Point-by-point response

We thank the reviewers for their comments to our manuscript. Below are the point-by-point responses to their comments.

Reviewer #1:

Remarks to the Author:

I apologize for redundancies or failures of coherence, as these comments were dictated from notes based on a general read of the manuscript.

This report by Toh et al’23 is basically a methods paper and falls short of providing significant insight into type II diabetes in the Nile rat, but it does present useful descriptive information related to the metabolomic data presented.

Indeed, our intent was to describe methods that could be applied to future studies in Nile rats that would focus on understanding the progression across diabetic phenotypes. The data provided in the manuscript showcases the utility of non-fasted blood collections for metabolomics and lipidomics studies.

The journal LabAnimal “encourages submissions of innovative methods and techniques that improve and expand the use of model organisms in the lab, and research that deepens our basic understanding of model organisms and the opportunities/challenges for modeling human health and disease." We feel that our manuscript is well suited for a journal that values sharing methods and techniques related to animal model studies.

In addition to validating methods for metabolomics, we found significant relationships between non-fasted metabolites and glucose tolerance, and several Nile rat metabolites found in our study have been reported in human studies of type 2 diabetes (see Fig 5E). We specifically focused on the *spectrum* of glucose tolerance (see Fig 1C) rather than grouped categories, like ‘diabetic’ or ‘non-diabetic’. This unique way of exploring the data helps to better link metabolites (or metabolic processes) to glucose tolerance as a continuous process rather than a on/off switch.

It is agreed that the Nile rat, and Israeli Sand rat, are superior diurnal model species considering their susceptibility to diet-induced type II diabetes; and it behooves science to elaborate on the mechanisms involved using these models to better understand the diet x gene interactions in progress here.

Metabolomics can clearly add to that understanding.

Thank you for those comments; we agree. To design those experiments using metabolomics to study diet x gene interactions, we first needed to evaluate the methods used for collecting samples and performing metabolomics studies. Because there was no existing metabolomics data evaluating non-fasted vs. fasted sampling in Nile Rats or other rodent models, and only a handful of studies have looked at non-fasted vs. fasted data collection in humans, we felt that this study was needed.

That said, the classic definition of diabetes is an elevated blood glucose, and estimates of long-standing chronic damage to tissues is defined historically by HbA1c and organ/tissue damage observed at necropsy and attributed to the chronic hyperglycemia prior to death. Thus, blood glucose is an easily measured early metabolite and a constant that has previously been assessed in humans and Nile rats, clearly showing what these data with metabolomics confirm, i.e. that high glucose values in diabetic Nile rats are the key, readily accessible metabolite for diagnosing their diabetes.

We agree.

Specifically, it has been previously demonstrated with more robust modeling in hundreds of Nile rats (see Bolsinger ’17, Subramaniam ’18 , ’19, ‘22) that the random blood glucose (RBG, ie. nonfasted) is superior to the fasting blood glucose [FBG] for defining the onset and extent of diabetes in this species. The 30’ -60’ time points during the oral glucose tolerance test [OGTT] were found to be the most sensitive predictor, but not that much better than RBG; and OGTT requires much more effort in sample collection, processing, etc. with minimal added value. Plus the 2004 ref on superiority of postprandial glucose for determining T2DM in humans is dated, and less informative than newer info on the importance of RBG in humans and in the Nile rat data cited above. FBG is still used by clinicians as the gold standard, but only because once it is elevated, the patient definitely requires medical treatment where the physician is obligated to intercede, not because FBG is a better index of diabetes onset. This background information needs to be integrated better into the current report because its absence suggests a superficial and incomplete reading/understanding of the literature.

Thank you for the additional references. The goal of this manuscript was to explore the utility of non-fasted sampling for metabolomics. We are familiar with the excellent prior observations about non-fasted sampling for better diagnostic power in Nile rats, however these prior papers did not analyze metabolites/lipids. We agree glucose is a good metric for diabetes, but broader metabolomics measurements can offer more context for understanding the underlying mechanism. To address your concerns, we have edited the title to: “Plasma metabolomics supports non-fasted sampling for metabolic studies across a spectrum of glucose tolerance in the Nile rat model for type 2 diabetes”. The new title emphasizes the utility of the method for metabolic studies rather than its potential use for diagnosing diabetes.

