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Assessing Whole-body Lipid Handling Capacity in Mice

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TITLE:

Assessing Whole-Body Lipid-Handling Capacity in Mice

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KEYWORDS:

Lipid metabolism, mouse, adipose tissue, serum, lipolysis, β3-adrenergic receptor

SUMMARY:

This paper provides three easy and accessible assays for assessing lipid metabolism in mice.

ABSTRACT:

Assessing lipid metabolism is a cornerstone of evaluating metabolic function, and it is considered essential for in vivo metabolism studies. Lipids are a class of many different molecules with many pathways involved in their synthesis and metabolism. A starting point for evaluating lipid hemostasis for nutrition and obesity research is needed. This paper describes three easy and accessible methods that require little expertise or practice to master, and that can be adapted by most labs to screen for lipid-metabolism abnormalities in mice. These methods are (1) measuring several fasting serum lipid molecules using commercial kits (2) assaying for dietary lipid-handling capability through an oral intralipid tolerance test, and (3) evaluating the response to a pharmaceutical compound, CL 316,243, in mice. Together, these methods will provide a high-level overview of lipid handling capability in mice.

INTRODUCTION

Carbohydrates and lipids are two major substrates for energy metabolism. Aberrant lipid metabolism results in many human diseases, including type II diabetes, cardiovascular diseases, fatty liver diseases, and cancers. Dietary lipids, mainly triglycerides, are absorbed through the intestine into the lymphatic system and enter the venous circulation in chylomicrons near the heart[1]. Lipids are carried by lipoprotein particles in the bloodstream, where the fatty acid moieties are liberated by the action of lipoprotein lipase at peripheral organs such as muscle and adipose tissue[2]. The remaining cholesterol-rich remnant particles are cleared by the liver[3]. Mice have been widely used in laboratories as a research model to study lipid metabolism. With

comprehensive genetic toolsets available and a relatively short breeding cycle, they are a powerful model for studying how lipids are absorbed, synthesized, and metabolized.

Due to the complexity of lipid metabolism, sophisticated lipidomics studies or isotopic tracer studies are usually used to quantify collections of lipid species or lipid-related metabolic fluxes and fates[4,5]. This creates a massive challenge for researchers without specialized equipment or expertise. In this paper, we present three assays that can serve as initial tests before technically challenging techniques are used. They are non-terminal procedures for the mice, and thus very useful for identifying potential differences in lipid-handling capacity and narrowing down the processes affected.

First, measuring fasting serum lipid molecules can help one ascertain a mouse's overall lipid profile. Mice should be fasted, because many lipid species rise after meals, and the extent of the increase is strongly affected by the composition of the diet. Many lipid molecules, including total cholesterol, triglyceride, and non-esterified fatty acid (NEFA), can be measured using a commercial kit and a plate reader that can read absorbance.

Second, an oral intralipid tolerance test evaluates lipid-handling capability as a net effect of absorption and metabolism. An orally administered intralipid will cause a spike in circulating triglyceride levels (1–2 hours), after which they eventually return to basal levels (6–8 hours). This assay offers information about how well a mouse can handle the exogenous lipids. Heart, liver, and brown adipose tissue are active consumers of triglycerides, whereas white adipose tissue stores it as an energy reserve. Changes in these functions will lead to differences in the test results.

Lastly, promoting lipolysis to mobilize stored lipids is considered a possible strategy for weight loss. The β3-adrenergic receptor signaling pathway in the adipose tissue plays an important role in adipocyte lipolysis, and human genetics have identified a loss-of-function polymorphism Trp64Arg correlated with obesity[6]. CL 316,243, a specific and potent β3-adrenergic receptor agonist, stimulates adipose tissue lipolysis and the release of glycerol. Evaluation of a mouse's response to CL 316,243 can provide valuable information on the development, improvement, and understanding of the efficacy of the compound.

Collectively, these tests can be used as an initial screen for changes in the lipid metabolic state of mice. They are chosen for the accessibility of the instruments and reagents. With the results derived from these assays, researchers can form an overall picture of the metabolic fitness of their animals and decide on more sophisticated and targeted approaches.

PROTOCOL

Animals are housed in standardized conditions following animal-care and experimental protocols approved by the Institutional Animal Care and Use Committee of the Baylor College of Medicine (BCM). Animals are fed a standard or special diet, water ad libitum, and kept with a 12-hour day/night cycle.

1. Measuring of fasting serum lipids

1.1 Transfer mice to a new cage after 5 PM and fast with free access to water, overnight (with around 16 hours of fasting before the experiment). The overnight fasting ensures complete emptying of the mice's stomachs.

NOTE: Mice eat their feces during fasting, so simple food withdrawal cannot ensure they are adequately fasted.

1.2 The next morning, make a superficial incision (nick) in the tail vein of the free-moving mouse, and draw 25 µL of blood from the incision into a glass capillary (filling about 1/3 of the capillary) without restraining the mouse. Quickly flow the blood into a microcentrifuge tube.

