Journal of Visualized Experiments

Measuring liver mitochondrial oxygen consumption and proton leak kinetics to estimate mitochondrial respiration in Holstein dairy cattle

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE58387R2
Full Title:	Measuring liver mitochondrial oxygen consumption and proton leak kinetics to estimate mitochondrial respiration in Holstein dairy cattle
Keywords:	Dairy cow liver biopsy, Liver mitochondria isolation, Mitochondrial oxygen consumption, Mitochondrial membrane potential, Mitochondrial protein leak kinetics, Respiratory control ratio, State 3 respiration, State 4 respiration, proton motive force
Corresponding Author:	Heidi Rossow University of California Davis School of Veterinary Medicine Tulare, CA UNITED STATES
Corresponding Author's Institution:	University of California Davis School of Veterinary Medicine
Corresponding Author E-Mail:	heidi.rossow@gmail.com
Order of Authors:	Heidi Rossow
	Gabriela Acetoze
	John Champagne
	Jon J Ramsey
Additional Information:	
Question	**Response**
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TITLE:

Measuring Liver Mitochondrial Oxygen Consumption and Proton Leak Kinetics to Estimate Mitochondrial Respiration in Holstein Dairy Cattle

AUTHORS & AFFILIATIONS:

Heidi A. Rossow[1], Gabriela Acetoze[2], John Champagne[1], Jon J. Ramsey[3]

[1]Veterinary Medicine Teaching and Research Center, University of California Davis, Tulare CA
[2]Archer Daniels Midland, Fresno, CA
[3]Department of Molecular Biosciences, University of California Davis, CA

Corresponding Author:

Heidi A. Rossow (Heidi.Rossow@gmail.com)
Tel: 559-688-1731 x230

Email Addresses of Co-Authors:

Gabriela Acetoze (gabriela.acetoze@adm.com)
John Champagne (jchampagne@vmtrc.ucdavis.edu)
Jon J. Ramsey (jjramsey@ucdavis.edu)

KEYWORDS:

Dairy cow liver biopsy, Liver mitochondria isolation, Mitochondrial oxygen consumption, Mitochondrial membrane potential, Mitochondrial protein leak kinetics, proton motive force, Respiratory control ratio, State 3 respiration, State 4 respiration

SUMMARY:

Here, we share methods for measuring mitochondrial oxygen consumption, a defining concept of nutritional energetics, and proton leak, the primary cause of inefficiency in mitochondrial generation of ATP. These results can account for 30% of the energy lost in nutrient utilization to help evaluate mitochondrial function.

ABSTRACT:

Oxygen consumption, proton motive force (PMF) and proton leak are measurements of mitochondrial respiration, or how well mitochondria are able to convert NADH and FADH into ATP. Since mitochondria are also the primary site for oxygen use and nutrient oxidation to carbon dioxide and water, how efficiently they use oxygen and produce ATP directly relates to the efficiency of nutrient metabolism, nutrient requirements of the animal, and health of the animal. The purpose of this method is to examine mitochondrial respiration, which can be used to examine the effects of different drugs, diets and environmental effects on mitochondrial metabolism. Results include oxygen consumption measured as proton dependent respiration (State 3) and proton leak dependent respiration (State 4). The ratio of State 3 / State 4 respiration is defined as respiratory control ratio (RCR) and can represent mitochondrial energetic efficiency. Mitochondrial proton leak is a process that allows dissipation of mitochondrial membrane potential (MMP) by uncoupling oxidative phosphorylation from ADP decreasing the efficiency of

ATP synthesis. Oxygen and TRMP+ sensitive electrodes with mitochondrial substrates and electron transport chain inhibitors are used to measure State 3 and State 4 respiration, mitochondrial membrane PMF (or the potential to produce ATP) and proton leak. Limitations to this method are that liver tissue must be as fresh as possible and all biopsies and assays must be performed in less than 10 h. This limits the number of samples that can be collected and processed by a single person in a day to approximately 5. However, only 1 g of liver tissue is needed, so in large animals, such as dairy cattle, the amount of sample needed is small relative to liver size and there is little recovery time needed.

INTRODUCTION:

Mitochondria are very sensitive to stress and their cellular environment can contribute to a wide variety of metabolic diseases. Oxygen consumption and proton leak in mitochondria are indicators of mitochondria health. The methods described in this paper estimate mitochondrial energy efficiency using RCR based on oxygen consumption with and without proton leak. These results can account for 30% of the energy lost in nutrient utilization[1]. Changes in oxygen consumption and proton leak can identify mitochondrial dysfunction which contributes to metabolic disease and results in decreased energy efficiency. These methods can also be used to examine the effect of different treatments on mitochondrial respiration. The overall goal of measuring mitochondrial oxygen consumption and proton leak kinetics is to assess mitochondrial function and energetic efficiency.

Hepatic mitochondrial dysfunction has been associated with several diseases in dairy cattle. The ability of cellular metabolism to switch between carbohydrate and lipid fuels when faced with an energy deficit in early lactation is influenced by the number and function of mitochondria in the cell[2]. Defects in the ability of mitochondria to adapt to an increased demand for energy and increased β-oxidation can lead to accumulation of intracellular lipid associated with insulin resistance and may lead to the formation of fatty liver in early lactation dairy cows. Mitochondria, as the site of ketone body production and use, can play a key role in ketosis in dairy cows[3]. A lack of mitochondria or mitochondrial dysfunction will impact fuel availability to the periphery and be reflected in changes in oxygen consumption or RCR.

Mitochondrial oxygen consumption changes in response to inflammation. Seven-day-old broilers were randomly assigned to a group infected with *Eimeria maxima* and a control group[4]. Broilers that did not undergo coccidiosis challenge had lower oxygen consumption due to proton leak and higher RCR indicating that liver mitochondria respond to an immune challenge by increasing proton leak. While proton leak and reactive oxygen species production was once considered a sign of mitochondrial membrane dysfunction and detrimental to energetic efficiency, now it is known that it is important for import of proteins and calcium into mitochondria[5], and for the generation of heat[1].

Electron leak from the respiratory chain makes mitochondria susceptible to reactive oxygen species production and oxidative damage to mitochondrial membrane proteins, lipids and mitochondrial DNA. As mitochondria age, damage can accumulate especially to mtDNA causing further dysfunction in mitochondrial metabolism[6]and greater susceptibility of the cow to disease.

In practice, many livestock animals are fed high levels of supplements such as Cu, Zn and Mn to boost antioxidant function. However, feeding high levels of Cu, Zn and Mn decreased milk production and increased oxygen consumption due to proton leak (State 4 respiration)[7].