We have also expanded the background information in the introduction to update references and better define where the current study fits into the prior work. See highlights below for new text:

“When considering experimental methods for studying diabetes, a majority of studies looking for metabolic changes will use blood that has been sampled under fasted state to avoid excess variability from unrestricted eating behavior. However, for the early progression of diabetes, it is known that postprandial hyperglycemia precedes fasted hyperglycemia, and thus is a more sensitive measurement for early diabetes demonstrated in human studies14–16 as well as in the Nile rat model11,17–19 In addition, there is some evidence that postprandial state might have reduced variability in blood metabolites20. For rodent models, non-fasted state likely represents a postprandial state given the high frequency of food intake. Yet, to date, no study has validated the use of non-fasted sampling for metabolomics studies in rodent models. Specifically, reproducibility and replicate variability between fasted and non-fasted states have not been sufficiently analyzed, thus this study compares metabolite variance between non-fasted and fasted blood sampling for studying progressive glucose intolerance in Nile rats.”

By studying mostly old rats [24 of 34 rats between 26-42wks] herein, the authors miss the point that early detection of the metabolomic profile should better separate the genetic aspect of the disease that identifies resistant and susceptible Nile rats, best detected during early development of their type II diabetes. By separating weanling rats for study, these two degrees of susceptibility might best reveal clusters of metabolites to predict which genes are involved early… and or chronically, if still active in older rats with advanced diabetes. Even among the 10 ‘young 8-12wk old males’ 7 or 8 of them already display diabetes based on their OGTT. So basically this study had no control rats for a ‘nondiabetes profile’...ie. metabolites specifically from nondiabetic rats. As this study unfortunately illustrates, it is a gamble to begin only with old, diabetic rats to try and demonstrate metabolites that might predict the diabetes. In essence, of the 34 rats studied here, it appears that 31-32 already had diabetes, so early metabolic signals have already passed …it’s like looking for tulips in a July flower bed. :>)

Our primary experimental cohort were 8-week-old Nile rats, the youngest age that is amenable to repeated sampling without being influenced by isoflurane anesthesia. In our past experience, 4-week-old Nile rats are vulnerable to isoflurane hyperglycemia.

Regarding our older validation cohort, we have stated in our results the following statement: “To select for animals developing diabetes, we collected weekly plasma samples and weekly RBG measurements from 20 euglycemic Nile rats starting at 24 weeks old and took samples from the first 11 Nile rats (5 males and 6 females) that developed non-fasted hyperglycemia.”

Nor does this elegant detailed metabolomics study identify or describe a metabolite more useful than blood glucose for the diagnosis of diabetes. Many metabolites described and identified as sugars, alcohols, lipids, etc. are fascinating in their own right, but it could be simply be descriptive of Nile rats as a species , or their diet, or gender, or age, or chronic diabetes characteristics per se. One has no way of telling for lack of proper controls. Even the ‘gender control’ is biased by age, as most females over 30wks old fed mouse chow 5008 have developed diabetes.

The objective of this study is to test the validity of using non-fasted sampling for metabolic studies. Rather than finding a diagnostic biomarker superior to blood glucose, obtaining a broad range of metabolites and lipids that relates to glucose tolerance provides important context for understanding the underlying mechanisms.

Rather than using control versus diabetes groups, we used linear regression to analyze the spectrum of glucose tolerance, more accurately treating it as a continuous variable. For our primary experimental cohort captured OGTT glucAUC values from 317-1004 h.mg/dL, representing animals from euglycemia to overt diabetes. In comparison, Nile rats in our validation cohort had OGTT glucAUC values ranging from 429-734 h.mg/dL. +

Beyond this, we designed the study so that each animal is its own control by comparing fasted versus non-fasted metabolites. Our primary research interest is validating non-fasted versus fasted for metabolomics analysis and our study design accomplishes this.

It would have been nice if a cluster of metabolites provided new insight into the genes/mechanisms underlying the inherent problem in type II diabetes for Nile rats. Early metabolites may differ from the chronic signals found in advanced diabetes. It might be helpful to assign clusters of the ‘66 highlighted metabolites’ to metabolic pathways, for example can gut metabolites be separated from the liver or muscle metabolites? Are specific nervous system or hormonal pathways involved earlier than others?

