Comparing the effect of fasting and feeding on type 2 diabetes glucose tolerance in Nile rat plasma using discovery metabolomics

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Abstract

Introduction

Diabetes is an urgent global health challenge with an accelerating incidence rate in recent decades. Currently, 537 million adults are living with diabetes and 541 million adults have impaired glucose tolerance with a high risk of developing type 2 diabetes[1](https://www.zotero.org/google-docs/?tULKVh). The Nile rat is a developing animal model of type 2 diabetes with key benefits over other rodent models[2](https://www.zotero.org/google-docs/?7L3HDm). Firstly, it is a cost-effective animal model where diabetes is rapidly induced in both sexes by a conventional laboratory rodent chow. The onset of diabetes starts around 1-2 months of age in some Nile rats and by 10 months old, approximately 90% of male and 50% of female Nile rats develop diabetes. These incidence rates of diabetes occur on a conventional laboratory rodent chow diet that is hypercaloric for Nile rat compared to its native diet of fibrous grass stems in Africa[3](https://www.zotero.org/google-docs/?k0dPJk). Secondly, diabetic Nile rats develop long-term diabetic complications recapitulating the natural progression of type 2 diabetes in humans[2,4,5](https://www.zotero.org/google-docs/?R1TLCB). For example, Nile rats develop retinal lesions in diabetic retinopathy similar to humans[6](https://www.zotero.org/google-docs/?0ufVMe), whereas other rodent models typically show limited asymptomatic structural differences in the retina[7](https://www.zotero.org/google-docs/?J76eWB). Third, in contrast to the Nile rat, common strains of laboratory mice and rats are relatively resistant to diet-alone induced diabetes, and are typically treated with additional chemical or genetic manipulations[8](https://www.zotero.org/google-docs/?ueVYNF). Fourthly, the Nile rat is an outbred species that models a wide range of diabetic phenotypes in the human population unlike inbred model organisms. Additionally, the diurnal chronotype of the Nile rat [9](https://www.zotero.org/google-docs/?MtfxuK) enables the investigation of how disrupted diurnal rhythm in humans is associated with type 2 diabetes. Finally, we can achieve high precision and accuracy in Nile rat genetic and epigenetic studies with the recent publication of a high quality haplotype-resolved reference genome.

People living with diabetes experience difficulty in maintaining glucose homeostasis resulting in post-prandial hyperglycemia, fasted hyperglycemia or both. Post-prandial and fasted-state hyperglycemia are driven by different biological mechanisms[10](https://www.zotero.org/google-docs/?2daMzV)[.](https://doi.org/10.2337/db10-1032) It is known that post-prandial hyperglycemia precedes fasted hyperglycemia, and thus is a more sensitive measurement for subjects with pre-diabetes or early diabetes[11](https://www.zotero.org/google-docs/?wNScyJ),[12](https://www.zotero.org/google-docs/?e0xRVR). Importantly, for predicting disease outcome, post-prandial hyperglycemia, more so than fasting hyperglycemia, is associated with a high risk of developing cardiovascular disease[13](https://www.zotero.org/google-docs/?yHQWNk),[14](https://www.zotero.org/google-docs/?YAJUGD),[15](https://www.zotero.org/google-docs/?iHpUUx),[16](https://www.zotero.org/google-docs/?ym9aDQ) and possibly associated with all-cause mortality[17](https://www.zotero.org/google-docs/?EXbk1y). To investigate the early pathogenesis of diet-induced diabetes, the post-prandial, non-fasted state may reveal findings that do not overlap when similarly interrogated in the fasted state. Currently, the majority of diabetic studies use fasted sampling to avoid uncontrolled variability from unrestricted eating behaviors. However, the common practice for *ad libitum* feeding of laboratory rodents results in frequently eating throughout their waking hours, unlike humans who eat large discrete meals. To date, the differences in variability and quality of metabolic changes between fasted and non-fasted states have not been sufficiently examined to inform decision making to support or refrain from using non-fasted sampling to study the early pathogenesis of diet-induced diabetes in rodent models.

In support of studying the metabolic differences between the fasted state and non-fasted state, we opted to study blood plasma in Nile rats using mass spectrometry-based metabolomics. LCMS metabolomics has been used extensively to analyze small molecules in plasma[18–20](https://www.zotero.org/google-docs/?QKHQp9), in rodent models[21](https://www.zotero.org/google-docs/?JA2sXu) and in humans to study diabetes[22](https://www.zotero.org/google-docs/?ObOSHH). To date, few studies have analyzed metabolites in Nile rats using mass spectrometry or other methods[23,24](https://www.zotero.org/google-docs/?rmeL02). Blood plasma is known to be a rich source of molecular biomarkers of diabetes, comprising thousands of polar metabolites and lipids[20,25](https://www.zotero.org/google-docs/?Ou5Agz), with many of these molecules being biomarkers for diabetes[26–28](https://www.zotero.org/google-docs/?11P8Ht). To both uncover metabolites associated with diabetes and fasting conditions in the Nile rat, and to set a baseline understanding of overall levels of metabolites in Nile rat plasma, we designed an experiment to measure plasma metabolites in both fasted and non-fasted states.