Previous research on the role of mitochondrial function in energy efficiency in cattle has focused on changes in mitochondrial oxygen consumption and proton leak. Very few studies have been published in dairy cattle and most papers compare production efficiency in the form of residual feed intake (RFI) to mitochondrial function in beef cattle. Variability in mitochondrial respiration rates were examined by measuring state 3, state 4 and RCR in livers from both lactating Holstein cows and lactating beef cows (Angus, Brangus and Hereford)[8]. The researchers did not find any correlation in mitochondrial respiration with growth or milking traits for beef cattle but did report a correlation between mitochondrial respiration and milking traits for Holsteins. In two studies, RFI was compared in beef cattle to mitochondrial respiration rates (state 3, state 4 and RCR) in muscle mitochondria[9,10]. Mitochondrial respiration rates changed in response to DMI and low rates were associated with less efficient beef steers. In another study, RFI of steers from high or low RFI bulls were compared with mitochondrial respiration rates and proton leak kinetics between the two groups of progeny[11]. Differences were due to gain confirming the conclusion that gain does not impact mitochondrial respiration in beef cattle.

In this paper, an experiment examining liver RCR in response to feeding 3 antioxidant minerals to lactating dairy cattle illustrates the use of methods to measure oxygen consumption during State 4 and State 3 respiration and PMF.

PROTOCOL:

All methods, protocol and studies described here were approved by the Institutional Animal Care and Use Committee (IACUC) of University of California, Davis.

1. Obtaining a Liver Biopsy from a Holstein Dairy Cow

Note: A liver biopsy should be performed by a licensed veterinarian. Liver biopsies can be performed on the dairy site where the cows are located. Lactating dairy cows can continue to be milked normally and milk does not need to be withdrawn from the food supply before or after the procedure. It is recommended that at least 4 people are needed to perform the liver biopsy on a dairy cow: a veterinarian to perform the biopsy, an animal handler to stand at the cow's hip to protect the biopsy area and veterinarian, a lab technician on the outside of the pen to transfer tools, materials and biopsy sample to and from the veterinarian and maintain the clean area, which can be in the back of a vehicle (**Figure 1**), and a technician to retrieve the liver sample and begin mitochondrial isolation.

1.1. One month prior to liver biopsies, give cows a clostridia vaccination. Create surgical packs by autoclaving surgical towels, biopsy instrument, scalpel holders and surgical equipment.

1.2. One day before the liver biopsy, inject the cow with Ceftiofur Hydrochloride 0.044 mL/kg bodyweight subcutaneously in the neck. Monitor cow temperature, intake and fecal scores to

use as a baseline for normal function.

1.3. Create mitochondria isolation media (MIM) containining 220 mM mannitol, 70 mM sucrose, 20 mM HEPES, 1 mM EDTA, and 0.1% (w/v) fatty acid free BSA, pH 7.4 at 4 °C. Approximately 30 mL per sample will be needed.

1.4. Restrain cow physically utilizing a headlock with a halter as needed (**Figure 2**). Using the halter, tie her head to the left side of the stanchion. If necessary, a chemical restraint (Xylazine hydrochloride 100 mg/mL IV at 0.010-0.015 mg / kg bodyweight) can be used.

1.5. The area of the biopsy is found at the right 10 - 11th intercostal space (**Figure 3**). Draw a straight line from the right tuber coxae to the point of the right shoulder. The biopsy site is where this line intersects with the 10-11th intercostal space. Sterilize the area of the cow to be biopsied by shaving a 10 cm square area (**Figure 4**). Wash area with 10% providone scrub (**Figure 5**) using a circular motion. Spray area with 70% ethanol solution (**Figure 6**). Repeat providone and ethanol washes.

Note: The liver is in a slightly different position in Holstein dairy cattle compared to beef cattle.

1.6. Inject 2% lidocaine HCl (10-15 mL) locally to the area to provide anesthesia of the skin and underlying muscle and connective tissue (**Figure 7**). Repeat providone and 70% ethanol washes.

Note: The nerve endings are in the skin and muscle, but not internal organs, so only a local anesthestic is needed. At most, the cow should only feel some pressure and no pain during the biopsy procedure.

1.7. Make a 1-2 cm stab-incision through the skin of the 10-11th intercostal space (**Figure 8**). Pass a Schackelford-Courtney bovine liver biopsy instrument through the skin and direct the biopsy instrument in a slight cranial direction while continuing through the diaphragm and into the liver (**Figure 9**, **Figure 10**). Obtain a 1 g sample of the liver and remove the instrument (**Figure 11**). Close the skin with suture placement (**Figure 12**).

1.8. Place liver sample in a conical tube with enough MIM to cover sample, on ice for immediate mitochondria isolation

1.9. Check incision for any redness, swelling, heat, or pain within 24 hours of biopsy and inject the cow with Ceftiofur Hydrochloride 0.044 mL/kg bodyweight subcutaneously in the neck once a day for the next 3 days (**Figure 13**). Monitor the cow's temperature, intake and fecal scores daily for 1 week after liver biopsy. If a fever develops, continue antibiotics at the discretion of the veterinarian.

Note: If a cow is exhibiting signs of pain, such as kicking at the incision site, recumbancy, redness, heat, or reaction to touch within 1 h after the liver biopsy, a 1 mg/kg bodyweight IV injection of flunixin meglumine can be used to alleviate pain and inflammation. A second

injection can be administered if necessary.

1.10. Remove sutures 7 days after biopsy.

2. Isolating Mitochondria from Dairy Cow Liver

2.1. As soon as possible after the liver sample is removed from the cow, wash the liver sample in MIM (step 1.3) to remove red blood cells and finely mince the sample with scissors. The liver should be minced in a chilled beaker containing enough isolation media to keep the tissue moist.

2.2. Place the minced liver into a 30 mL glass vial with a teflon pestle of 0.16 mm clearance incubated in ice and containing MIM (1:4 w/v).

2.3. Homogenize liver sample in teflon pestle at 500 rpm for a min with 4 strokes/min.

Note: The liver homogenate is kept in an ice-packed beaker in MIM during the entire process, and all of the following centrifugation steps are completed at 4 °C

2.4. Centrifuge homogenate at 500 x g for 10 min, discard pellet, transfer supernatant to a chilled centrifuge tube and then centrifuge the resulting supernatant at 10,000 x g for 10 min to obtain the mitochondrial pellet.

2.5. Resuspend and wash the pellet in 10 mL of MIM with fatty acid free BSA and centrifuge at 8100 x g for 10 min. Discard supernatant.

2.6. Resuspend and wash the pellet in 10 mL of MIM without fatty acid free BSA and centrifuge at 8100 x g for 10 min. Discard supernatant.

2.7. Suspend the pellet in 200 µL of isolation media and place on ice until used for oxygen consumption and proton leak kinetic assays.

2.8. Determine protein concentration of the pellet suspension (1/100 dilution) using Bicinchoninic acid (BCA) kit per manufacturer's protocol with BSA as the standard. All protein is considered to be mitochondrial protein.

3. Measuring Mitochondrial Oxygen Consumption (State 3 and State 4)

3.1. Create oxygen consumption media (OCM) from 120 mM KCl, 5 mM KH_2PO_4, 5 mM $MgCl_2$, 5 mM Hepes and 1 mM EGTA, pH 7.4 at 30 °C with 0.3% defatted BSA. Approximately 3 mL per sample will be needed. Also prepare a solution of 8 µg/mL oligomycin in ethanol.