We performed class-level annotation of metabolites (Fig 2A-B), because this is an alternative categorization (vs. pathways). Unfortunately assigning tissue specificity to circulating metabolites is not well established, however, we have added KEGG identifiers and KEGG pathways for each metabolite (where possible) into Supplemental Table 5.

While these data represent a detailed methodology for overall study of metabolomics in Nile rats, not much new information is presented about blood glucose [specifically RBG] and its usefulness in detecting the diabetes, nor is any insight provided about metabolomics related to the previously described striking difference between mouse chow versus rabbit chow during diabetes induction from this lab [Toh et al ‘20]. The concluding statement in the abstract simply confirms what has already been reported for the Nile rat [and Sand rat], ie. that diabetes can be followed readily by monitoring blood glucose alone. It has been well established (including their own previous experiment) that dietary fiber protects the developing Nile rat from type II diabetes. Why was not a small group [5-6 rats] fed a fiber-rich diet like rabbit chow included as simple control, just to shoe it works to separate metabolites between diabetes or no diabetes?

We agree that we did not add any additional information about blood glucose; we acknowledge that it is a well-established marker of diabetes. We intended to validate metabolomics as an investigative tool to probe underlying mechanisms of glucose tolerance.

We also chose to limit the diet to only rodent chow because we wanted to avoid the confounding effect of having two different diets. Adding the rabbit diet will invoke large changes to the metabolic profile including those unrelated to diabetes progression; using one chow helps eliminate the confounding effect. We used rodent chow to provoke diabetes in these Nile rats to explore metabolic signatures related to different glucose tolerance levels. We hope to follow up with additional work in Nile rats to explore greater extent of metabolic changes occurring with diet and time.

Many informed metabolomic reports use software to identify clusters of metabolites to suggests the key metabolic pathways that are affected by treatment or disease process…which help predict which genes are involved in the disease process being studied.

It is not surprising that the fasting metabolome was less informative compared to the postprandial situation, in part because fasting allows the gut and liver to return to baseline dynamics, which is in turn pushed to the limit during eating and recovery from food intake…and thus more apt to expose any distortions in metabolism.

Thank you for the comment. We sought to represent the data in the most honest way possible, using statistics that are simple and appropriate given our cohort size and study design.

No data are provided on the diet or food intake other than the fact that all rats were fed mouse chow 5008 which is known to be diabetogenic from a young age in this species. And a high glycemic load enhances the diabetes, and individual rats that consume the most food develop the most severe diabetes. One must assume that at this late stage of diabetes 26-42 weeks for 24/34 rats, most of the rats are overconsuming energy and that their circulating metabolites in the nonfasting RBG and postprandial condition [OGTT] are distorted to the upside. There might be better insight related to the metabolites had there been some effort to distinguish between early and late onset vs advanced cases of diabetes, which likely were present in all of the older rats.

Thank you for the comments. We recognize the potential inaccuracy when we use the term “early diabetes” and have made edits to the manuscript to reiterate that we are studying a spectrum of glucose tolerances in these animals.

For example, our 26-42 week cohort is not selected for late-stage diabetes. We selected them based on their trajectory of RBG (please see Supplemental Figure 4A). In the figure 6 caption, we say “mature males and females displaying signs of early diabetes”, and we have changed this to say “progressive glucose intolerance.”

Regarding your comment on diet, we acknowledge that we do not have measurements of food intake. We have added this to our caveats in the discussion: “Since our focus was to develop an optimal method for reproducible plasma metabolite measurements in the Nile rat model, this study is limited in its ability to discover diabetes biomarkers due to the small study size and short sampling timeline. In addition, rodent chow is diabetogenic in Nile rats[4,28](https://www.zotero.org/google-docs/?bCYbpT) and this choice of diet skews the population toward higher glucose intolerance. Future studies to investigate the underlying mechanisms of glucose tolerance could be enhanced by incorporating food intake and additional metrics of diabetes; however these data were not captured here due to our priority on the metabolomics data. In summary, the method presented in this manuscript enables larger studies that could use metabolomics to explore diabetes progression, analyze the effects of different diets, and define the genetic and epigenetic contributions to diabetes in Nile rats. ”