1.3 Stop the bleeding using septic powders, refill the feed in the cage, and make sure the mice show no signs of extreme stress.

1.4 Complete the blood withdrawal for all the mice.

1.5 Allow the blood to clot by leaving it undisturbed at room temperature for 1 hour. Spin the clotted blood samples at 2,000 x g at 4 °C for 10 minutes in a refrigerated benchtop microcentrifuge, and transfer supernatant (serum) for analysis.

NOTE: Serum can be stored at –20 °C for several weeks until analysis. For long-term storage, keep the serum at –70°C.

1.6 Analyze each lipid metabolite using the manufacturer's provided protocol.

2. Oral Intralipid Tolerance Test

2.1 After 5 PM, weigh the mice for the calculation of the intralipid volume to be given to them the next day. Then, transfer the mice into a new cage and fast them overnight (16 hours).

2.2 The next morning, mark tails of the mice housed in one cage to help identify them in the subsequent bleeding steps.

2.3 Make a nick in the tail vein and draw 15 µL of blood from the incision into a glass capillary (filling about 1/5 of the capillary), and quickly blow the blood into a microcentrifuge tube for T = 0 serum.

2.4 Gavage mice 20% intralipid using an 18G gavage needle at a ratio of 15 µl per gram of bodyweight, using the pre-fasting bodyweight. Stack each mouse by 1 minute.

NOTE: Researchers who are inexperienced with oral gavage or tail bleeding techniques can stack each mouse by 2 minutes or even longer.

2.5 Draw blood at T = 1, 2, 3, 4, 5, and 6 hours: Draw 15 µL of blood (1/5 capillary) per mouse through tail bleeding, and quickly blow the blood into a microcentrifuge tube.

NOTE: There is no need to stop the bleeding during the assay unless the mice show excess bleeding.

2.6 Spin the blood samples at 2,000 x g at room temperature for 10 minutes in a microcentrifuge. Transfer the supernatant, including the floating fat layer, to a PCR tube for storage. The supernatant can be stored at –20 °C for several weeks until analysis.

NOTE: The supernatant should be plasma. If some samples have already clotted by the time of centrifugation, it does not affect triglyceride measurement.

2.7 After the last blood collection, stop the bleeding using septic powders, refill the feed in the cage, and make sure the mice show no signs of extreme stress.

2.8 Load 2 µL of triglyceride standard and collected supernatants into a 96-well plate.

2.9 Add 200 µL of triglyceride reagent and let the plate incubate for 5 minutes at 37 °C for color development.

2.10 Measure the absorbance at 500 nm with a reference wavelength of 660 nm in a laboratory plate reader, and calculate the sample's concentration.

3. β3 Adrenergic Receptor Agonist CL 316,243 Stimulated Lipolysis Assay

3.1 Prepare CL 316,243 as a stock solution of 5 mg/mL (50x) in phosphate-buffered saline (PBS), and store at –20°C until use.

3.2 In the morning, weigh the mice to calculate the amount of diluted CL 316,243 solution needed for the experiment. The mouse will receive 10 µL per gram of bodyweight of diluted CL 316,243, for a final dose of 1 mg/kg bodyweight.

3.3 Transfer the mice into a new cage with free access to water, and fast them for 4 hours.

3.4 Make enough 1x CL 316,243 solution from 50x stock using PBS. The final concentration of 1x CL 316,243 solution is 0.1 mg/mL. Use PBS for the control treatment group.

3.5 Mark the tails of the mice housed in the same cage for easy identification during the bleeding steps.

3.6 Make a nick in the tail vein, and draw 15 µL of blood from the incision into a glass capillary (filling about 1/5 of the capillary), and quickly flow the blood into a microcentrifuge tube for T = 0 sample.

3.7 Inject diluted CL 316,243 solution (or control if included in the experiment) intraperitoneally at a volume of 10 µL/g bodyweight. Stack each mouse by 1 minute. Use a maximum of 5 mice for each 60-minute experiment, or 10 mice for a two-person team.

3.8 Draw blood at T = 5, 15, 30, 60 minutes: Draw 15 µL of blood (1/5 capillary) per mouse through tail bleeding.

NOTE: There is no need to stop the bleeding during the assay unless the mice show excess bleeding.

3.9 After the last blood collection, stop the bleeding using septic powders, refill the feed in the cage, and make sure the mice show no signs of extreme stress.

3.10 Spin blood samples at 2,000 x g at 4 °C for 10 minutes in a refrigerated microcentrifuge. Transfer the supernatant to a PCR tube for storage. The supernatant can be stored at –20°C for several weeks until analysis.

3.11 Load 1 µL of 2x serially diluted glycerol standards (0.156, 0.312, 0.625, 1.25, and 2.5 mg/ml Trioleine-equivalent concentrations) and collected supernatants into a 96-well plate. Add 100 µL of free glycerol reagent, and let the plate incubate for 5 minutes at 37 °C for the color to develop.