3.2. Incubate OCM at 30 °C. Set up respiration chamber, pump and oxygen electrode according to manufacturer directions (oxygraph system). The oxygraph software should already be installed on the computer.

3.3. Place 1 mL of OCM into the respiration chamber and stir vigorously. This will help insure that the solution becomes saturated with air.

3.4. Add 0.35 mg protein of mitochondrial protein to the respiration chamber and maintain temperature at 30 °C.

3.5. Add 1.25 µL of 4 mM rotenone solution to inhibit Complex I and then add 5 µL of 1 M succinate solution to reach a final concentration in the respiratory chamber of 5 mM succinate.

3.6. Record oxygen consumption for approximately 5 min. The oxygraph system records oxygen concentration so as respiration increases, oxygen concentration decreases. When oxygen consumption becomes constant (a decreasing straight line), record oxygen consumption (slope of line = concentration of oxygen/time). This is baseline oxygen consumption.

3.7. Add 1 µL of 100 mM ADP solution to reach a final concentration in the respiration chamber of 100 µM. Oxygen concentration will decrease (increased respiration) and then after approximately 5 min becomes a straight line. Record oxygen consumption (slope of line = concentration of oxygen/time). This is State 3 respiration.

3.8. Add 0.56 µL oligomycin solution for a final concentration of 2.8 µg oligomycin/0.35 mg of mitochondrial protein to inhibit ADP utilization and induce State 4 respiration. Record respiration for about 5 min (approximately). When oxygen consumption becomes constant, record oxygen consumption. This is State 4 oxygen consumption.

3.9. Optional: At the end of the run, add FCCP (0.2 µM total volume) to induce maximal respiration. Record respiration for about 5 min (approximately). When oxygen consumption becomes constant, record oxygen consumption. This is maximal oxygen consumption.

3.10. Calculate Respiratory Control Ratio (RCR) using the equation State 3 oxygen consumption / State 4 oxygen consumption.

3.11. Aspirate all of the solutions out of the respiration chamber. Rinse the chamber several times with double deionized water.

4. Measuring Mitochondrial Membrane Potential (MMP) and Proton Motive Force (PMF)

4.1. Prepare solution of 80 ng/mL nigericin in ethanol.

Note: These chemicals are dissolved in ethanol, and every effort should be made to limit the amount of ethanol that is added to less than 1 µL, since ethanol can uncouple the electron transport system and cause mitchondrial dysfunction.

4.2. After thoroughly rinsing the chamber with double deionized water, place 1 mL of the OCM

into the respiration chamber and stir vigorously with a magnetic stir bar. This will help insure that the solution becomes saturated with air. Add methyl-triphenyl-phosphonium (TPMP+) sensitive electrode to chamber setup. TPMP+ electrode should be connected to a pH meter and values are read from the pH meter.

4.3. Add 0.35 mg of mitochondrial protein to the respiration chamber.

4.4. Add 1.25 µL of 4 mM rotenone solution to inhibit Complex I. Record respiration for 2-5 min (approximately). When oxygen consumption becomes constant, record oxygen consumption.

4.5. Add 0.56 µL of 8 µg/mL oligomycin solution for a final concentration of 2.8 µg oligomycin /0.35 mg of mitochondrial protein to inhibit ADP utilization and induce state 4 respiration. Record respiration for 2-5 min (approximately). When oxygen consumption becomes constant, record oxygen consumption.

4.6. Add 0.112 µL 80 ng/mL nigericin solution to abolish the pH gradient across the mitochondrial inner membrane. Record respiration for 2-5 min (approximately). When oxygen consumption becomes constant, record oxygen consumption.

Note: Rotenone and oligomycin are used to block electron transport chain at Complex I and ATP synthase, respectively. Nigericin is added to convert transmembrane H+ gradient to a K+ gradient that can be measured with an electrode.

4.7. Prepare a standard curve for TPMP+ by adding 5 µL of 10 mM TPMP+ solution to the mitochondrial incubation. Repeat this step four more times until a total concentration of 2.5 µM TPMP+ has been added.

4.8. Initiate respiration by adding 5 µL of 1M succinate to the chamber.

4.9. Record respiration until you have achieved a stable trace, and then titrate the system by adding malonate. Additions of malonate should be 0.5 µL, 1 µL, 1.5 µL, 3.0 µL, 6.0 µL, 9.0 µL, then 12.5 µL of 0.1 mM Malonate solution to achieve successive additions of malonate concentrations in the incubation chamber of 0.1, 0.2, 0.3, 0.6, 1.2, 1.8, and 2.5 mM.

4.10. Collect data from the two electrodes (oxygen and $TPMP^+$). Data acquisition software from the oxygraph system can be used to collect simultaneous measurements of mitochondrial oxygen consumption and mitochondrial membrane potential and observe changes in oxygen consumption in real time. **Figure 14** shows how the oxygraph system records oxygen consumption as the experiment progresses.

4.11. Calculate MMP in mV based on the Nernst equation:

MMP = 61.5 log ([TPMP+] added – external [TPMP+]) x TPMP+ binding correction/ (0.001 x mg of protein/mL x [TPMP+])

A TPMP+ binding correction of 0.4 µL/mg of mitochondrial protein^{-1} is used.

Example calculation based on concentrations in protocol:
MMP = 61.5 x log (5 µM – 2 µM) x 0.4/ (0.001 x 0.35 mg mitochondrial protein/mL x 2 µM)
MMP = 198.9 mV

4.12. Estimate PMF by plotting a graph of MMP *vs.* oxygen consumption (**Figure 15**). PMF is reported as oxygen consumption at a membrane potential of 165 mV.

Note: Titrating the electron transport chain with malonate (0.1 to 2.5 mM) shows the kinetic response of proton leak to MMP. Then, plotting MMP against oxygen consumption determines proton leak kinetics. PMF is determined by calculating oxygen consumption at a common membrane potential (165 mV).

4.13. At the end of the last run of the sample, add FCCP (0.2 µM total volume) to induce maximal respiration and release TPMP+ for baseline correction.

4.14. Aspirate all of the solutions out of the respiration chamber. Rinse the chamber several times with double deionized water. At the end of the day, the chamber should also be rinsed a few times with ethanol.

REPRESENTATIVE RESULTS:

Positive results showing RCR and proton leak kinetics are shown in **Table 1** and **Figure 15**, respectively. In this study[7], RCR and protein leak kinetics were measured in Holstein dairy cows at 70 days in milk after cows had been fed 1 of 5 different levels of Cu, Zn and Mn for 28 days. State 4, maximum proton leak-dependent respiration, had a tendency to be affected by mineral intake of Cu, Mn and Zn ($p < 0.1$). State 3 respiration (maximum ATP stimulated respiration) and RCR = State 3 / State 4 (respiratory control ratio) was not affected by mineral intake. State 4 respiration was highest in LowMn and lowest in Control, indicating that Mn plays an important role in minimizing proton leak dependent respiration. Manganese, through the enzyme Mn Superoxide Dismutase is known to reduce reactive oxygen species in the mitochondrial matrix and reduce proton leak[12]. Higher State 4 respiration was associated with lower milk and milk protein yield. Since proton leak is an important component of energy efficiency, reducing State 4 respiration through Mn supplementation could improve efficiency.