The 2004 reference to postprandial glycemia in humans being superior to FBG for diagnosis of T2DM, is somewhat dated at this point, as new data indicate the full story. Although transient, the postprandial glycemia is best at capturing insulin resistance or limited beta cell production of insulin, which is reflected in the RBG all day long. Thus, RBG estimates the glucose exposure of tissues throughout the day, especially the micro vasculature expressing diabetes in terms of diabetic pathology, while FBG becomes elevated only once insulin resistance and limited insulin availability are in effect in chronic diabetes, which happens much later in the disease process. OGTT detects and anticipates the earliest rise in RBG, but OGTT is much more demanding assay and not that much more effective than RBG in terms of its diagnostic/clinical usefulness.

Thank you for your comment, we have added more recent references, same as what you mentioned previously.

It's noteworthy that the pages are not numbered in this manuscript. But in the methods section, the dose of glucose administered should be listed as 2.0g per kilogram of body weight, not just per kilogram.

Thank you for catching this. We have modified the methods text to be: “2g of dextrose per kilogram body weight was introduced via oral gavage.”

It is important to reiterate that this is not really ‘early diabetes’ that described by the metabolites, as the rats are mostly 26 to 42 weeks old, most of which would be advanced diabetes for the Nile rat fed mouse chow ( 5008 chow) , a low-fat, high-carb rodent chow in terms of energy percent.

We have edited all instances of “early diabetes”, see comments above.

The three groups of animals tested unintentionally reflect selection bias in terms of diabetes in this species. 10 young males were tested between 8 and 12 weeks of age, which is old enough to have significant diabetes in progress [demonstrated here in their OGTT graphs]. Diabetes begins in males on mouse chow at roughly 6 to 8 weeks old and can be quite extensive by 12 or 14 weeks. In fact, seven of the 10 young male rats already have distorted OGTT, indicating their advanced stages of diabetes. Thus none of the data, other than 2-3 young male animals, appeared to be resistant to diabetes and are, thus, actually displaying any data related to diabetes resistance described in this species. The 10 males range from 50 to 75 fasting blood glucose with one at about 150 mg/dL. Only two out of 10 are actually normal based on their 30 to 60 minute OGTT, and most rats [six out of 10] are already insulin depleted or insulin limited, weighing 82 -115 gr at eight weeks .. full-adult weights, and the 105- 220 g at 12 weeks. These these are mature rats well on their way to diabetes when fed mouse chow, thus making interpretation of the results somewhat limited to diabetic rats only.

We agree that this study looks at a range of glucose tolerances and have edited the text to reflect this. See other comments above.

Discussion

It was already pointed out above that this study is not really describing ‘early diabetes’ as claimed. Do not mislead the naïve reader. In fact, even among the 10 males 8 to 12 weeks old, all but two or three of them have semi-advanced diabetes, and we can assume all of the 26 to 42 week old adult rats were likely in advanced stages of diabetes with RBG in excess of 125 mg/dL, although there’s no baseline RBG offered? for these older animals, so one cannot accurately assess their diabetes status. Furthermore, this age bias and slant towards advanced diabetes in most animals renders the rest of the figure somewhat biased because one cannot adequately distinguish between the Fed /fasting focus as even that outcome is not distinct here being in the 54% to 46% range…statistically, but not biologically different enough to rely on…other than for its methodological consistency.

We addressed ‘early diabetes’ in prior comments. The RBGs for this older cohort can be found in supplemental figure 4A.

Regarding fed versus fasted, the expected outcome is that non-fasted variance would be higher, however we found that each method has similar variance. This runs counter to the field’s accepted wisdom and is a valuable finding for our future work.

