Dear Dr. Rossow,  
  
Your manuscript, JoVE58387 Measuring liver mitochondrial oxygen consumption and proton leak kinetics to estimate mitochondrial respiration in Holstein dairy cattle, has been editorially and peer reviewed, and the following comments need to be addressed. Note that editorial comments address both requirements for video production and formatting of the article for publication. Please track the changes within the manuscript to identify all of the edits.  
  
After revising and uploading your submission, please also upload a separate rebuttal document that addresses each of the editorial and peer review comments individually. Please submit each figure as a vector image file to ensure high resolution throughout production: (.svg, .eps, .ai). If submitting as a .tif or .psd, please ensure that the image is 1920 pixels x 1080 pixels or 300 dpi.  
  
To submit a revision, go to the [JoVE submission site](http://www.editorialmanager.com/jove) and log in as an author. You will find your submission under the heading "Submission Needing Revision".  
  
**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. **DONE**  
2. Please revise lines 289-291, 305-309, 322-326, 330-332 to avoid previously published text. **DONE**  
3. Please obtain explicit copyright permission to reuse any figures/Tables from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account.

Figures 1-13 have never been published

Figure 14 is raw data from the Oxygraph software and has never been published

Figure 15 has never been published (I can provide the paper if you need proof- (Acetoze, G., J. Champagne, J.J. Ramsey, **H.A. Rossow**. 2017. Liver mitochondrial oxygen consumption and efficiency of milk production in lactating Holstein cows supplemented with Copper, Manganese and Zinc. J Anim Physiol Anim Nutr (Berl), DOI: 10.1111/jpn.12836)

Figure 16 was published in Acetoze G, K. L. Weber, J. J. Ramsey, **H. A. Rossow**. 2015. Relationship between liver mitochondrial respiration and proton leak kinetics in low and high RFI steers from two lineages of RFI Angus bulls. ISRN Vet Sci 194014 <http://dx.doi.org/10.1155/2015/194014>. The axis were inverted from the copy that I submitted. This journa l is open access and copyright statement is at https://www.hindawi.com/copyright/

Table 1 is a combination of tables 3 and 4 in the publication Acetoze, G., J. Champagne, J.J. Ramsey, **H.A. Rossow**. 2017. Liver mitochondrial oxygen consumption and efficiency of milk production in lactating Holstein cows supplemented with Copper, Manganese and Zinc. J Anim Physiol Anim Nutr (Berl), DOI: 10.1111/jpn.12836). Copyright statement is at https://onlinelibrary.wiley.com/page/journal/14390396/homepage/permissions.html

go down the page to **AUTHORS**

Table 2 is from the same journal article listed for Figure 16. This journal is open access and copyright statement is at https://www.hindawi.com/copyright/

4. Please upload each Table individually to your Editorial Manager account as an .xls or .xlsx file. **DONE**  
5. Figure 15: Please label y-axis and provide both numbers and units. **DONE**  
6. Figure 16: The uploaded figure is improperly cut off. Please revise. Please label y-axis and provide both numbers and units. **DONE**  
7. Please rephrase the Introduction to include a clear statement of the overall goal of this method. **DONE**  
8. Please use SI abbreviations for all units: L, mL, µL, µM, h, min, s, etc. **DONE**  
9. Please include a space between all numbers and their corresponding units: 15 mL, 37 °C, 60 s; etc. **DONE**  
10. Please place the ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution. **DONE (line 127)**  
11. 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. **DONE**  
For example: Sontech®, Oxygraph system, Hansatech Oxygraph System, etc.  
12. 2.1: Please specify the isolation media used here.**DONE**  
13. 2.4: What happens to the pellet after the first centrifuge step? **DONE**  
14. 2.8: Please add more details to this step. This step does not have enough detail to replicate as currently written**. There are several methods to measure protein concentration in a solution (Bradford assay, Lowry method, etc.) We now use a kit based on BCA protein assay and so I referenced the kit instead of going into detail on how to run the Lowry assay. I also added the kit to the materials list.**15. 3.10, 4.10, 4.11: Please write the text in the imperative tense. **DONE**  
16. Line 347: Should it be Figure 16 instead of Figure 2? Figure 2 shows restraint of the cow using a halter tied to a cross pole of the head lock. Yes, thanks **DONE**  
17. References: Please do not abbreviate journal titles. Please include volume and issue numbers for all references. **DONE**  
  