3.12 Measure the absorbance at 540 nm using a laboratory plate reader, and calculate the sample's concentration.

REPRESENTATIVE RESULTS

We show with three excerpts that each assay offers valuable information about the mice's lipid metabolism. For C57BL6/J male mice, challenged by eight weeks of high-fat-diet (HFD) feeding starting at eight weeks of age, total cholesterol levels were significantly elevated, while serum triglyceride and NEFA were not (**Table 1**), suggesting that triglyceride and NEFA in the blood are not predominantly regulated by a dietary fat challenge. In the second cohort of mice, C57BL6J and C57BL6/NJ substrains of C57BL6 were fed the HFD for eight weeks, starting at eight weeks of age. Their serum triglyceride levels were compared after an oral intralipid challenge. The results demonstrated a striking difference between 6N and 6J substrains, with 6J having a significantly higher peak, indicating an enhanced absorption or a much slower triglyceride clearance (**Figure 1**). Lastly, for eight-week-old male C57BL6/J mice fed on normal chow (NC), a single CL 316,243 treatment dose (1 mg/kg bodyweight) led to a significant increase in serum glycerol. However, daily intraperitoneal treatment of mice with 1 mg/kg bodyweight CL 316,243 for one week led to a blunted reaction, suggesting the development of resistance to CL 316,263 in those mice (**Figure 2**).

FIGURE AND TABLE LEGENDS

Table 1: **Fasting lipid species in mice fed on normal chow (NC) or high-fat diet (HFD) for eight weeks.** Data are expressed as mean values ± SEM. N = 6 for NC group, n = 12 for HFD group. *P*-value was determined using 2-tail Student's *t*-test.

Figure 1: **An example of results demonstrating the difference in serum triglyceride of C57BL6 substrains after an oral intralipid challenge.** Data are expressed as mean values ± SEM. N = 5 for each group. *P*-value was determined using 2-tail Student's *t*-test at each time point. * $p < .05$, ** $p < .01$.

Figure 2: **An example of results demonstrating the development of resistance to CL 316,243 treatment in C57BL6/J mice after one week of daily CL 316,243 treatment.** Data are expressed as mean values ± SEM. N = 5 for each group. *P*-value was determined using 2-tail Student's *t*-test at each time point. ** $p < .01$.

DISCUSSION

The three assays described function robustly in the lab, with a few critical considerations. Overnight fasting is strongly recommended for determining serum lipid levels. Overnight fasting is also used for the oral intralipid tolerance test. After drawing blood from the mice, it is critical to spin it at room temperature to obtain supernatant for triglyceride measurement. At the 1- and 2-hour time points, there is likely to be a fat layer on the top; it is important not to discard this. Make sure to transfer the supernatant with the lipid layer, and pipet gently to mix them together.

Interpretation of the fasting serum lipid levels

Fasting has been shown to lower total cholesterol levels in mice[7], whereas chronically high dietary fat content usually increases total cholesterol levels[8]. There are two main types of cholesterol: high-density lipoprotein -cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C). HDL-C is regarded as the "good" lipid in humans. It carries cholesterol and transports it to the liver to be flushed out of the system. LDL-Cs make up most of the cholesterol in the human serum, and they can build up in arteries, leading to major artery diseases. However, mice lack an important enzyme, cholesteryl ester transfer protein (CETP)[9], that mediates the exchange of triglycerides for esterified cholesterol between HDL and apoB-lipoproteins[10]. This gives mice a completely different lipoprotein particle profile, with HDL being the main species. As a result, a change in total serum cholesterol levels primarily reflects changes in HDL-C levels.

In both mice and humans, high serum triglyceride levels can increase low-grade inflammation and may impair cardiac function[11,12]. However, HFD does not increase serum triglyceride levels, and genetic factors may play a dominant role in serum triglyceride levels over metabolic conditions[13]. NEFA in the blood can be avidly absorbed and utilized by many organs, suppressed by insulin and feeding, and increased by epinephrine[14]. HFD feeding does not change serum NEFA level, suggesting the hormonal cue dominates the regulation of serum NEFA levels.

Interpreting oral intralipid clearance test

An orally administered intralipid is absorbed by the intestinal epithelial cells and carried in lipoprotein particles in the bloodstream, where it is liberated and used by peripheral organs. Changes in LPL activity, peripheral-tissue triglyceride uptake, and oxidation will affect the dynamics of serum triglyceride levels. For example, brown and beige adipocytes avidly oxidize fatty acids for heat production. Cold exposure significantly increases brown and beige adipocyte activity, accelerating plasma clearance of triglycerides[15]. The oral intralipid tolerance clearance test was crucial for evaluating the effects of cold exposure on triglyceride metabolism, as demonstrated in the paper[15].