Methods

Animal studies

All animal experiments were approved by the University of California, Santa Barbara, Institutional Animal Care and Use Committee, and conducted in accord with the NIH Guide for the Care and Use of Laboratory Animals. The Nile rats were fed *ad libitum* on a regular rodent diet (Diet 5008; Newco Speciality, Rancho Cucamonga, CA, USA) [[29](https://www.nature.com/articles/s41374-019-0264-3#ref-CR29)], and housed in a 12-hour, 10am to 10pm, light cycle room. To perform oral glucose tolerance test (OGTT), Nile rats were fasted for 16 hours from 6pm to 10am, and 2g of dextrose per body weight was introduced via oral gavage. Fasted plasma samples were collected around 10am to 11am and non-fasted samples were collected around 3 to 4pm, in the middle of the light-on duration. Blood collections were done under fasted and non-fasted conditions in triplicates spaced apart weekly. Each week, non-fasted samples were collected first and fasted samples 3 days after. After the last collection, the rats were recovered for 2 weeks before OGTT. All plasma samples were stored at -80˚C.

Glucose and insulin measurements

Blood glucose was measured by a Contour Next glucometer using blood from a tail prick. Plasma insulin was measured using the Ultrasensitive Mouse Insulin ELISA Kit (Crystal Chem Inc., Reference 90080) according to standard protocol.

Lipidomics and metabolomics sample preparation

Plasma samples were thawed once prior to the second thawing on ice for subsequent lipidomic and metabolomic sample preparation. Each sample was extracted with 500 µL 6:2:2 n-butanol:acetonitrile:water[29](https://www.zotero.org/google-docs/?tANmgc) . Samples were vortexed for 10 s and then centrifuged at 14,000 x g for 2 min at 4 °C to precipitate the protein. 100 µL of extract was dried down in an amber autosampler vial with glass insert by a SpeedVac evaporator. For lipidomics, each extract was resuspended in 50 µL 9:1 Methanol:Toluene. For metabolomics, each extract was resuspended in 25 µL 1:1 Acetonitrile:Water then analyzed on the mass spectrometer.

HILIC-LC-MS Metabolomics

Sample analysis was performed on a ZIC-pHILIC HPLC column held at 50 °C (100 mm x 2.1 mm x 1.7 μm particle size; Millipore) using a Vanquish Binary Pump (150 μL/min flow rate; Thermo Scientific). Mobile phase A consisted of 10 mM ammonium acetate in ACN:H2O (10:90, v/v) containing 0.1% ammonium hydroxide. Mobile phase B consisted of 10 mM ammonium acetate in ACN:H2O (95:5, v/v) containing 0.1% ammonium hydroxide. Mobile phase B was initially held at 95% for 2 min and then decreased to 30% over 18 min. Mobile phase B was held for 6 min at 35%, then raised to 95% over 1 min. The column was re-equilibrated at 95% mobile phase B for 8 min. 2 µL of extract was injected by a Vanquish Split Sampler HT autosampler (Thermo Scientific).

The LC system was coupled to a Q Exactive-HF Orbitrap mass spectrometer through a heated electrospray ionization (HESI II) source (Thermo Scientific). Source conditions were as follow: HESI II and capillary temperature at 350 °C, sheath gas flow rate at 40 units, aux gas flow rate at 15 units, sweep gas flow rate at 1 units, spray voltage at |3.0 kV| for both positive and negative modes, and S-lens RF at 50.0 units. The MS was operated in a polarity switching mode acquiring positive and negative full MS and MS2 spectra (Top10) within the same injection. Acquisition parameters for full MS scans in both modes were 60,000 resolution, 1 × 10e6 automatic gain control (AGC) target, 100 ms ion accumulation time (max IT), and 70 to 900 m/z scan range. MS2 scans in both modes were then performed at 45,000 resolution, 1 × 10e5 AGC target, 100 ms max IT, 1.0 m/z isolation window, stepped normalized collision energy (NCE) at 20, 30, 40, and a 30.0 s dynamic exclusion.

The resulting LC-MS data were processed using Compound Discoverer 3.3, with the Untargeted metabolomics template.