[Insert **Table 1** here]

Mitochondrial proton leak is a process that dissipates MMP through the movement of protons across the mitochondrial inner membrane without production of ATP[14]. Proton leak kinetics are assessed by calculating rates of oxygen consumption at a common membrane potential of 165 mV. A lower membrane potential means that protons are 'leaking' across the mitochondrial membrane, which results in lower ATP synthesis (**Figure 15**). In the Holstein cow study, hepatic proton leak dependent respiration was greatest in LowMn and lowest in Control, which agrees

with results in **Table 1**, that State 4 respiration was greatest in LowMn and lowest in Control.

[Insert **Figure 15** here]

Negative results are illustrated in **Table 2** and **Figure 16**. Feed efficiency (RFI) was higher in Angus steers born from low RFI bulls than high RFI bulls, but this was not reflected in mitochondrial RCR (**Table 2**) or proton leak kinetics (**Figure 16**). There were no differences in mitochondrial respiration and proton leak kinetics between groups of steers but there was a difference in RFI. There were also no differences (p = 0.88) in hepatic mitochondrial proton leak in high and low RFI steers (**Figure 16**). There were large standard errors associated with mitochondrial respiration measurements, and proton leak kinetic curves were flat. Liver samples from this study were obtained after steers were slaughtered, a process that delayed liver sample collection and processing by an hour. Variation in mitochondrial respiration measures may reflect mitochondrial respiration degradation due to tissue death. Proton leak kinetic lines were flat because oxygen consumption measurements did not begin until 8 min when plateau had already been reached due to an equipment malfunction.

[Insert **Table 2** and **Figure 16** here]

FIGURE AND TABLE LEGENDS:

Figure 1: Clean area for surgical and biopsy materials located in the back of a vehicle and outside of the cow pen.

Figure 2: Restraint of the cow using a halter tied to a cross pole of the head lock.

Figure 3: The area of the cow to clean for the biopsy and location of biopsy at the right 10 - 11th intercostal space found by drawing a straight line from the right tuber coxae to the point of the right shoulder. The biopsy site is where this line intersects with the 10-11th intercostal space.

Figure 4: Shaving a 10 cm area of the cow to prepare to sterilize for the biopsy.

Figure 5: Wash biopsy area of the cow with 10% providone scrub using a circular motion.

Figure 6. Spray biopsy area area with 70% ethanol solution.

Figure 7: Inject 2% lidocaine HCl (10-15 mL) locally to the area to provide anesthesia of the skin.

Figure 8: A 1-2 cm stab-incision through the skin of the 10-11th intercostal space to insert biopsy tool.

Figure 9: Insertion of bovine liver biopsy instrument through the skin.

Figure 10: The biopsy instrument should be directed in a slight cranial direction while continuing

through the diaphragm and into the liver.

Figure 11: A 1 g sample of the liver being moved from biopsy instrument to Falconer tube for transport to mitochondrial isolation station.

Figure 12: Suturing the skin to close biopsy incision.

Figure 13: Injection of the cow with Ceftiofur Hydrochloride 0.044 mL/kg bodyweight subcutaneously in the neck.

Figure 14: Oxygraph software results showing oxygen consumption responses to addition of each substance to measure mitochondrial membrane potential (MMP) and proton motive force (PMF).

Figure 15. Proton leak kinetics in Holstein cows fed different amounts of Cu, Mn and Zn. This graph is based on data from Acetoze *et al.* 2017[7].

Figure 16. Proton leak kinetics for progeny of high and low RFI Angus bulls. This graph has been adapted from Acetoze *et al.* 2015[11].

Table 1: Effect of Cu, Mn and Zn supplementation on liver mitochondrial oxygen consumption and milk production from dairy cows at 70 days in milk. This table has been adapted from Acetoze *et al.* 2017[7].

Table 2: Performance and mitochondrial respiration of high and low Residual Feed Intake (RFI) Angus bull progeny. This table has been adapted from Acetoze *et al.* 2015[11].

DISCUSSION:

The most critical point in the protocol is obtaining a representative liver tissue sample and beginning the isolation of mitochondria as soon as possible after biopsy. Variation in respiration measurements is low (**Table 1**) due to a short transport time from cow to laboratory. To reduce transport time, a small laboratory was set up in the office of the dairy, and liver samples were driven to the office laboratory as each was collected so that mitochondria were isolated within 10 min of biopsy. Setup and testing of the respiration chamber and electrodes (oxygen, TPMP+) with the pH meter used to record differences in proton gradients the day before collecting and processing samples can avoid malfunctions such as missing early measurements in proton leak kinetics (**Figure 16**).

Because of the need for fresh liver samples and rapid isolation of mitochondria, the number of samples that can be collected and processed in a day is limited. Each sample takes approximately 5-6 h to complete; therefore, only about 5 samples per day can be collected and analyzed per respiration chamber. This is not a high throughput method; the sample size for treatments is limited and small errors can increase variability associated with results and the ability to detect significance.

The isolation technique may exclude some mitochondria that are associated with some cell components and remain embedded in the pellet or smaller mitochondria may be lost in the pellet washes during the centrifugation steps. This may lead to results that do not reflect the complete population of mitochondria. Mitochondria can change in size and density depending on physiological states such as starvation and exercise (training)[15]. Estimating mitochondrial number with enzyme activity using citrate synthase[16] or succinate dehydrogenase[17] to corroborate findings may be needed.

No modifications were made to the original techniques established in rodents for mitochondrial isolation[18], respiration[19] and proton leak kinetics[20]. Modifications to this technique can be made depending on tissue source of mitochondria and experimental treatments. BSA (defatted) is used to bind free fatty acids in tissue. If the tissue has a lot of fatty acids (greater than 10%) associated with it, more defatted BSA can be added because free fatty acids will interfere with the mitochondrial measurements.

Measuring mitochondrial oxygen consumption and proton leak kinetics using this technique is the standard procedure. Liver has been the tissue of choice primarily because it has a lot of mitochondria, they are fairly easy to extract, and the liver is the primary site of nutrient processing. Modifications of this technique have been used to measure oxygen consumption in other tissues such as muscle and mammary. However, mitochondrial isolation techniques must be modified to fit the tissue. For instance, in muscle, mitochondria are embedded in muscle fibers and so the isolation procedure must include a protein digestion and the digestion must be controlled to ensure that mitochondrial function is not disrupted.