Evaluation of compounds targeting adipose tissue lipolysis

Activation of lipolysis is conveyed by the sympathetic nervous system, endocrine factors, and various metabolites. Many compounds have been put into development by pharmaceutical companies to promote adipose tissue lipolysis[16,17]. Assessing their efficacy in pre-clinical animal models such as mice is critical for facilitating the development process. Here we use a β3-adrenergic receptor agonist, CL 316,243, as an example to illustrate how we can assess how a mouse responds to the compound and whether there are any differences in its sensitivity to the compound in different metabolic states. As seen in the exemplary results, repeated use of CL 316,243 caused increased tolerance in the mice. We used CL 316,243 to illustrate how we could assess a mouse's response to acute treatment; more importantly, this concept and design can be easily applied to other molecules targeting adipose tissue lipid metabolism.

Limitations

A few selected lipid species offer limited information about lipid metabolism in mice. Due to the small amount of serum available from tail bleeding, this protocol measures only total cholesterol and does not distinguish HDL-C and LDL-C, as those assays require significant amounts of blood. Because mice are unique in the way they lack the CETP, cholesterol is a good approximation, and more HDL-C in mice does not indicate a healthy lipid profile, so the additional information obtained by distinguishing the cholesterol in different lipoprotein particles is limited.

Serum lipid levels, including triglyceride levels, are usually a net effect of absorption and excursion by many organs acting in a very dynamic way. Interpreting the results usually requires an experimental setup with only one variable. As shown in the exemplary result, no specific conclusion regarding the lipid absorption or excursion can be made between C57BL6J and C57BL6/NJ substrains of C57BL6 mice. However, in the cited cold exposure study[15], prior knowledge and sometimes assumptions can be used to exclude contributions from other variables, and authors were able to pin down to a specific tissue and discovered that brown adipose tissue contributed to the enhanced triglyceride clearance.

Lastly, metabolism is a dynamic process. The change of one metabolite in a lipid metabolic pathway provides only a snapshot of the overall state. To understand the flow, a more sophisticated flux study using isotope-tracing techniques is required.

In summary, the simplicity is both the power and weakness of this protocol. The three assays presented here are not designed for the study of specific lipid metabolism pathways, but rather

to provide an initial screening or a starting point for evaluating lipid metabolism in general nutrition and obesity research.

ACKNOWLEDGMENTS

This work is supported by the National Institutes of Health (NIH), grant R00-DK114498, and the United States Department of Agriculture (USDA), grant CRIS: 3092-51000-062 to Y. Z.

DISCLOSURES:

The authors have nothing to disclose.