LC-MS Lipidomics

Sample analysis was performed on an Acquity CSH C18 column held at 50 °C (100 mm x 2.1 mm x 1.7 μm particle size; Waters) using a Vanquish Binary Pump (400 μL/min flow rate; Thermo Scientific). Mobile phase A consisted of 10 mM ammonium acetate in ACN:H2O (70:30, v/v) containing 250 μL/L acetic acid. Mobile phase B consisted of 10 mM ammonium acetate in IPA:ACN (90:10, v/v) with the same additives. Mobile phase B was initially held at 2% for 2 min and then increased to 30% over 3 min. Mobile phase B was further increased to 50% over 1 min, then raised to 85% over 14 min, and finally raised to 99% over 1 min and held at 99 % for 7 min. The column was re-equilibrated with mobile phase B at 2% for 1.75 min before the next injection. 10 µL of extract was injected by a Vanquish Split Sampler HT autosampler (Thermo Scientific).

The LC system was coupled to a Q Exactive HF Orbitrap mass spectrometer through a heated electrospray ionization (HESI II) source (Thermo Scientific). Source conditions were as follow: HESI II and capillary temperature at 300 °C, sheath gas flow rate at 25 units, aux gas flow rate at 15 units, sweep gas flow rate at 5 units, spray voltage at |3.5 kV| for both positive and negative modes, and S-lens RF at 90.0 units. The MS was operated in a polarity switching mode acquiring positive and negative full MS and MS2 spectra (Top2) within the same injection. Acquisition parameters for full MS scans in both modes were 17,500 resolution, 1 × 106 automatic gain control (AGC) target, 100 ms ion accumulation time (max IT), and 200 to 1600 m/z scan range. MS2 scans in both modes were then performed at 17,500 resolution, 1 × 105 AGC target, 50 ms max IT, 1.0 m/z isolation window, stepped normalized collision energy (NCE) at 20, 30, 40, and a 10.0 s dynamic exclusion.

The resulting LC–MS data were processed using Compound Discoverer 3.1 (Thermo Scientific) in conjunction with LipiDex [30](https://www.zotero.org/google-docs/?a3v7dq). All peaks with a 0.4 min to 21 min retention time and 100 Da to 5000 Da MS 1 precursor mass were aggregated into distinct chromatographic profiles (i.e., compound groups) using a 10-ppm mass and 0.2 min retention time tolerance. Profiles not reaching a minimum peak intensity of 5x105, a maximum peak-width of 0.25 min, a signal-to-noise (S/N) ratio of 1.5, and a 3-fold intensity increase over blanks were excluded from further processing. MS/MS spectra were searched against an in silico generated lipid spectral library comprising 35,000 unique molecular compositions from 48 lipid classes. Spectral matches with a dot product score greater than 500 and a reverse dot product score greater than 700 were retained for further analysis. Lipid MS/MS spectra which contained no significant interference (<75 %) from co-eluting isobaric lipids, eluted within a 3.5 median absolute retention time deviation (M.A.D. RT) of each other, and found within at least 2 processed files were then identified at the individual fatty acid substituent level of structural resolution. If individual fatty acid substituents were unresolved, then identifications were made with the sum of the fatty acid substituents.

Data processing and statistical analysis

Lipidomics: Compound Discoverer 3.1 parameters. LipiDex and parameters. Filtering steps for lipids.

Metabolomics: Compound Discoverer 3.3 parameters. Filtering steps in CD33.

All the statistical methods once analysis pipelines are finalized.

Results

To compare non-fasted to fasted metabolites and lipids associated with the early diet-induced diabetes, we analyzed Nile rat plasma collected in triplicates at 8, 9, and 10 weeks old, where each replicate was spaced one week apart (**Figure 1A**). Within each week, the non-fasted plasma was collected on day 0 and the fasted plasma on day 3. When Nile rats reached age 12 weeks, we measured their glucose tolerance as a surrogate marker for diabetes progression, using  an oral glucose tolerance test (OGTT). From our data, the 60-minute post glucose load (1hPG) appeared more sensitive within the range of milder impaired glucose tolerance whereas the 120-minutes post glucose load (2hPG) appeared more sensitive within the range of more severe impaired glucose tolerance (**Figure 1B**). In human studies, 1hPG is not a diagnostic measurement like 2hPG, but it has been repeatedly proven to detect pre-diabetes or predict diabetes in high-risk populations [31–33](https://www.zotero.org/google-docs/?4ylQ5f). We then ordered the identity of the Nile rats A to J based on glucAUC (**Figure 1C**). When we compare the two sampling conditions, only non-fasted blood glucose, but not fasted blood glucose, show a non-zero relationship with subsequent glucAUC (**Figure 1D**). Plasma insulin levels during an OGTT revealed that Nile rat C was hyperinsulinemic, indicating that it was either pre-diabetic or at an early stage of diabetes. On the other hand, Nile rats H, I and J were relatively hypoinsulinemic during OGTT, indicating that these rats were more advanced in the diabetes spectrum (**Figure 1E and 1F**). When we looked at the longitudinal weight data from 8 to 12 weeks, we found two weight groups, one with prediabetic Nile rats A to C and another with diabetic rats D to J (**Figure 1G**). Though the growth rates were similar in both groups, the diabetic group started out being heavier at 8 weeks. The two groups of growth curves agreed with the average non-fasted blood glucose levels from Figure 1D.