While the data make the point that RBG is better than FBG, that conclusion has been documented for several years in Nile rats [J Bolsinger 2017, A Subramaniam 2018, 2019, and 2022). The distribution of ‘fed metabolites’ is interesting, but not providing much insight that can be assigned to specific molecules relative to diabetes onset or severity, so the usefulness relative to type II diabetes is limited. We don’t know but what the molecules detected were related to the diet [as mouse chow] the age of the animals, or the gender of the rat. It’s well-known that young males develop diabetes much more rapidly than young females, so simply using older animals for both the RBG in fed /fasting condition, and the gender identification, is of limited value here because the analysis was conducted too far into the age bracket where diabetes was apt to be firmly entrenched in most rats. The reader wants to know what the metabolites look like BEFORE and AFTER diabetes begins and how long those profiles persist into old age…at least 1y.

We agree that random sampling of blood glucose is well-established as the superior marker for diabetes. We disagree that fed metabolites do not provide insight into diabetes, as triglycerides in particular are very highly correlated to glucose tolerance and reflect dysregulation in lipid metabolism. We further note that the modern crop of diabetes drugs are not those that most reduce blood glucose, but rather target other parts of the complex etiology of diabetes. Given that our focus in this work is establishing non-fasted metabolomics as a method, we have not yet performed the extended longitudinal diabetic profiling. Despite this, we think this work provides guidelines for these studies and our metabolomics provides a baseline understanding of the Nile rat’s plasma metabolome.

While this paper provides significant novel information on techniques and adjustments for volume size for study of Nile rat metabolomics, other inferences are less useful. The point about ‘there being less variance in fed rats’ is limited to Nile rat with diabetes, as nondiabetic controls are not identified in terms of metabolomics, rendering the data of minimal clinical value. One can assume it might apply to nondiabetic resistant rats as well, but the study would have done better to conduct this pilot methodology in younger rats with and without diabetes and expended more effort focused on the diabetes rather than on the technique. Even though 60 samples were accumulated in the test mode for the 10 younger males, the study still only has a biological n=10… replicants don’t count in terms of biological endpoints. If Time and $$$ was the issue, the authors would have done better with an n= 20 with only one non-fasted RBG sample per rat to provide an overall n= 20 to get a better idea of animal variation rather than focus on sample technique and variation introduced by the method. This could have been anticipated based on the hundreds of data points in Nile rats already published that indicate the RBG was better than FBG and more easily applied than the OGTT test, which does not provide an advantage over the RBG in terms of diagnostic usefulness…ie. relative to the disease observed at necropsy.

In figure 1 we point out that 2 out of 10 Nile rats are not diabetic by OGTT, and these rats also have low variance. We are studying the spectrum of glucose tolerance and centered on the progression towards overt diabetes.

Regarding your points on using n=20 non-fasted Nile rats, we are studying the variance caused by the sampling method, not analyzing the variance across animals.

Prior studies on RBG variance do not analyze plasma metabolites, and these data cannot be used to infer the variance of metabolites in metabolomics studies.

To clarify, we do not propose that other plasma metabolites will provide a diagnostic advantage over glucose, rather that they provide a system-wide context for the underlying mechanisms.

Thus the Discussion should list the caveats associated with this study, less the naïve reader overinterpret the results. First, point out that the extreme variation in diabetes already present among the 10 control males greatly limits the interpretation of all results because NO true control for diabetes was established. Note that only 2 of the 10 males were relatively normal, so the elegant array of metabolites clustered as sugars, lipids, alcohols, etc. are not assignable to diabetes per se, but rather simply describe metabolites in non-fasted Nile rats at various stages of diabetes, despite the wide ranges in age from 8wk to 42wks.

We agree, we have clarified the text across the manuscript to address this.

In fact, all the 26 to 42wks- old rats would likely be severely diabetic (no RBG was offered in the latter that could be used to describe the diabetes in these rats). Closer attention to the literature on type II diabetes in the Nile rat would have indicated that the controls should have been six weeks old males, with a greater number of rats sampled.

We showed the RBGs of the older cohort in supplemental figure 4A. We clarified that we are studying Nile rats across a spectrum of glucose tolerance rather than grouping into strict diabetic versus non-diabetic.

Second, it should be pointed out that all the data collected here represent one diet source which is known to be diabetogenic in this species. Previous data from the Toh lab [‘20] demonstrated the benefit of feeding fiber in the form of rabbit chow. A group with that dietary history would have been a better control group, even if the number was small.

We have added this point to the caveats paragraph in the discussion.