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Figure_1

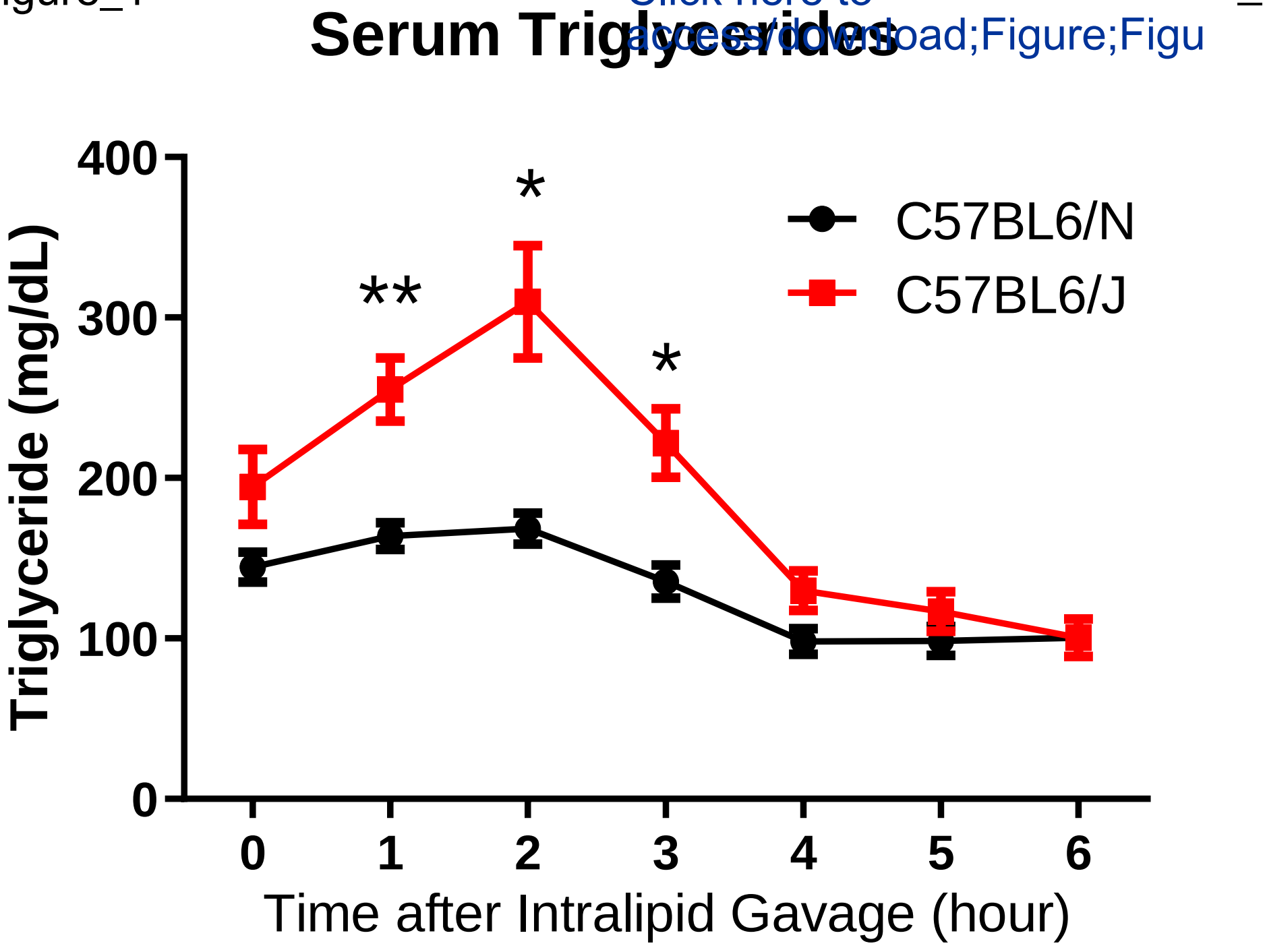

Figure_2

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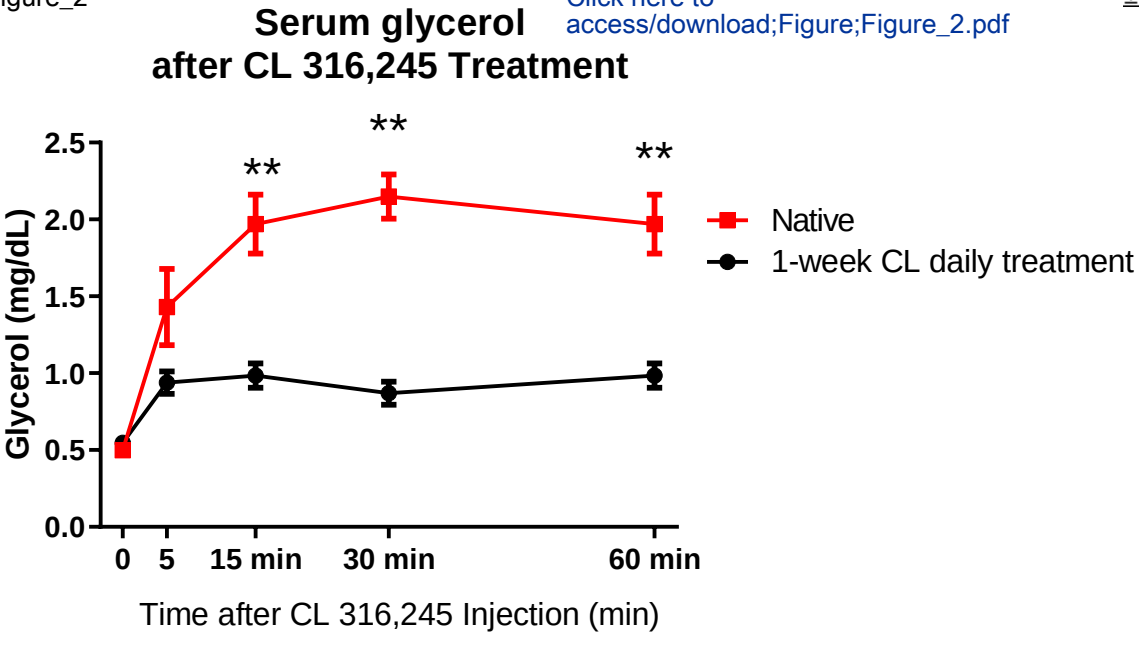

Table_1

Serum Parameters	NC	HFD	*P* Value
Cholesterol (CHOL) (mg/dL)	132.7±10.3	202.3±8.4	0.0002
Triglyceride (TG) (mg/dL)	91.7±9.1	79.3±4.5	0.26
Non esterified fatty acids (NEFA) (mmol/L)	1.47±0.12	1.48±0.08	0.73

Table of Materials

Click here to access/download;Table of Materials;Table of Materials.xlsx

Materials	Vendor	Catalog Number
20% Intralipid	Sigma Aldrich	I141
BD Slip Tip Sterile Syringes 1ml	Shaotong	B07F1KRMYN
CL 316,243 Hydrate	Sigma-Aldrich	C5976
Curved Feeding Needles (18 Gauge)	Kent Scientific	FNC-18-2-2
Free Glycerol Reagent	Sigma Aldrich	F6428
Glycerol Standard Solution	Sigma	G7793
HR SERIES NEFA-HR(2)COLOR REAGENT A	Fujifilm Wako Diagnostics	999-34691
HR SERIES NEFA-HR(2)COLOR REAGENT B	Fujifilm Wako Diagnostics	991-34891
HR SERIES NEFA-HR(2)SOLVENT A	Fujifilm Wako Diagnostics	995-34791
HR SERIES NEFA-HR(2)SOLVENT B	Fujifilm Wako Diagnostics	993-35191
Ketamine	Vedco	50989-161-06
Matrix Plus Chemistry Reference Kit	Verichem	9500
Micro Centrifuge Tubes	Fisher Scientific	14-222-168
Microhematrocrit Capillary Tube, Not Heparanized	Fisher Scientific	22-362-574
NEFA STANDARD SOLUTION	Fujifilm Wako Diagnostics	276-76491
Phosphate Buffered Saline	Boston Bioproducts	BM-220
Thermo Scientific Triglycerides Reagent	Fisher Scientific	TR22421
Total Cholesterol Reagents	Thermo Scientifi	TR13421
Xylazine	Henry Schein	11695-4022-1