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**Figure 1. Metabolomic study design and characterization of glucose tolerance**

(**A**) Overview of study design analyzing plasma metabolites and lipids at 8-10 weeks and measuring glucose tolerance by OGTT at 12 weeks. (**B**) OGTT curve with blood glucose levels taken at  0, 10, 20, 30, 60 and 120 minutes after ingesting glucose delivered by gavage. (**C**) Area under the curve calculated from glucose OGTT (glucAUC). Nile rats ordered by increasing glucAUC. (**D**) The correlation between glucAUC at 12 weeks and mean blood glucose levels at 8-10 weeks, measured in fasted versus non-fasted state of the Nile rats. (**E**) OGTT curve with plasma insulin levels taken at  0, 10, 20, 30, 60 and 120 minutes after ingesting glucose. (**F**) Area under the curve calculated from insulin OGTT (insAUC). Nile rats ordered by increasing glucAUC. (**G**) Growth chart from 8 to 12 weeks based on whole body weight for Nile rats A to J. Abbreviations: OGTT oral glucose tolerance test.

To characterize the plasma biomolecules in Nile rats A to J under fasted and non-fasted conditions, we performed discovery metabolomics and lipidomics analysis using a biphasic extraction method[34](https://www.zotero.org/google-docs/?9u2cSz) with two analytical platforms, an LC-MS/MS lipidomics method using reversed phase chromatography and an LC-MS/MS polar metabolomics method using HILIC chromatography. We annotated 363 lipids across 5 super classes[21,35](https://www.zotero.org/google-docs/?Idlbgn), including phospholipids, glycerolipids, sphingolipids, fatty acyls, and sterol lipids; approximately 500 lipid chromatographic features remain unannotated but were included in some of the downstream analyses (**Figure 2A**). Of the annotated lipids, 200 were identified at species level and 163 were at molecular species level[36](https://www.zotero.org/google-docs/?rTKLgV). For polar metabolites detected by the HILIC method, we annotated 80 compounds from 6 compound classes, including organic alcohols, amino acids, amino acid derivatives, nitrogen heterocycles (such as nucleotide bases and derivatives) carbohydrates and organic acids. (**Figure 2B**). Of these, approximately 400 chromatographic features remained unannotated. Principal component analysis (PCA) of all combined annotated and unannotated lipid and polar metabolite features reveals two distinct clusters separated on the first principal component by sampling conditions with fasted samples on the left and non-fasted samples on the right (**Figure 2C**). Within the non-fasted cluster, the samples appeared to be ordered by glucose tolerance of the Nile rats, as demonstrated by the colorimetric gradation from top to bottom where animals with increasing impaired glucose tolerance nearer to the bottom. On the contrary, this order was absent in the fasted cluster. Thus, the combined relative quantification of plasma biomolecules varied with the level of glucose tolerance only in the non-fasted samples, not the fasted samples.

As a high-level overview, the heatmap in **Figure 2D** shows how non-fasted plasma metabolites and lipids differ from fasted in Nile rats. To demonstrate the deviation of each metabolite abundance in non-fasted samples to the baseline fasted samples, log2 fold change is calculated as the difference between each metabolite’s abundance from the average abundance in fasted. Each rat contributed 3 plasma samples in both fasted and non-fasted conditions, and the metabolite abundance across the 3 samples is averaged into 1 column. Columns are ordered by increasing OGTT glucose AUC within each sampling type to uncover relationships between glucose tolerance and metabolite clusters. Finally, rows are hierarchically clustered based on non-fasted samples to show metabolites with similar profiles, and we see glycerolipids (represented by purple) forming two clusters near the bottom and middle of the map.