**Reviewers' comments:**  
**Reviewer #1:**  
Manuscript Summary:  
Procedures are described to perform liver biopsies and measure liver mitochondrial oxygen consumption and proton leak kinetics to estimate mitochondrial respiration in cattle. The procedures are clearly described with helpful pictures.  
  
Major Concerns:  
None  
  
Minor Concerns:  
The manuscript is generally well-written but requires careful editing. Be certain to use metric units (e.g., kg rather than lb) and proper abbreviations (e.g., mL rather than mls, cm for centimeter, h for hour, etc.) throughout the manuscript. **DONE**  
  
Page 7, line 340 - It is unclear to which study you are referring when you say "in the present study". **DONE**  
  
Page 7, lines 341, 342 - Please check to be certain the following statements are correct. The two statements appear to be contradictory. "hepatic mitochondrial proton leak kinetics were greatest in Control and lowest in LowMn which agrees with results in Table 1, that proton leak was greatest in LowMn and lowest in Control". Corrected - thanks **DONE**  
  
  
**Reviewer #2:**   
The manuscript contained valuable information, however, needs to be reconstructed for a complete full text. I make some points for consideration. **I made changes that were suggested but did not see any other area that needed additional references. Other figures are pictures and example output from the oxygraph system. and do not include text. Figure titles are short sentences to match the portion of the protocol they represent. I am happy to alter these but I think this format is following the journal instructions.**

1) The key concerns of the manuscript lack scientific evidences or supports. For example, (L.30) "These resuls can account for 30% of energy lost...". (L.73) "Up to 30% of glucose is supplied by amino acids in early lactation". A r**eference was added and I edited the text around line 73.**

2) A number of incomplete sentences and grammatical errors in figures that need to be worked on to improve the flow of the manuscript. For example, figure 15 lacks units for horizontal and vertical axis, and the units for oxygen consumption should be nmol O2/mg protein/min. **These were cut off from the original in converting the images of the graphs to pdf format. This has been corrected in the eps format**  
  
**Reviewer #3:**  
Manuscript Summary:  
The paper describes a technique for harvesting mitochondria from the liver of cattle and assessing their function.  
  
General Concerns:  
The grammar needs to be fixed to be sure that the meaning of sentences matches with their intent. Very often these do not appear to match. The word "therefore" is used too much and sometimes inappropriately. There are places in the manuscript where the phrase preceding "therefore" does not contain an explanation or reason for the phrase after "therefore". **Deleted most therefores**  
  
Specific Concerns:  
l. 33 This is not an abstract; it is an introduction, and a very good one because it introduces some important concepts regarding membrane potentials and respiration states. An abstract summarizing the paper should be provided.

**The instructions do not call for a typical abstract. The instructions require a purpose statement for the method (line 39- 41), a detailed over view of the method (Lines 34-39 ), a brief summary of the methods (lines 46 - 49) advantages, limitations and applications (lines 49-54) and general types of results acquired (lines 41-46).**

l. 57-64 This paragraph is not needed.

**This paragraph explains why you would want to use this method and contains a purpose statement which was required by the editor**

l. 66 Indicate "hepatic" mitochondrial dysfunction. **DONE**  
l. 68 Suggest replacing "negative energy balance" with "occurs". **DONE**

l. 74 Add reference about amino acid use in gluconeogenesis. **Removed**   
l. 75 "… will impact fuel availability" to the periphery "and be…" **DONE**  
l. 77 Was it seven day-old broilers or seven-day-old broilers? **DONE**  
l. 84 The Wallace and Fan (2010) reference does not discuss proton leak in relation to protein and calcium import or heat generation during inflammation.