We thank the editor and reviewers for your constructive feedback on our manuscript. We have revised our manuscript accordingly, and below, we provide a point-by-point response to all the comments.

Overall changes to the protocol:

Assay 1: Measuring of fasting serum lipids

To stress this protocol's accessibility and to serve as an initial screen, we changed the lipid measurement methods to commercial kits. We also removed HDL-C and LDL-C measurement since it requires 100 µl of serum and will force this experiment to be terminal.

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues and ensure the use of American English. Also, please define all abbreviations at first use.

Response: We have revised and proofread the manuscript to ensure it complies with the JoVE publishing guidelines.

2. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

For example: VITROS® 5600 in the introduction, 20% Intralipid (Sigma, I141), glass capillary (Fisher, Cat # 22-362574) etc

Response: We have revised the manuscript accordingly.

3. Please revise the Introduction to include all of the following:

a) The advantages over alternative techniques with applicable references to previous studies

Response: We have emphasized the accessibility of the collection of assays we present in this paper over traditional lipidomics or isotope tracing studies, which usually requires specialized equipment or expertise that are not widely accessible to general labs. Our methods hereby serve as initial tests before the use of more technically challenging or informative techniques.

4. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes.

Response: We have adjusted the numbering accordingly.

5. Please use µL instead of ul throughout the manuscript.

Response: We have edited the manuscript accordingly.

6. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g. button clicks for software actions, numerical

values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.
Response: We have substantiated the protocols so that they can be easily replicated. We focused on the mouse experiment part rather than assaying specific lipid molecules using a commercial kit as different labs may be equipped with different equipment for absorbance reading.

1.1: What is the age/gender/strain of the mouse used?
Response: We updated this information in *Representative Results*. The protocol is deemed suitable for mice of any gender at any age.

1.2: Is the mouse immobilized for the bleeding? Is any anesthesia used? What happens after centrifugation? Transfer the serum?
Response: Details have been updated to the manuscript.

Please specify a step or a citation for the serum analysis, the triglyceride measurement, and the glycerol/NEFA analysis. If this step is to be filmed, we need explicit details for the scripting.
Response: These measurements will be carried out using the manufacturer's protocol. We have incorporated triglyceride measurement for assay 2 and glycerol measurement for assay 3.
What happens to the mouse after? Please expand the animal treatment details during and after the experiment.
Response: Mice will be returned to the cage. This detail has been updated in the manuscript.

7. Being a video based journal, JoVE authors must be very specific when it comes to the humane treatment of animals. Regarding animal treatment in the protocol, please add the following information to the text:
Please specify the euthanasia method.
Please do not highlight any steps describing euthanasia.
Response: There is no euthanasia of mice in the updated protocol.

8. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Any limitations of the technique
Response: We have added a section to discuss the limitation of the protocols presented at the end of the paper.

9. Please write journal titles fully in the reference list.

Response: We used the endnote style file directly downloaded from the JoVE website. Could the editorial office send us an updated endnote style file?

10. Please rename Table 2 as Table 1 and Table 1 as the Table of Materials. Please sort the

Materials Table alphabetically by the name of the material.
Response: We have reorganized the Materials Table and revised the manuscript accordingly.

_____

Reviewers' comments:

Reviewer #1:

The manuscript submitted by Mathew and colleagues entitled "Assessing Whole-body Lipid Handling Capacity in Rodents" aims to provide accessible protocols for testing aspects of lipid metabolism in mice. Three experimental tests (1. Fasting circulating lipid concentrations, 2. Oral intralipid tolerance test, 3. β3 adrenergic receptor agonist CL 316,243-stimulated lipolysis assay) are recommended. In addition, anticipated results and data interpretation, critical steps within the protocols, and lipid handling physiology are briefly discussed. Concerns are:

Title:

1. The authors use rodents in the title. However, the manuscript data and discussion only refer to mice. This is important as the metabolism of mice differs from other rodents and the specifics of the experimental protocols may not always be directly transferred to other rodents. Similarly, the manuscript often refers to "research subjects". This may not be the most appropriate term for studies involving mice.

Response: we have changed the title to “Assessing Whole-body Lipid Handling Capacity in Mice” to correctly reflect the subject we worked on and discussed in the manuscript. Other unspecific words such as “research subjects” have been replaced with “mice.”