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**Figure 2. Non-fasted sampling showed a greater dynamic range of metabolite values.**

**(A)** A summary of counts of 363 identified lipids, divided into 5 lipid superclasses on the inner circle, and lipid class on the outer circle. **(B)** A summary of counts of 80 total identified polar metabolites within 6 classes. **(C)** Principal component analysis (PCA) of all plasma samples using all features from both HILIC and reversed phase LC-MS methods. Principal components (PC) 1 and 2 are shown. 95% confidence interval (CI) ellipses are displayed around points based on fasted or fed sampling. Points are colored according to Nile rat OGTT glucose AUC with identical animal colors used in Figure 1. **(D)** Heatmap of all identified lipids and polar metabolites, separated by fasted (left side) and non-fasted (right). Each column is the average of 3 weeks of Nile rat’s fasted or non-fasted plasma samples. Rows are clustered based on euclidean distance with complete linkage for each metabolite profile across only non-fasted plasma samples. Lipid superclass and metabolite class are given in vertical colorbars. Metabolite abundances are given as log2 fold change of the difference from feature-wise mean of fasted samples. Abbreviations: AC acylcarnitine; FA fatty acyl; CE cholesteryl ester; Cer[NS] ceramide non-hydroxylated fatty acyl sphingosine; SM sphingomyelin; PE phosphatidylethanolamine; PC phosphatidylcholine; PI phosphatidylinositol; DG diacylglycerol; TG triacylglycerol.

To quantify the difference between repeated measures of plasma metabolites in fasted versus non-fasted, we calculated percent relative standard deviation differences (%RSD) for each compound [37](https://www.zotero.org/google-docs/?KzKvkc). The per-compound %RSDs were summarized into groups based on animal and compound class (**Figure 3**). A lower %RSD shows better repeatability of quantitation and is necessary for confidence in the sampling method. While it is expected that non-fasted sampling would lead to much greater variance than fasted sampling[38–40](https://www.zotero.org/google-docs/?LqyPso), 5 out of 10 animals showed lower median variance per compound in replicate non-fasted sampling versus replicate fasted sampling. **Figure 3A** gives the median % RSD for all metabolites in each Nile rat between fasted and non-fasted. When grouped by compound class, 11 out of 16 lipid compound classes have lower median % RSD, and all 6 polar metabolite classes have lower median % RSD (**Figure 3c-d**). It is notable that triacylglycerols (TG) show higher RSDs while non-fasted. Given that feeding generally upregulates TGs in blood, and that their abundance is strongly influenced by diet and quantity of calories consumed, it is expected that TG variance will be higher following [41](https://www.zotero.org/google-docs/?4rxFpF)random-feeding[42](https://www.zotero.org/google-docs/?M7u6yl).

To compare the variance in metabolite quantitation to the variance in random blood glucose (RBG)[43](https://www.zotero.org/google-docs/?eYA1sP), we measured RBG every hour over a 12 hour period for a cohort of 13 different Nile rats (**Figure 3B**). Repeated measures of RBG shows that all hours of the day show approximately equal variance around the Nile rat’s median quantitation, except for 10 am which may be an indicator of the dawn phenomenon of RBG in diabetes. Additionally, RBG variance is approximately the same across all Nile rats regardless of average RBG. Importantly, 3B and 3C show that Nile rats do not exhibit the same spike in RBG that accompanies non-random feeding of large meals like what occurs in humans[44](https://www.zotero.org/google-docs/?KWmM3Q).

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**Figure 3. Non-fasted plasma metabolites show similar variance compared to fasted sampling within animal and within metabolite class.** (**A**) For each polar metabolite and lipid, the variance is calculated within-animal by percent relative standard deviation (% RSD) of each quantitation over the 3 weeks of plasma sampling at age 8, 9 and 10 weeks. Plasma samples are separated by non-fasted and fasted conditions. The median of all metabolite % RSDs is calculated for each rat and given on the left 10 columns. 5 out of 10 Nile rats show lower median repeated measures RSD for all lipids and polar metabolites in non-fasted condition versus fasted. On the right side, within-animal % RSDs are combined across all animals and all metabolites within metabolite molecule class. 17 out of 22 molecule classes show lower median repeated measures RSD in non-fasted versus fasted.

(**B**) Random blood glucose was measured for a separate cohort of 13 Nile rats every hour over 12 hours from 10 am to 10 pm. The percent difference between each hour’s measurement and the rat’s average RBG is plotted over time.  (**C**) The RBG measurements of all 12 hours are shown for each rat to show the breadth of RBG levels for all rats. Abbreviations: DG diacylglycerol; PI phosphatidylinositol; SM sphingomyelin; PE phosphatidylethanolamine; CE cholesteryl ester; PC phosphatidylcholine; AC acylcarnitine; Cer[NS] ceramide non-hydroxylated fatty acyl sphingosine; FA fatty acid; TG triacylglycerol; RBG random blood glucose.