There are other methods for measuring mitochondrial respiration that require an analyzer specifically designed to measure respiration. Cells must be harvested from tissue and fixed to incubation plates. The analyzer measures whole cell oxygen consumption rate (OCR; basal), ATP linked OCR (associated with mitochondria), nonmitochondrial OCR, and maximal OCR. However, since mitochondria are inhibited during some of the incubation, isolated mitochondria measurements are not possible. This method has been used to examine OCR changes with disease and drug interventions[21] in humans.

Current and Future Applications

The contribution of proton leak to energy requirements of the animal can be large and indicative of the physiological state of the animal including growth, lactation and disease. In the past, this technique has been primarily used to examine the association of mitochondrial oxygen consumption and contribution of proton leak to feed or energetic efficiency. However, as our understanding of the role of mitochondria in metabolism expands, the importance of this technique will also increase particularly in combination with other mitochondrial measures such as Electron Transport Chain enzyme activities, calcium dynamics in apoptosis and enzyme activities of the TCA cycle.

ACKNOWLEDGMENTS:

This research was supported by Alltech and USDA Hatch funds through the Center for Food Animal Health at UC Davis School of Veterinary Medicine.

DISCLOSURES:

The authors have nothing to disclose.

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Figure1

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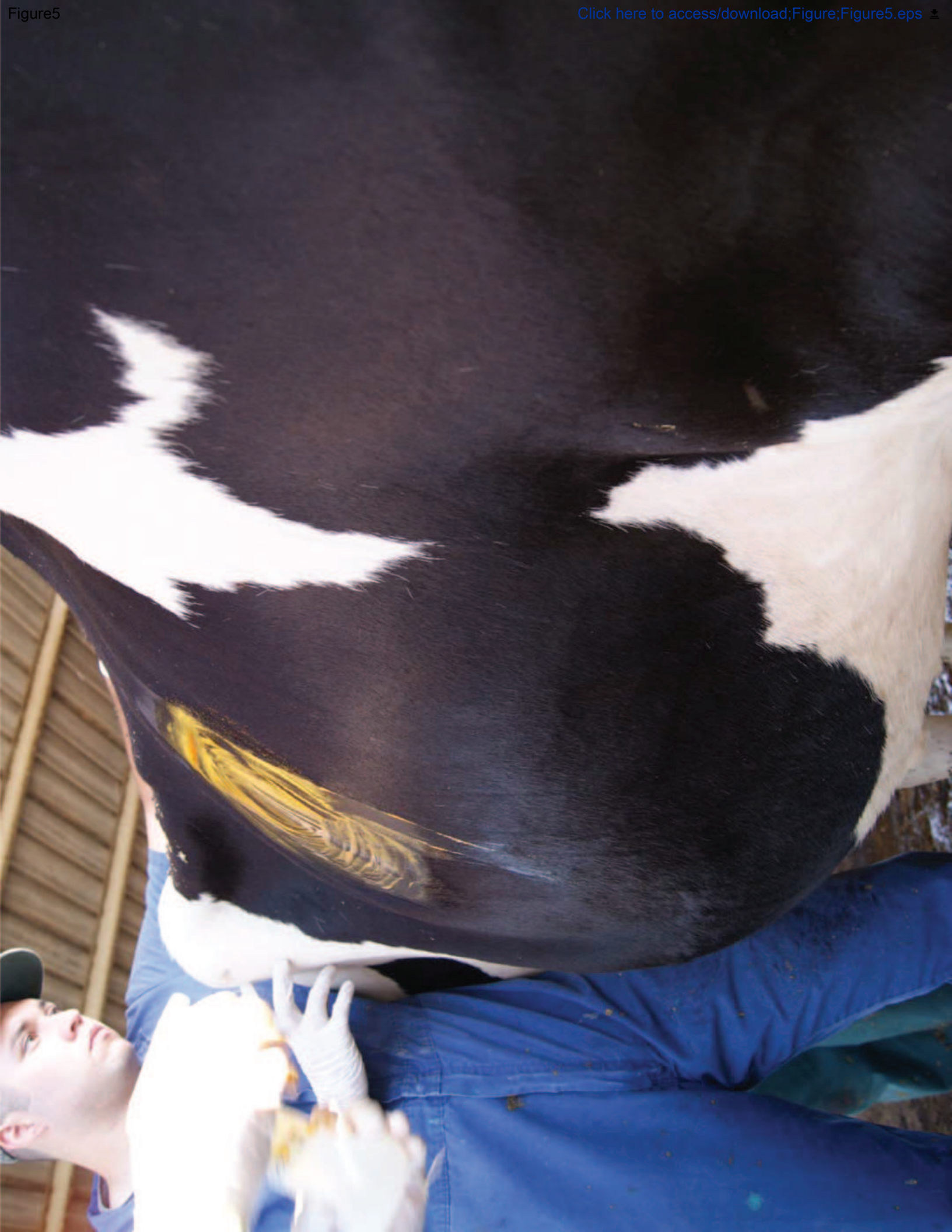

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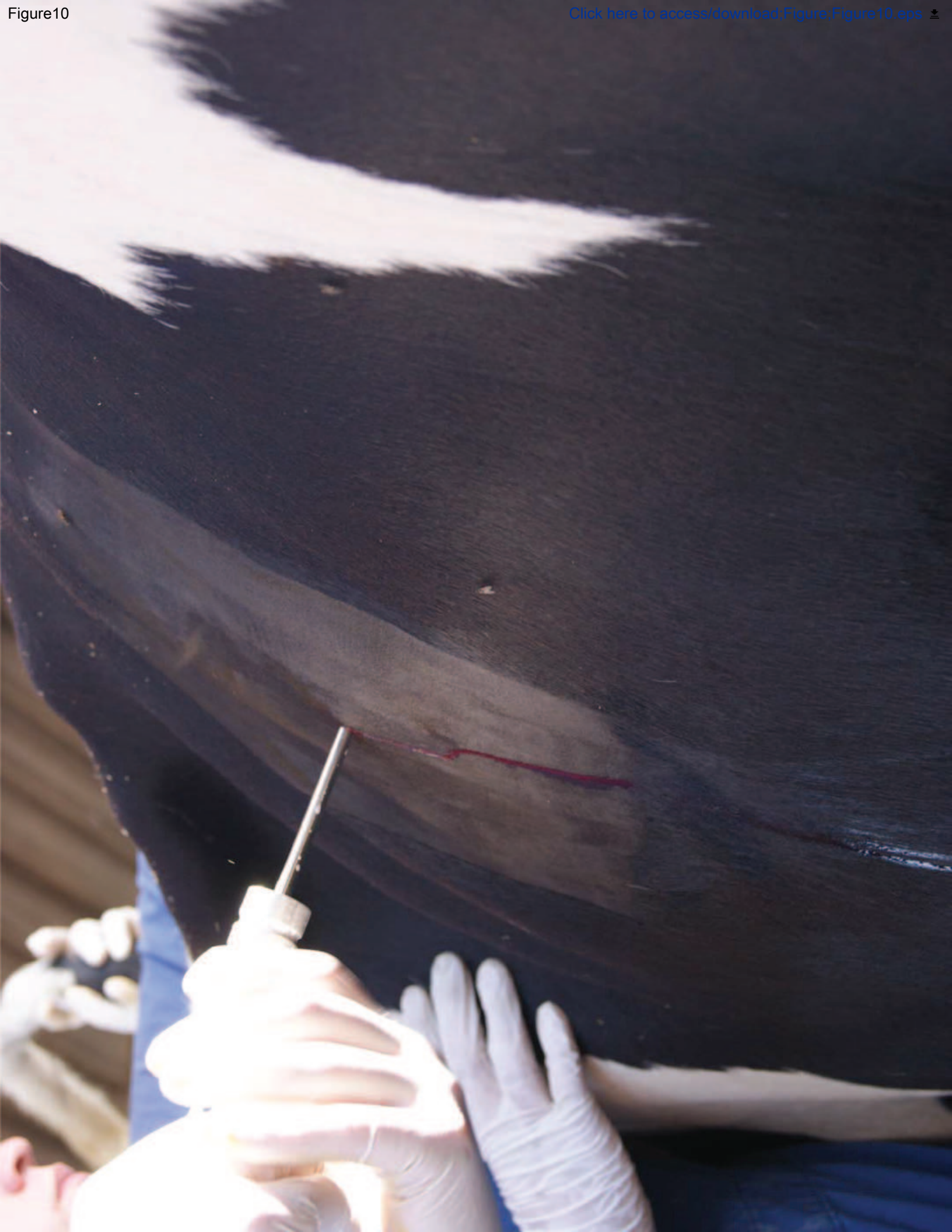