**Edited references and for calcium import see wallace and Fan section 2.4 and section on BDNF**

l. 90-91 Suggest moving "such as Cu, Zn and Mn" to after "high levels of supplements" **DONE**  
l. 93-94 Sentence not necessary. Should the conclusion be that Cu, Zn and Mn do not improve mitochondrial function as expected?

**No, the conclusion was that feeding high levels of Cu,Zn and Mn did not improve mitochondrial function . Deleted sentence**

l. 97-98 Remove "to examine differences in feed efficiency"**DONE**  
l. 101 Were the beef cows also lactating? **Yes (see edits)**  
l. 108-109 Remove "with higher intakes" **DONE**  
l. 109-114 Suggest summarizing more succinctly. Instead of listing individual results in this paragraph, go straight to the main conclusions of all these studies. **DONE**  
l. 115 Add reference after "milk yield". Remove rest of sentence because causation cannot be concluded.

**Deleted due to previous comment**

l. 120 Fix the wording referring to RCR

**Not sure what you mean but deleted RCR**

l. 120 Remove "to examine efficiency and the impact of mitochondrial function on milk production in dairy cattle" **DONE**  
l. 122 Somewhere in the paper, maybe here at the end of the introduction, reference should be made to previously published liver biopsy methods

**Other papers and videos describing liver biopsy methods for mitochondria experiments refer to methods in beef cattle. The dairy cattle method we used is a little different as the liver is located in a different place. see NOTE line 161**

l. 125 Replace "have been" with "were" **DONE**  
l. 127 Why should the procedure be performed by a licensed veterinarian? Many, if not most, liver biopsies on cattle are not performed by licensed veterinarians.

**Notice that we said 'should'. Most IACUC committees and dairy producers that I am aware of would want the biopsy performed by a veterinarian especially considering that is a research experiment and biopsies were performed on cows in a commercial dairy at a commercial dairy.**

l. 127-128 Suggest "on the site where cows are housed" instead of "on the dairy site where the cows are located"

**This is important because the liver biopsies were performed at the commercial dairy site in the pen and cows were not moved to a hospital, sterile site or even to a squeeze chute for the procedure.**

l. 128 Replace "milked" with "subjected to their normal milking routine" **changed, but the word 'subjected' has negative connotations.**

l. 129 Withdrawal depends on the antibiotic used and the country in which the regulations for that antibiotic are set. Yes.

**Given the drugs that are used in this protocol, US labels on drugs allow cow's milk to still be in the food supply.**

l. 131 Phrase the number 4 as a recommendation. **DONE**  
l. 138 "clostrida" spelling **DONE**  
l. 142 Give Ceftiofur dose in SI units **DONE**  
l. 146 Replace "from" with "containing". How much media should be prepared per biopsy sample? **DONE**  
l. 154 Remove second "to be biopsied"**DONE**  
l. 160 Replace "skin/hide and" with "skin and muscle but" **DONE**  
l. 168-171 Move this note to l. 174 after checking incision for redness, swelling, etc. **DONE**  
l. 169 Replace "painful to the" with "or reaction to" **DONE**  
l. 175 SI units **DONE**  
l. 181 In this section, it would be really helpful if the additions to the chamber were given in volume units of a concentration to be prepared beforehand. Something like, "add 10 uL of 5 M succinate" instead of "add succinate to a final concentration of 5 mM". All these rotenone, succinate, oligomycin, nigericin,. etc. solutions that should be prepared beforehand could be given around paragraph 1.3. If they are to be prepared fresh on the day of biopsy, inform the user. If they are to be prepared fresh for every sample coming in, also inform the user.

**This information is included in the materials spreadsheet that lists all of the chemicals, solutions, and manufacturers of chemicals and equipment. Added amounts of chemicals to add to the manuscript**

l. 210 Should OCM be prepared beforehand and listed after paragraph 1.3? How much should be prepared per biopsy sample? Replace "from" with "containing".

**This information is included in the materials spreadsheet that lists all of the chemicals, solutions, and manufacturers of chemicals and equipment**.

l. 214 Give manufacturer name and address. The oxygraph system, because it is an integral part of the procedure being described, should be introduced around line 120.