To show the combined effect of glucose tolerance and fasted versus non-fasted state on metabolite abundance, we demonstrate this effect on one metabolite, 3-hydroxybutyrate (3HB) in **Figure 4A and 4B**, then show the effect on all identified polar metabolites in **Figure 4C and 4D.** In **Figure 4A**, the abundance of 3HB is divided into quantitations from fasted and non-fasted plasma samples. These 2 groups can be analyzed by t-test to show the differential abundance at an average fold change of 1.5 higher in fasted, with significance level p < 0.0001. However, this bulk analysis of fasted versus non-fasted obscures the effect that glucose tolerance has on the quantitation of 3HB within each Nile rat in each sampling group. These results are shown for 3HB in **Figure 4B**, which plots the abundance of 3HB versus the glucose tolerance of each rat, separated by sampling. We see that fasted and non-fasted show a linear relationship with 3HB abundance, except that fasted shows a negative relationship, whereas non-fasted shows a positive correlation. The analysis on 3HB in **4A and 4B** was repeated for all identified polar metabolites to yield the volcano plot in **Figure 4C** and the glucose tolerance slope plot in **Figure 4D**, with 3HB highlighted in each plot to show how the data give rise to its location in the plot. In **4C**, organic acid species such as quinic acid and hydrocinnamate, metabolites associated with consumption of plant matter[45](https://www.zotero.org/google-docs/?3M8nVL), are highly up-regulated in non-fasted samples. Additionally, the branched chain amino acids (BCAA) leucine and isoleucine are commonly implicated in diabetes[46](https://www.zotero.org/google-docs/?YPMGTG) and are up-regulated in non-fasted samples. Their effect in regards to glucose tolerance is most pronounced in non-fasted sampling, with a positive slope approximately twice as high in non-fasted compared to the negative slope in fasted sampling as given by their position in **4D**.

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**Figure 4 - Polar plasma metabolites display differential behavior between fasted and non-fasted.**

(**A**) The abundance of 3-hydroxybutyrate is plotted and summarized in a box plot, with group based on quantitation in fasted or non-fasted plasma samples. (**B**) The abundance of 3-hydroxybutyrate is plotted against Nile rat OGTT glucose AUC for all plasma samples. Each dot represents 3HB abundance in one plasma sample versus the Nile rat’s 12-week OGTT glucose AUC value. Samples are grouped by their fasted or non-fasted state, and linear regression is fitted to both groups. The slope of the two regression lines gives the position of the metabolite in **4d**. (**C**) A volcano plot for all identified polar metabolites shows -log10 of FDR-corrected p-value versus the log2 fold change of non-fasted minus fasted. Selected metabolites are annotated with identifications, and 3-hydroxybutyrate is highlighted in bold.  (**D**) The slope of quantitation versus OGTT is plotted for all identified polar metabolites in non-fasted samples (x-axis) and fasted samples (y-axis). Selected metabolites are annotated with identifications, and 3-hydroxybutyrate is highlighted in bold. The position of 3-hydroxybutyrate is based on the slope of each line as shown in **4B.**

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**Figure 5 – Triacylglycerols with greater number of fatty acyl unsaturations are elevated in fasted sampling.** (**A**) Volcano plot showing log2 fold change between mean of non-fasted and fasted lipid quantitations, highlighting select outliers with identifications given in annotation. (**B**) Regression coefficients of each identified lipid’s linear regression slope when trained on non-fasted data (x-axis) and fasted data (y-axis). Selected lipids have identifications given in annotation. (**C**) Longer and more unsaturated TGs are more abundant in fasted plasma. All identified TGs are plotted by the number of unsaturations in their fatty acyl tails against the number of carbons in their fatty acyl tails. The point color represents the log2 fold change of average non-fasted abundance minus average fasted abundance. Different TGs with the same sum composition (i.e. the same unsaturation:carbon count) are plotted as overlapping dots. (**D**) The abundance of TG 20:5\_22:6\_22:6 for all plasma samples is plotted against the OGTT glucose AUC of the respective Nile rat. Fasted and non-fasted data are separated, and a linear regression is fitted to each sampling type. This TG is highlighted with gray background in A, B and C.