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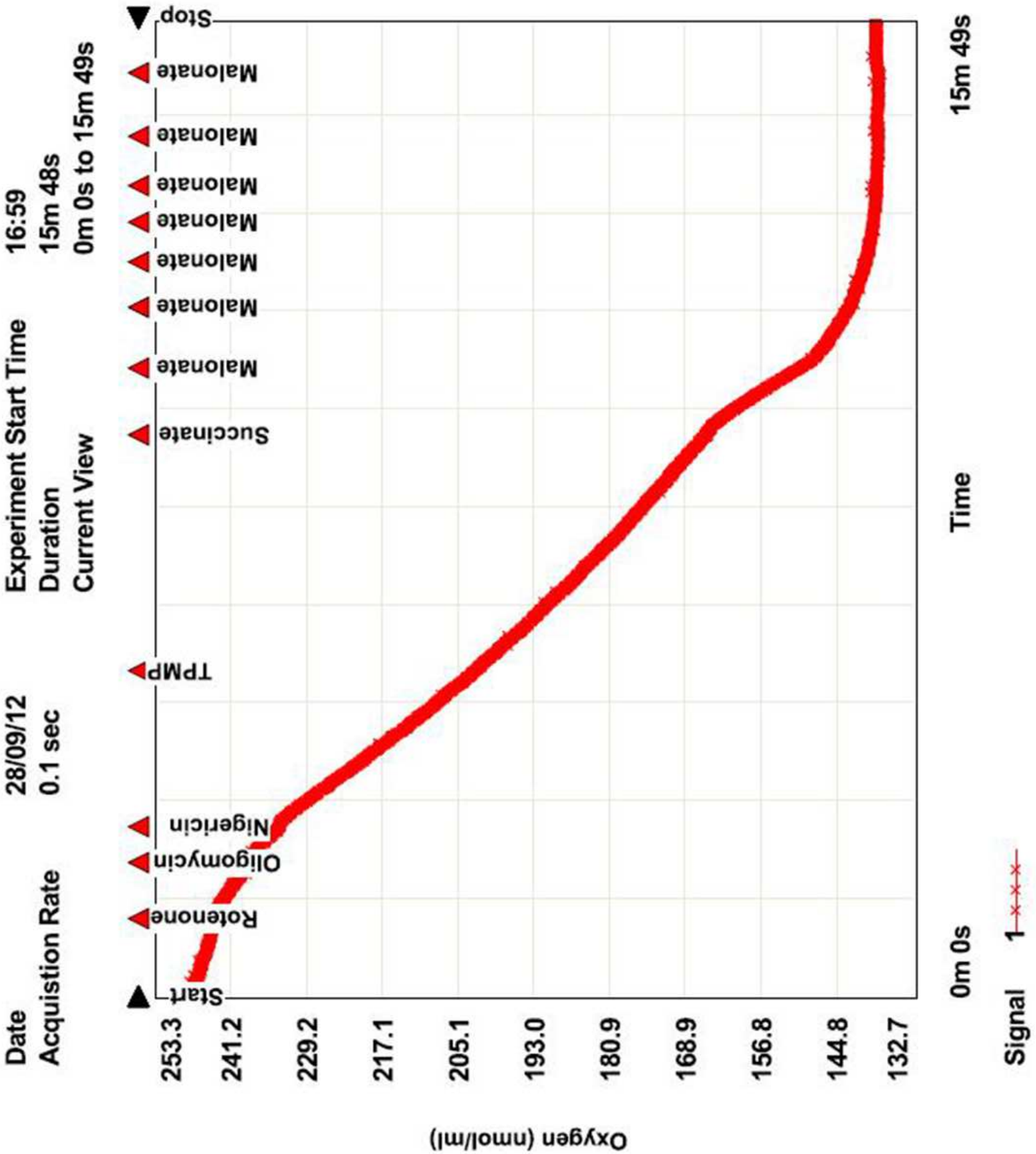