**This information is given in the materials spreadsheet and the instructions to authors state not to put that information in the protocol**

l. 223 This is a final concentration of 5 uM or addition of 5 uM? Either way, the volume of rotenone solution to be added should be given.  **DONE**  
l. 226 This set of phrases is repeated throughout: "Record respiration for about 5 min (approximately). When oxygen consumption becomes constant, record oxygen consumption." I get the feeling the 2 sentences are not consecutive instructions but are repetitive (although I'm not sure). Respiration and oxygen consumption are the same thing? So what the user should do is record oxygen concentration long enough to get at least 3 min of a linear decrease? How is linear defined? By eye? Algorithmically by Oxygraph software? These seem like pretty important instructions to be left vague.

**Linear is defined by eye looking at the oxygraph system output real time - Manuscript edited.**

l. 232 What is meant by "the respiration rate should increase"? The previous sentence says it should be constant.

**Explained in manuscript**

l. 242 Replace "by" with "as" **DELETED**  
l. 250-254 Provide more details such as: how do you make sure that each addition is 1 ul or less? what is the limit on ethanol inclusion?

**Concentrations added. Basically, we need to keep the volume of ethanol low so it doesn’t disrupt the mitochondria membrane. Since all these solutions are ethanol based, each reagent should be added at volumes of 1µL or less.**

l. 259 Remove "a outlet on" **DONE**  
l. 276 The PMF is composed of a pH gradient and a membrane potential (MMP). As an H+/K+ ionophore, nigericin abolishes the pH gradient in a KCl-based medium so that PMF = MMP. I suggest either replacing "MMP to mV" with "PMF to MMP" or replacing "pH component of MMP to mV allowing PMF to be measured" with "transmembrane H+ gradient to a K+ gradient that can be measured with an electrode". **DONE**  
l. 289-291 This should be given in the intro as suggested for l. 214. **See comments above (DONE)**  
l. 297 Use of this equation to interpret Fig. 14 should be shown in the results section.

**There is not enough information in Figure 14 to supply all the numbers for the calculation. So, we have added an example to the protocol.**

l. 302-303 Insert "reported as" after "PMF is" and delete "common" **DONE**  
l. 311 Last run of the day for each sample or after all samples have been run? **DONE**  
l. 319/347 Not in favor of describing users' feelings about the results with adjectives "positive" and "negative**". Positive and Negative do not refer to feelings. They refer to whether RCR or proton leak changed with treatment or not.**   
l. 321 "… had been fed" 1 of "5 different …"**DONE**  
l. 329 Remove "due to decreased energy efficiency due to increased proton leak" because the cause of the lower milk and milk protein yields was not shown. **DONE**  
l. 358 Is it necessary to show results of an equipment malfunction in a paper describing a technique? **YES, That is part of what is included in paper instructions**  
1. 424-425 Worded as if samples complete the analyses. **DONE**  
l. 427 Replace "are" with "is" **DONE**  
l. 428 "… with results and" the ability to detect "significance." **DONE**  
l. 435 Enzyme activity and amount are synonymous. The difference between these two references is in which enzyme was used as a marker. **DONE**  
l. 442 What constitutes a lot of fatty acid? A concentration threshold should be given. **DONE**  
l. 448 Liver is chosen when liver function is specifically of interest, not as an indicator of general mitochondrial function in an animal. This misconception that hepatic mitochondrial function says something about mitochondrial function in other tissues permeates throughout the manuscript and the authors need to make sure it is not implied. **We do not mean to imply that liver indicates general mitochondrial function in other tissues. This method is specific for liver and our biopsy methods and statements regarding nutrient processing are specific to cattle. Liver, in ruminants, is the primary site of nutrient processing particularly in regards to propionate and acetate from rumen fermentation and that is why we focus on liver. This has important health consequences for ruminants and dairy cattle in particular. This paper has stated that this method is for liver tissue only and if you think it is implied anywhere else, I will be happy to change it.**l. 454 Replace "are also" with "is". **DONE**  
l. 475 "… through the Center" for "Food Animal…" **DONE**