Abstract:

1. The authors state: "In contrast to using glucose or hemoglobin A1c to assess glucose metabolism, assessment of lipid metabolism lacks one or two simple parameters,…". The manuscript then goes on to describe more than one or two tests. Perhaps, it is more appropriate to not focus on the number of tests but the accessibility of these tests for researchers without specialized equipment or expertise.

Response: We have revised the manuscript to focus on the accessibility of these tests.

Introduction:

1. The authors state: "They offer valuable insight into the mechanism on how lipids are absorbed, synthesized, and metabolized due to the similarity of their genome and physiology to the human's". This statement is bold. The metabolism of mice is quite different than that of humans. The power of mice as a model for metabolic disease research is the ability to manipulate the genome for experimental purposes and the relatively inexpensive cost of husbandry.

Response: We thank the reviewer for the review and constructive critiques. We have revised the text accordingly.

2. The authors state: "In this paper, we will present three simple assays that offer an overall view of a subject's lipid metabolism". Indeed, for most metabolic research laboratories, these metabolic tests are routine or "simple". For, those new to metabolic research or mouse work, these tests may be technically challenging. Also, access to chemical analyzers may be limiting. Perhaps, it is more appropriate to indicate that these tests are initial tests in the algorithm of lipid metabolism testing. They are useful in identifying potential differences in lipid handling and help narrow down the processes impacted. They should be used prior to more technically challenging or informative techniques such as the use isotopic tracers to quantify lipid-related metabolic fluxes and fates.

Response: We thank the reviewer for the suggestion. We revised our protocol to adopt the commercially available colorimetric kits instead of chemical analyzers and incorporated the comments into the manuscript.

3. The authors appropriately provide a brief background on lipid metabolism related to each test. However, given that the information provided is a brief summary, it is recommended that references be included so that the reader can readily access more detailed information on the processes discussed regarding lipid metabolism.

Response: Most of the basics for lipid metabolism were discoveries decades ago, if not centuries ago. We searched many pieces of literature; none of them can provide a comprehensive summary of the background. But instead, a google search can lead to specific websites, paid databases, or Wikipedia pages that usually offer better-curated information. So we decide to leave readers to perform an internet search.

Protocol:

1. For tests 1 and 2, the authors recommend an overnight fast. An overnight fast is particularly challenging for mice. Please specify why this is recommended/advantageous for the experiment aims. Also, what does overnight refer to? (i.e. a 12-, 18-, 24-hour fast?).

Response: The argument to advocate for a shorter fasting period in mice is due to their high metabolic rate, an overnight fast results in a “starvation”, rather than a “fasting” state. However, in spite of a high metabolic rate, the digestive system of a mouse is comparatively slow, a fact usually under-appreciated. 4 hours of fasting for mice is considered not long enough to allow the stomach to be fully emptied.

2. For test 1 (Measuring of fasting serum lipids), the authors indicate that blood sampling via cardiac puncture is recommended. What volume of blood do you recommend to complete all the lipid metabolite measurements referred to within the protocol? Is 15 µl enough for one lipid metabolite? Does this include multiple measurements (i.e. duplicate or triplicate)? What anesthesia (and dose) are recommended? Can this anesthesia method affect lipid

measurements? Why recommend cardiac puncture over tail bleed or other sampling sites for sample acquisition?

Response: To focus on the tests' accessibility, we no longer propose using the *Vitros* chemical analyzer to analyze the serum lipid levels. Instead, commercial kits will be used. HDL and VLDL measurements need 100 µl of serum, so a terminal bleeding method is required. Since these assays will serve as initial screens, we want to present them as non-terminal procedures, so HDL and VLDL measurements have since been removed from this protocol.

3. For test 1 (Measuring of fasting serum lipids), the authors state: "Carefully transfer the serum for analysis with a laboratory chemical analyzer". For those without a chemical analyzer, how should the samples be stored until use in one of the recommended kits?

Response: Serum samples can be stored at -20 °C for several weeks until analysis.

4. The authors refer to test 2 as the triglyceride clearance test. While differences in clearance may impact the lipid excursion measured over the 6-hour period, it is not the only factor. As previously mentioned in the manuscript, these other factors include absorption, turnover, hormonal response. It is recommended that authors use a different name to refer to this test. Perhaps, oral intralipid tolerance test is appropriate?

Response: We have changed the name of the second test to the “oral intralipid tolerance test.”

5. What is the sampling site for test 2 (triglyceride clearance test)?

Response: The sampling site for test 2 is the tail. We have updated this information in our protocol.

6. It is unclear what an EP tube is. Are there specific tubes recommended for collecting storing serum and/or plasma? Are there advantages/disadvantages to serum or plasma for these measurements?

Response: EP tubes refer to Eppendorf Tubes, a commonly used microcentrifuge tube. There is nothing special about it. We changed EP tubes in the protocol to microcentrifuge tubes.