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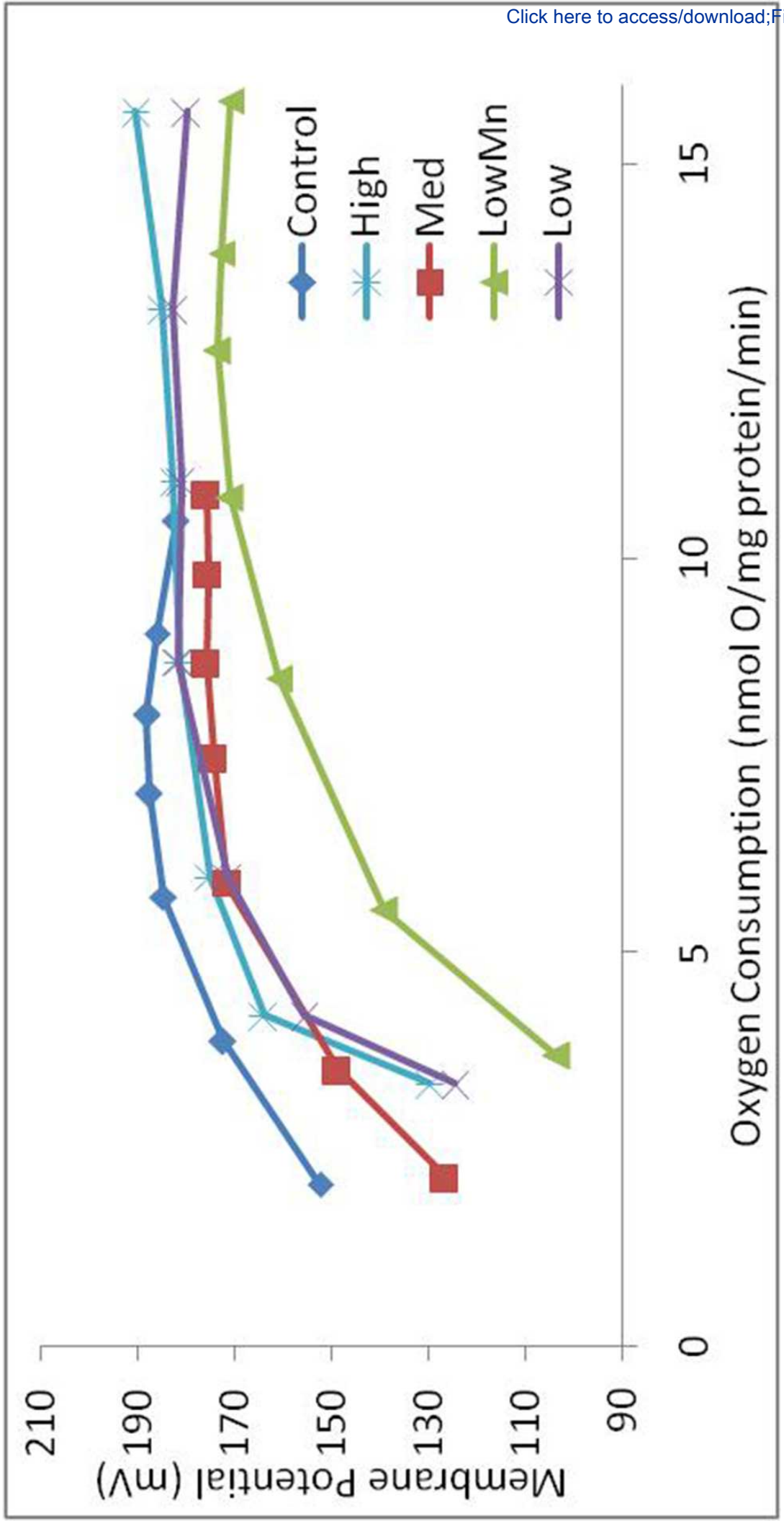
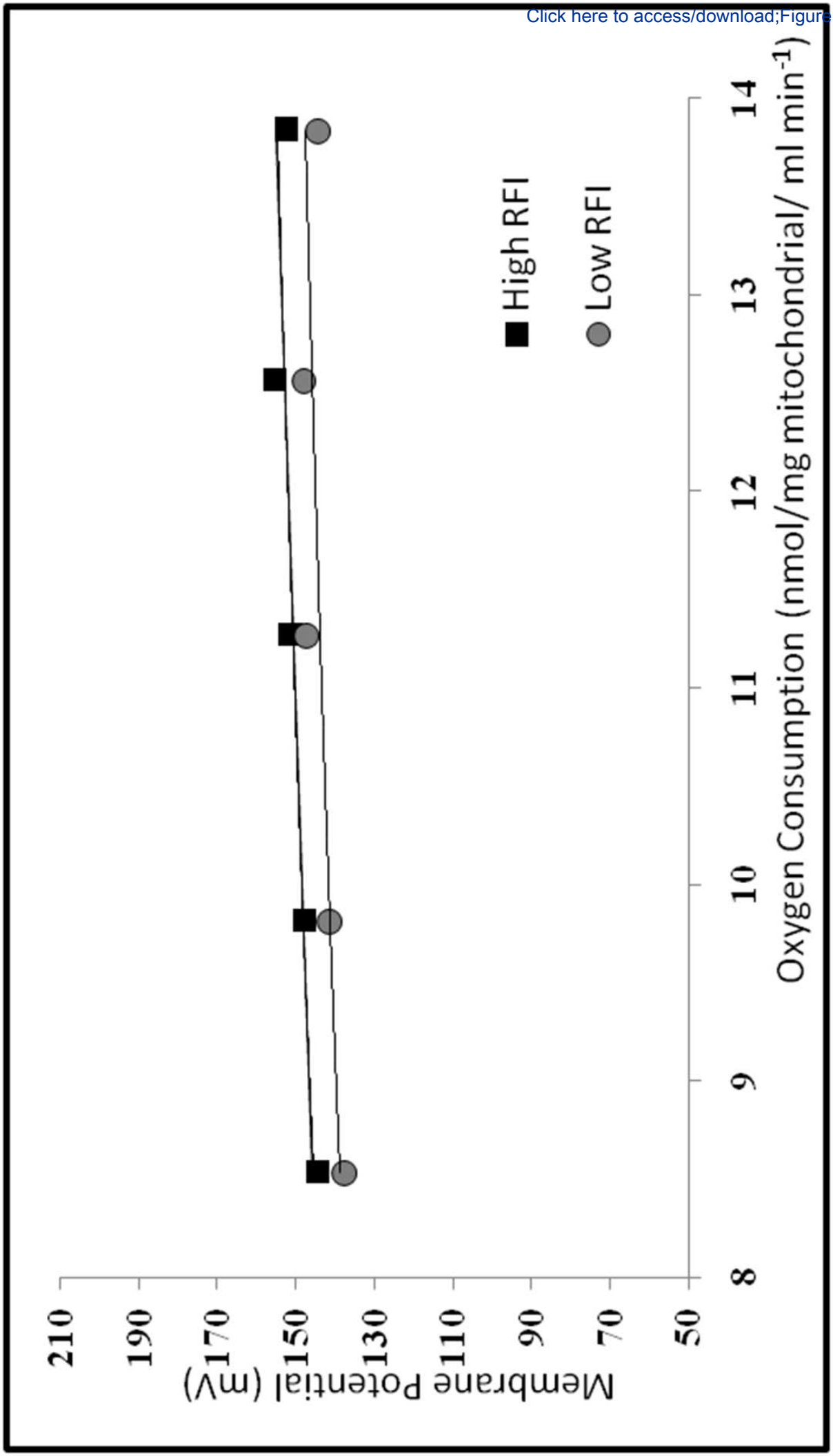


Figure16



	Treatments[1]					
	High	Med	Low	LowMn	Control	SEM
Milk, kg	47.4[ab]	50.9[a]	46.0[ab]	43.6[b]	49.7[a]	2.9
Milk protein, kg	1.38[ab]	1.44[a]	1.40[ab]	1.23[b]	1.43[a]	0.09
State 3	75.8	64.4	78.2	73	64.1	13
State 4	26.2[ab]	22.6[ab]	25.9[ab]	27.1[a]	22.0[b]	3
RCR	2.89	2.76	2.98	2.65	2.83	0.27

[a b] Means within a row not followed by the same superscript letter are significantly different ($P < 0.1$).