Third, point out that the extreme age in the two older groups precludes any robust conclusions about sex differences in their metabolome, as age trumps gender in this species when they become aged and essentially all become diabetic. On that note one cannot tell if the metabolite clusters described are simply Nile rat characteristics or due to diet, or age, or degree of diabetes present. Alert the naive reader that this is the case.

We agree we cannot make definitive conclusions about sex differences from our data. The key conclusion from Figure 6 is that the replicate sampling (n=3) variance is no worse than in the younger cohort. We have added a caveat about small sample size to the discussion.

The 2004 reference on FBG versus OGTT is dated, both for humans and for the Nile rat, considering the extensive data showing RBG is much better than the FBG as an indicator of early diabetes onset in this species. Although the 30’ and 60’ OGTT oral is slightly more sensitive as an index of diabetes than RBG, the OGTT is not worth the extra effort related to the sample collection and processing.

We agree with you that RBG is better for assessing diabetes, and it is unfortunate that others in the field do not seem to agree with us: “Fasted glucose levels tend to provide less variable results, as you remove the food source for a designated amount of time for all of the mice involved.” Quotation from <https://www.jax.org/news-and-insights/jax-blog/2019/september/5-common-questions-for-diabetic-models>

Although this manuscript has its limitations identified above, it does offer potential insight into metabolites in the non-fasted Nile rat as a useful approach to study various disease entities related to nutrition in this species, including type II diabetes.

We thank you for your helpful comments on this paper.

Reviewer #2:

Remarks to the Author:

The reviewer has the following comments regarding the manuscript.

The authors give only very few info on the reason how/why this rats develop type 2 diabetes.

Thank you for pointing this out, we have added a new paragraph in the results section that describes how the Nile rats develop T2D. The new paragraph is copied here:

“Nile rats have been well-described to develop glucose intolerance when consuming conventional rodent chow 5008, in part due to the glycemic load of 500811,17–19,28. Additionally, males progress toward glucose intolerance more rapidly than females on the same dietary challenge28. Because the Nile rat model is genetically diverse, they display a spectrum of glucose intolerance. To evaluate plasma metabolites that show trends over a spectrum of glucose tolerance, we selected juvenile males fed rodent chow.”

As far as I can read in the manuscript diabetes is developing in the Nile rats under regular rodent diet. This is in contrast to what I read in literature that a high carbohydrate diet is necessary to develop diabetes.

High carbohydrate diet can promote diabetes. A recent paper (Subramaniam 2022) describes how glycemic load contributes to glucose intolerance in the Nile rat. “The Nile rat, a diurnal North African desert rodent, develops ‘spontaneous’ diabetes when housed in captivity and fed typical commercial mouse/rat chow low in fiber, i.e., Lab Diet 5008; 65:8:27 as CHO:fat:protein %energy with 3% fiber.” This manuscript has been cited in the additional text in the results section (also see above).

Reference:

Subramaniam A, Park B, Raphael D, Landstrom M, Hayes KC. Dietary Carbohydrate as Glycemic Load, Not Fat, Coupled with Genetic Permissiveness Favoring Rapid Growth and Extra Calories, Dictate Metabolic Syndrome and Diabetes Induction in Nile Rats (Arvicanthis niloticus). Nutrients. 2022 Jul 26;14(15):3064. doi: 10.3390/nu14153064. PMID: 35893924; PMCID: PMC9331090.

The reviewer has some doubts regarding the significance of the results since questions can be raised regarding the usefulness of this animal model, which is mainly due to the high variance in age at which type 2 diabetes onset is observed. It seems not doable to screen Nile rats during their whole life for diabetes onset using the expensive metabolomics technique.

We believe that the diabetes onset variance is actually an advantage. Humans also show variability in diabetes onset age due to various factors that include genetics. Similarly, the Nile rat is a model system with high genetic diversity that mimics genetic diversity in humans.

We agree that screening Nile rats for diabetes using metabolomics is excessive, especially since metrics such as random blood glucose (RBG) reliably measure levels of glucose tolerance in the animals. We propose that metabolomics studies using the method validated here can be used to study the underlying mechanisms of glucose homeostasis. Additionally, non-fasted sampling methods are underutilized in the field and could potentially offer new perspectives about diabetes.