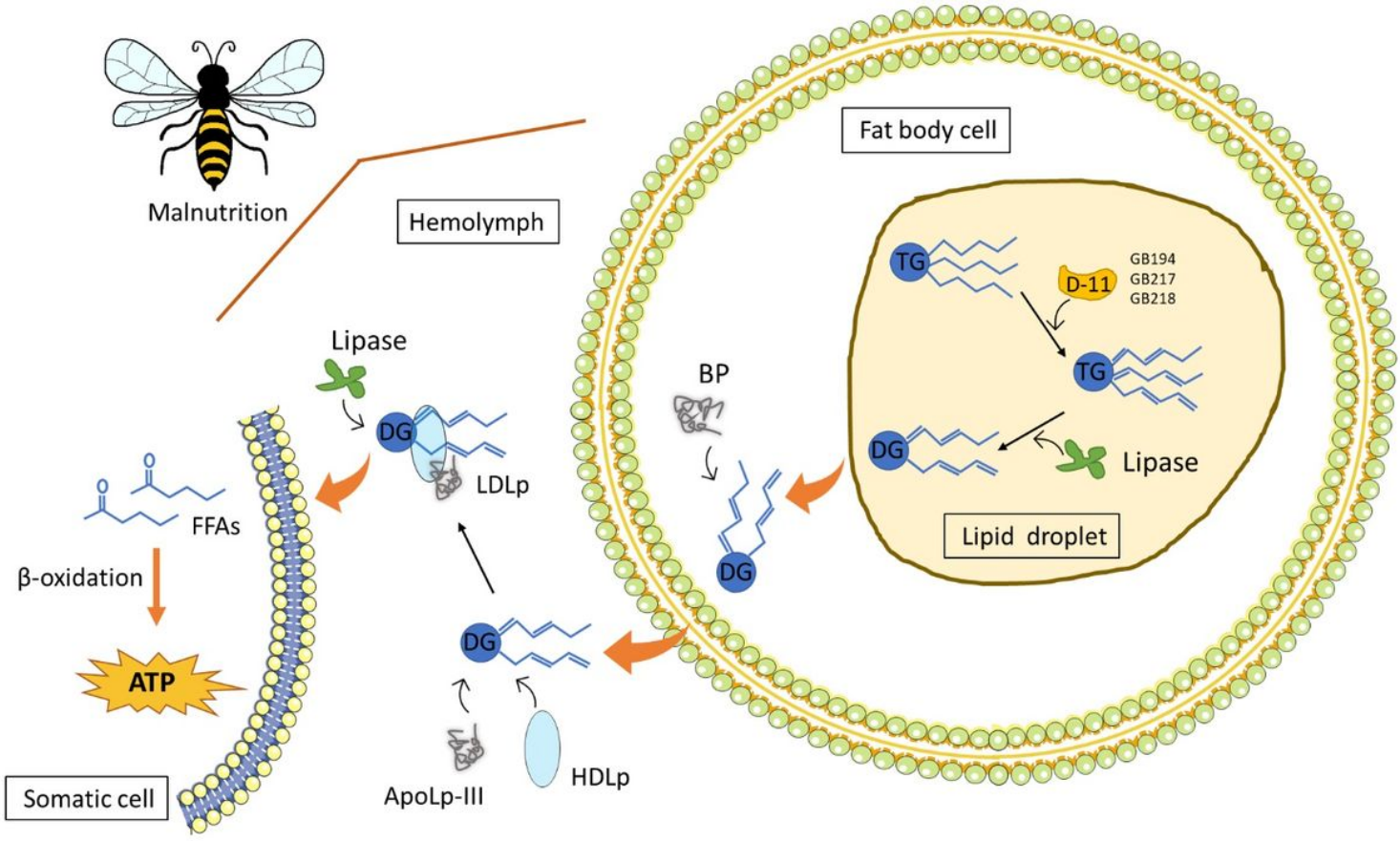

Figure 8

Schematic overview of proposed lipid mobilisation in honey bees during nutritional stress. TGs reserves previously accumulated in the fat body increase unsaturations by the addition of double bonds mediated by the D11-desaturases GB194, GB217, and GB218. DGs are then formed from TGs with the action of lipases, to be further mobilized out of the lipid droplet and the adipocyte with the help of binding proteins (BP.) DGs are carried through the hemolymph by the association of apolipophorin 3 (apoLp-III) and high-density lipophorin (HDLp), forming low-density lipophorin (LDLP). Finally, by the action of lipases, DGs get hydrolysed into FFA which are imported into a somatic cell and used in the production of energy through β-oxidation. Figure redrawn and modified from Canavoso et al.[69] and Sinclair & Marshall[28].

Supplementary Files

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