In a similar vein to Figure 4, we explored the relationship between sampling type, glucose tolerance and metabolite abundance for plasma lipids in Nile rats. **Figure 5A** shows the average abundance of identified lipids in non-fasted versus fasted. The left side of the volcano plot gives lipids which tend to be up-regulated in fasted samples, and lipids with highest up-regulation are dominated by free fatty acids, acylcarnitines and TGs with acyl tails that tend to be longer and highly unsaturated, for example FA 22:6 docosahexaenoic acid (DHA) and FA 20:5 eicosapentaenoic acid (EPA). This trend in TGs is likely driven by the fasting state liberating long, unsaturated fatty acyls from primarily structural phospholipids into energy producing metabolism[42](https://www.zotero.org/google-docs/?labMmq). Lipids on the right of 5A are up-regualted in non-fasted and tend to have shorter and less saturated fatty acyls.

By plotting each identified lipid against OGTT glucose AUC in the fasted and non-fasted condition (**Figure 5B**), the majority of lipids show a positive correlation with glucose tolerance in non-fasted samples as shown by the high number of species that are on the positive side of the x-axis. We see distinct clusters of phospholipids with mildly positive slopes, and a strong tendency for glycerolipids (purple points; the majority being TGs) to have steeper positive slopes versus OGTT glucose AUC. Because TGs comprise a significant portion of the plasma lipidome and have numerous links to physiological and disease conditions[47](https://www.zotero.org/google-docs/?WWghhs), we explored the relationship between average quantitation in fasted versus non-fasted to the fatty acyl length and fatty acyl unsaturation count in TGs (**Figure 5C**). As is suggested in Figure 5A, the overall trend is sustained in 5C where longer, more unsaturated TGs tend toward upregulation in the fasted state in Nile rat plasma, with less saturated TGs being upregulated in non-fasted samples. The most significant difference in average quantitation is shown by TG 20:5\_22:6\_22:6, with an average log2 fold change of 3, a substantial 8-fold increase in fasted plasma samples. The abundance versus OGTT for this TG is given in **Figure 5D**, which confirms this average quantitation trend as shown by the fasted sample points being almost universally higher than any non-fasted sample, yet showing the significant difference in slopes versus glucose tolerance, where both slopes are positive, but non-fasted is much greater in magnitude (slope of 11 versus 2).

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**Figure 6 – Linear regression modeling trained on non-fasted plasma samples achieves superior performance in predicting OGTT glucose AUC.** (**A**) Median R2 was compared for 6 different machine learning model architectures trained on fasted versus non-fasted plasma metabolites. Across all models, non-fasted data provided higher median R2 values. Regularized linear models (LASSO, ridge and elastic net) achieved lower R2 than the non-regularized linear regression, with elastic net achieving nearly equal R2 while also enabling significant coefficient shrinkage. The 6 models were categorized based on the underlying mechanism of prediction, divided between parametric, latent space and non-parametric methods. (**B**) Normalized feature importance is given for the top 30 lipids from the elastic net model trained on non-fasted samples. The top 10 features based on normalized importance are listed.

To understand the difference between the ability of fasted and non-fasted plasma metabolites to predict glucose tolerance, we constructed statistical models that were trained on either fasted or non-fasted metabolite data to predict OGTT glucose AUC. Model performance was assessed by calculating coefficient of determination (R2) in regression to each Nile rat’s 12-week OGTT glucose AUC. To ensure a fair and randomized assessment of each model’s performance, we performed 200 repetitions of 6-fold cross validation[48](https://www.zotero.org/google-docs/?qt2Lrg) totalling 1200 individual models and give their median R2 values in **Figure 6A.** Overall, models trained on non-fasted data yielded higher median R2 over the same model trained on fasted data. It is important to note the difference between the regression models used here versus the linear regression models used in Figure 4B and 5B. Here, we perform multivariate regression to create a bulk model where all identified metabolite abundances are used as predictor variables to model OGTT as response as shown in eq. 1.

OGTT glucose AUC ~ Metabolite1 + Metabolite2 + ... + Metabolite\_n

Whereas in Figures 4 and 5, OGTT glucose AUC was used as the predictor variable to model the strength of correlation to the abundance of each metabolite, totalling 443 single metabolite linear models, as shown in eq 2:

Metabolite ~ OGTT glucose AUC

The single metabolite linear models give information at the level of the individual metabolite, while the bulk model is used to compare metabolite predictive performance against each other.