[1] High treatment contains highest levels of Cu, Zn and Mn all well above requirements[13], Med treatment co

ntains intermediate levels of Cu, Zn and Mn above requirements, Low treatment contains lower levels c

of Cu, Zn and Mn but still above requirements, Low Mn treatment contains the lowest levels of Mn (and

lower levels of Cu and Zn) but still above requirements and Control treatment contains the lowest levels

s of Cu and Zn, which are close to requirements.

	Low RFI (n=7)	High RFI (n=8)	SEM	*P* Value
RFI	-0.58	-0.01	0.1	0.05
State 3	31.3	30.8	9.42	0.9
State 4	9.76	10.4	3.23	0.8
RCR	3.05	3.03	0.24	0.93

Table of Materials

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Liver Biopsy			
Equipment			
Schackelford-Courtney bovine liver biopsy instrument	Sontec Instruments Englewood CO	1103-904	
Suture	Fisher Scientific	19-037-516	
Suture needles	NA	NA	Included with Suture
Scalpels	Sigma - Aldrich	S2896 / S2646	# for handle and blades
Surgery towels	Fisher Scientific	50-129-6667	
Falcon tubes 50 mL	Fisher Scientific	14-432-22	
Tweezers	Sigma - Aldrich	Z168750	
50 mL syringes	Fisher Scientific	22-314387	
Injection needles (22, 2 1/2)	VWR	MJ8881-200342	
Cow halter	Tractor Supply Co.	101966599	
Cotton swabbing	Fisher Scientific	14-959-102	
cotton gauze squares (4x4)	Fisher Scientific	22-246069	
Medical scissors	Sigma - Aldrich	Z265969	
Chemicals			
Coccidiosis Vaccine 0.75 bottle/cow			Provided by Veterinarian
Clostridia Vaccine			Provided by Veterinarian
Liver biopsy antibiotics excenel 2 cc/100 lbs for 3 days			Provided by Veterinarian
Providone Scrub	Aspen Veteterinary Resources	21260221	
Ethanol 70%	Sigma - Aldrich	793213	
Xylazine hydrochloride 100 mg/mL IV at 0.010-0.015 mg/kg bodyweight			Provided by Veterinarian
2% lidocaine HCl (10-15 mL)			Provided by Veterinarian
1 mg/kg IV injection of flunixin meglumine			Provided by Veterinarian
Isolation of Mitochondria (liver)			
Equipment			
Wheaton vial 30 mL with a Teflon pestle of 0.16 mm clearance	Fisher Scientific	02-911-527	
Homogenizer Motor	Cole Parmer	EW-04369-10	
Homogenizer Probe	Cole Parmer	EW-04468-22	
Auto Pipette (10 mL)	Cole Parmer	SK-21600-74	
Beaker (500 mL) with ice	Fisher Scientific	FB100600	
Refrigerated microfuge	Fisher Scientific	75-002-441EW3	
Microfuge tubes (1.5 mL)	Fisher Scientific	AM12400	
Chemicals			
Bicinchoninic acid (BCA) protein assay kit (microplates for plate reader)	abcam	ab102536	
Sucrose	Sigma - Aldrich	S7903-1KG	
Tris-HCl	Sigma - Aldrich	T1503-1KG	
EDTA	Sigma - Aldrich	EDS-1KG	
BSA (fatty acid free)	Sigma - Aldrich	A7030-50G	
Mannitol	Sigma - Aldrich	M4125-1KG	
Deionized water	Sigma - Aldrich	38796	
Hepes	Sigma - Aldrich	H3375-500G	
Use to create mitochondria isolation media: 220 mM mannitol, 70 mM sucrose, 20 mM HEPES, 20 mM Tris-HCl, 1 mM EDTA, and 0.1% (w/v) fatty acid free BSA, pH 7.4 at 4 °C, will last 2 days in refrigerator			
Mitochondrial Oxygen Comsuption			
Equipment			
Oxygraph Setup + Clark type oxygen electrode	Hansatech (PP Systems)	OXY1	
Thermoregulated Water Pump	ADInstruments	MLE2001	
Clark type Oxygen electrode	NA	NA	
Autopipette (1 mL)	Cole Parmer	SK-21600-70	Included with Oxy1
Small magnetic stir bar	Fisher Scientific	14-513-95	
Micropipette (10 µL)	Cole Parmer	SK-21600-60	
pH meter	VWR		
Chemicals			
KCl	Sigma - Aldrich	P9333-1KG	
Hepes	Sigma - Aldrich	H3375-500G	
KH2PO4	Sigma - Aldrich	P5655-1KG	
MgCl2	Sigma - Aldrich	M1028-100ML	
EGTA	Sigma - Aldrich	E3889-100G	
Use to make mitochondrial oxygen consumption media: 120 mM KCL, 5 mM KH2PO4, 5 mM MgCl2, 5 mM Hepes and 1 mM EGTA, pH 7.4 at 30 °C with 0.3% defatted BSA			
Rotenone (4 mM solution)	Sigma - Aldrich	R8875-5G	
Succinate (1 M solution)	Sigma - Aldrich	S3674-250G	
ADP (100 mM solution)	Sigma - Aldrich	A5285-1G	
Oligomycin (solution of 8 µg/mL in ethanol)	Sigma - Aldrich	75351	
FCCP	Sigma - Aldrich	C2920	
Mitochondrial Membrane Potential and Proton Motive Force			
Equipment			
TPMP electrode	World Precision Instruments.	DRIREF-2	
Chemicals-solutions do not need to be fresh but they do need to be kept in a freezer between runs			
Malonate (0.1 mM solution)	Sigma - Aldrich	M1296	
Oligomycin (8 µg/mL in ethanol), keep in freezer	Sigma - Aldrich	75351	
Nigericin (80 ng/mL in ethanol), keep in freezer	Sigma - Aldrich	N7143	
FCCP	Sigma - Aldrich	C3920	
TPMP	Sigma - Aldrich	T200	
TPMP solution: 10 mM TPMP, 120 mM KCL, 5 mM Hepes and 1 mM EGTA, pH 7.4 at 30 °C with 0.3% defatted BSA			

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Author(s): HA ROSSIUS, GACÉTOZÉ, J. CHAMPAGNE, JJ RAMSEY

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Department: POPULATION HEALTH & REPRODUCTION

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

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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/

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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.**
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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**

Dear Dr. Rossow,

Response to editorial comments for JoVE58387R1 "Measuring liver mitochondrial oxygen consumption and proton leak kinetics to estimate mitochondrial respiration in Holstein dairy cattle"

Editorial comments:

1. Abstract: Please remove the citation from the abstract. **DONE**
2. Figure 14: Please consolidate into one page. Also, please remove the "Hansatech" logo, as well as "Oxygraph/Oxytherm," as they are all commercial language (a few mentions of the latter in the manuscript itself are fine, though). **DONE**
3. Figure 15: "potenial" is a typo; please fix. **DONE**