7. In the abstract, the authors state: "assessment of lipid metabolism lacks one or two simple parameters, and usually involves assessment of several lipid species in different metabolic states, or after some forms of challenge". It is somewhat confusing as to why the authors then recommend to perform test 3 in a 4-hour fasted mouse. Isn't this a different metabolic state compared to the other two tests which are recommended to be performed in the overnight fasted mouse? Why do the authors recommend a 4-hour fast for this test?

Response: The comparison is always intended between groups for a specific assay; there is no intention to perform cross-comparison between assays. The particular reason to perform assay 3 in a 4-hour fasted mouse is that: 1). Overnight fasting will fully deplete glycogen and strongly activates lipolysis, which may not be furthered elevated by CL 316,243; 2). Shortly after a meal, there will be a surge in blood insulin, which is a potent inhibitor for adipose tissue lipolysis. Mice usually have random bouts of feeding during the day, and as a result, insulin levels vary during the day without fasting.

8. Does the intralipid or CL 316,243 solution dosing change if experimental groups differ in body weight or adiposity?

Response: We do not have enough data to support differential dosing for obese mice. The response to CL316,243 reduces when mice age and become obese, but the difference is never as dramatic as we showed after repeated CL316,243 dosing.

9. What glycerol assay is recommended. It does not appear to be provided in Table 1.

Response: Free Glycerol Reagent from sigma Aldrich will be used, the catalog has been updated to the Table for Materials.

10. Is 10 ul of blood enough for both the NEFA and glycerol assay?

Response: Yes, each assay only needs 1-3 µl of serum.

Discussion:
1. The authors state: "For example, mice from C57BL6 and FVB background behave significantly differently to metabolic challenges". While a description of the metabolic differences between mouse strains is beyond the scope of this manuscript, is there a reference(s) for reader to access more details.

Response: We have dropped this discussion in the revised manuscript.

2. A whole paragraph is devoted to circulating triglyceride clearance. As previously discussed, the oral intralipid delivery experiment and resulting lipid excursion profile is influenced by multiple processes. Broadening the paragraph to include these other factors may be more appropriate.

Response: As for most physiological experiments, many factors contribute to the experiment's outcome. However, the experiment can be set up with only one variable, or in a way that the experimenter can use prior knowledge to exclude other variables' contributions. We have revised the manuscript to include this statement.

3. Given the reduced glycerol excursion following multiple CL 316,243 administrations, do the

authors recommend mice only undergo this test a single time rather than longitudinal studies within the same mouse employing this method?

Response: The authors do not recommend performing this test repeatedly within a short period. However, if needed, a two-week recovery time will suffice based on our experience.

Figures:
1. Intralipid is misspelled in Figure 1

Response: We have corrected the spelling.

Reviewer #2:
Manuscript Summary:
The paper shows the protocol of lipid metabolism assessment in mice such as serum lipids analysis, oral fat load test, and pharmacological lipolysis assay. The authors describe step-by-step procedures including blood sampling, serum separation, oral gavage, intraperitoneal administration, and lipid assay using commercial kits.

Major Concerns:
Introduction (page 2, line 1)
The authors mention the similarity in mouse and human. But, as the authors described in the discussion, there are many different features of lipid metabolism in mouse and human. The sentence should be modified.

Response: We have removed the text mentioning the similarity between mouse and human. We have since focused on their differences and how that would affect the interpretation of the data.

Introduction (page 2, line 11)
Friedwald's equation has been defined to estimate human LDL-C. Do you have evidence that the equation can be applied to mice with different plasma lipoprotein compositions or different lipid composition of lipoproteins compared with human? The negative value of LDL-C has been shown in your experiment.

Response: We have seen many papers using Friedewald's equation for mice, but its validity has not been well-documented. LDL-C levels in mice usually are not informative, as we have discussed in the paper that because mice lack the CETP enzyme. We have modified the protocol to drop the HDL-C measurement as a). it requires a large quantity of serum; 2). Its levels are close to total serum cholesterol levels, as mice have very low levels of other types of cholesterol. As a result, Friedewald's equation is no longer used in the revised manuscript.

Introduction (page 2, lines 11-12)
"LDL" should be "LDL-cholesterol (LDL-C)". "HDL" should be "HDL-cholesterol (HDL-C)".

Response: Thank you for pointing this out. We have corrected all spellings in our paper.

Protocol 1.1 (page 3, line 2)
Please defined the fasting duration time.

Response: We have updated this information in the protocol.

Protocol 2.3 (page 3, lines 13-15)
Blood clotting time should be defined.

Response: We have updated this information in the protocol.

Representative result (page 4, line 4) and Fig 2
The text, NEFA, is inconsistent with Fig 2, glycerol.
Response: We have revised the manuscript accordingly.

Minor Concerns:
Please add SD or SE in Table 2, Fig 1, and Fig 2 legends.

Response: We have revised the manuscript accordingly.

Reference 12 seems incomplete.

Response: The original reference is a book chapter, and the Endnote software cannot handle it well. We replaced this reference with a recent review.