The superior performance suggests that non-fasted plasma samples have metabolic insight that better correlates with the rat’s OGTT glucose AUC. While linear regression was most performant (R2 = 0.71), it is worth noting that our data falls under the paradigm of having more variables than observations that is common in -omics studies. This is problematic due to the high number of features that show multicollinearity (supplemental figure X), which occurs when a feature’s abundance across plasma samples is highly correlated with multiple other features. This is prominent in TGs and phospholipids, where many of the lipid species undergo similar biological regulation and are therefore strongly multicollinear. This effectively diminishes the coefficient for each multicollinear feature, effectively diluting its actual importance. Techniques exist for overcoming multicollinearity by using coefficient shrinkage via regularization[citation], bootstrapping [random forest in biology citation] or transformation into latent spaces that capture the signal present in data in fewer dimensions [PLSr citation]. To accomplish this, we trained 5 regression models that use one of these techniques (all models except linear regression). To summarize, LASSO uses L1 regularization [citation] and performed poorly by R2. Ridge regression uses L2 and performed similarly to linear regression, however the model failed to significantly shrink the coefficients, and is therefore nearly identical to linear regression. Elastic net combines the methods in LASSO and ridge, and achieved nearly identical R2 to linear regression (0.67) while creating significant shrinkage of coefficients. Partial least squares regression (PLS-r) achieved good R2 but did not significantly shrink model coefficients. Random forest regression likely struggled due to problems interpolating OGTT glucose AUC between observations.

Gauging which metabolites most contribute to the prediction is calculated by averaging the model’s regression coefficients (or Gini importance in the case of random forest) across all 1200 repetitions of cross validation. These averaged coefficients can be normalized such that the highest importance is 1, and the top lipids from the elastic net model trained on non-fasted data are shown (Figure 6B). Despite the dominance of TGs slope versus OGTT in Figure 5B (i.e. the large number of purple dots far from the origin), all other lipid classes are represented in the top 30, and the top 2 features are a plasmanyl-PC and a sphingomyelin. Similar feature importance plots for both polar metabolites and lipids for all 6 models in both non-fasted and fasted datasets are given in Supplemental Figure X, along with R2 performance for many other machine learning model architectures. Notably, Supplement Figure XA shows that fasted models tend to rely more heavily on polar metabolites for prediction, whereas non-fasted models show more lipid features.

Chart

Description automatically generated

**Figure 7 – Validation of findings from age 8-10 week Nile rats in mature 20-34 week Nile rats.**

(**A**) A cohort of male and female Nile rats were measured for non-fasted random blood glucose starting after week 20. Rats that began to display elevated random blood glucose (hyperglycemia) were selected for non-fasted plasma sampling. n=6 female rats and n=5 male rats RBGs are averaged and RBG over time is shown as the number of weeks prior to OGTT. OGTT occurred between weeks 28 and 34 of age [double check numbers]. Plasma samples collected between 2 to 4 weeks prior to OGTT underwent LC-MS lipidomics and polar metabolomics as shown with gray background. (**B**) Similar to Figure 3, the repeated measures metabolite variance for the validation cohort of mature Nile rats is assessed via % RSD, with median RSD reported in percent, and 95% C.I. given by error bars. (**C**) Comparison of female versus male OGTT slopes for identified lipid species. (**D and E**) Two ceramide species that show strong trends versus OGTT in mature Nile rats.

Discussion

In this study, we revealed that Nile rat plasma metabolites show large differences in abundance between fasted and non-fasted dependent on glucose tolerance. Despite these large differences, the week-to-week variance of individual plasma metabolites in the non-fasted state is similar to the variance in fasted. Overall, these changes indicate that the choice of sampling blood plasma in fasted versus non-fasted condition can influence the quantitation of important biomarkers for type 2 diabetes.

This work is the first analysis of plasma metabolites in the Nile rat species. It is notable that this work accomplished plasma analysis with several key benefits. First, we performed separate lipidomics and polar metabolomics to provide broader coverage of the diversity of polarities of molecules present in plasma, from hydrophobic triacylglycerols to hydrophilic carbohydrates. In addition, we used small amounts of plasma, less than 20 microliters for each plasma sample. With such small quantities, this enables further analysis of plasma in smaller Nile rats, such as in weanlings, allowing for the monitoring of plasma metabolites in even younger cohorts.

In the context of diabetes, comparing the interaction effect between diabetic glucose tolerance and the fasted or non-fasted state is uncommon, yet can lead to key insights into diabetes disease etiology. This work shows the effect of glucose tolerance as measured by OGTT glucose AUC across a wide range of AUC values to show the extreme effect of diabetic glucose tolerance on metabolite quantitations. We also show these differences in different Nile rat age groups (10 week versus 30 week and in both sexes. While our data are enough to serve as a foundational baseline for future work on Nile rats, future studies will benefit from several strategies to increase statistical power in these associations and improve confidence in conclusions, namely to include greater numbers of Nile rats and using more females in cohorts. Studies will also benefit from a wider range of genetics from many different Nile rat litters due to the genetic diversity of these animals. Further study will benefit from continuing to use LCMS metabolomics to perform biomarker analysis and investigate disease associations with diabetes.

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