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# Integrated assessment of mitochondrial respiration and NADH redox state reveals tissue-specific bioenergetic phenotypes in murine cardiac and skeletal muscles

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## INTRODUCTION

Mitochondria are energy hubs within cardiac and skeletal muscle cells, where cellular energy demand is met by integrating substrate oxidation and the reduction of electron carriers (i.e., $NAD^+ \rightarrow NADH$) largely through the Krebs Cycle that are essential for generating the proton motive force and subsequent ATP synthesis through oxidative phosphorylation (OXPHOS) within the Electron Transport System (ETS)[1, 2]. The mitochondrial matrix NADH redox state critically regulates the Krebs Cycle and the ETS. Cardiac muscle sustains continuous contractile activity and depends heavily on mitochondrial OXPHOS, whereas skeletal muscles display considerable metabolic heterogeneity mainly associated with differences in the contractile and functional properties[3-6]. These distinct energetic environments provide a physiological framework in which to investigate how mitochondrial respiratory function and redox state are coordinated across these two divergent striated muscles.

High-resolution respirometry is an important approach for comprehensively characterizing mitochondrial respiratory function[7, 8]. However, oxygen consumption alone provides an incomplete representation of mitochondrial bioenergetics. Electron flow through the ETS is coupled with oxidation of reducing equivalents, particularly NADH, generated through substrate oxidation. The mitochondrial $NADH/NAD^+$ redox couple therefore represents a critical interface between substrate oxidation and respiratory control. Mitochondrial NADH redox state reflects the balance between NADH generation and its oxidation by complex I of the ETS, providing complementary information for understanding respiratory control[9-12]. Simultaneous assessment of mitochondrial respiration and NADH redox state may reveal differences in mitochondrial regulation not apparent from respiratory capacity alone or NADH redox balance measured in non-physiological respiratory control states. The Oroboros NextGen-O2k enables simultaneous measurement of mitochondrial respiratory function and NADH redox state in physiologically relevant respiratory control states.

A technical consideration when simultaneously measuring respiratory function and NADH redox state is obtaining high-quality mitochondrial preparations of comparable functional integrity across tissues with different structural and mechanical

characteristics. Isolating mitochondria from striated muscle requires sufficient tissue disruption to release mitochondria from the contractile apparatus while minimizing membrane damage. Excessive homogenization can compromise outer mitochondrial membrane integrity, whereas insufficient homogenization may reduce mitochondrial recovery and bias selection of the mitochondrial population studied. These considerations become particularly important when comparing cardiac muscle with skeletal muscles of differing size, fiber composition, and structural integrity. Rigorous assessment of mitochondrial membrane integrity, respiratory coupling, and ultrastructure is necessary before tissue-specific differences in mitochondrial respiration or redox state can be interpreted as biological rather than methodological.

Here, we aimed to optimize and validate a mitochondrial isolation approach for murine left ventricle (LV) and gastrocnemius (gastric) for simultaneously measuring mitochondrial respiratory function and NADH redox state using the Oroboros NextGen-O2k. We subsequently investigated tissue-specific mitochondrial respiratory and NADH redox phenotypes in LV and gastroc.

## MATERIALS AND METHODS

### *Animals, Experiments and Design*

Male C57BL/6J mice, 12-14 weeks of age, were used for all experiments. All animal procedures were approved by the Institutional Animal Care and Use Committees of Pennington Biomedical Research Center and LSU Veterinary Medicine. Following euthanasia, LV and gastroc tissues were excised, washed in ice-cold PBS to remove excess blood, and immediately placed in ice-cold Biopsy preservation solution (BIOPS) until mitochondrial isolation.

### *Optimization and Validation of Mitochondrial Isolation*

Optimization experiments were performed using an independent set of mice. LV and gastroc were obtained from the same animals and used to evaluate the mitochondrial isolation procedure. The effects of PTFE pestle clearance and homogenization on mitochondrial preparation quality were assessed by comparing preparations obtained using a commercially available PTFE pestle with those using an in-house modified PTFE pestle. The preparations were evaluated for outer mitochondrial membrane integrity, respiratory coupling, OXPHOS capacity, and ET capacity using high-resolution respirometry. Cytochrome c responsiveness and coupling-control efficiency were used as functional indices of preparation quality.

### *Mitochondrial Isolation Buffer*

Mitochondrial isolation buffer (Buffer A) contained 100 mM KCl, 50 mM Tris base, 5 mM $MgCl_2 \cdot 6H_2O$, 1.8 mM ATP, and 1 mM EDTA. The pH was adjusted to 7.2 using HCl. Buffer A was aliquoted and stored at −20°C until use [13]. Immediately before mitochondrial isolation, Trypsin EDTA was added to a subset of Buffer A to prepare Buffer A1 at a final Trypsin concentration of 0.025%.

### *Isolation of Mitochondria from Left Ventricle and Gastrocnemius*

All mitochondrial isolation procedures were performed on ice (0-4°C) using prechilled instruments. Freshly collected tissue was transferred to ice-cold Buffer A, and visible connective tissue and fat were removed. Approximately 80–100 mg of starting tissue was finely minced using vascular scissors and transferred to a precooled 5 mL Potter–Elvehjem homogenizer containing 2 mL of ice-cold Buffer A1

and incubated for 7 min. Following incubation, 2 mL of ice-cold Buffer A without trypsin was added, and the suspension was gently mixed. Tissues were homogenized with a PTFE pestle at 150 rpm for 10 min. The homogenizer tube slowly moved vertically relative to the pestle, with approximately 30-40 s for each upward or downward movement without grinding the tissue. The homogenate was passed through a 100-µm cell strainer into a precooled 50-mL centrifuge tube. The filtrate was brought to a final volume of 15 mL with ice-cold Buffer A and centrifuged at 700×g for 8 min at 4°C. The supernatant was transferred to a fresh precooled tube and centrifuged at 9,000×g for 10 min at 4°C. Following this, the supernatant was removed, and the pellet was resuspended in 1 mL of ice-cold Buffer A, brought to a total volume of 10 mL, and centrifuged again at 9,000×g for 10 min at 4°C. The mitochondrial pellet was gently resuspended in MiR05 medium without bovine serum albumin (BSA). For gastroc, a resuspension volume of approximately 2 µL per mg of starting tissue was initially used. For LV, a higher resuspension volume of approximately 8 µL per mg of starting tissue was used. Aliquots of the mitochondrial suspension were stored at -80°C for protein determination and citrate synthase activity. The isolated mitochondrial suspension rested on ice for 15 mins before 10 µL of mitochondrial suspension was added to each respiratory chamber using a Hamilton syringe in duplicate.

### *High-Resolution Respirometry and NADH Redox Measurements*

Mitochondrial respiratory function was assessed using the Oroboros NextGen-O2k in MiR05 at 37°C with an $O_2$ concentration between 50-190 µM using Datlab8. Respiratory function was measured using a modified substrate–uncoupler–inhibitor titration (SUIT) protocol based on SUIT-008, enabling the sequential assessment of LEAK respiration, oxidative phosphorylation (OXPHOS) capacity, electron transfer (ET) capacity, and ROX. Outer mitochondrial membrane integrity was evaluated by assessing the respiratory response to exogenous cytochrome c, while mitochondrial respiratory coupling was determined using flux control ratios derived from the measured respiratory states. Oxygen flux ($J_{O2}$, pmols·s$^{-1}$·µg$^{-1}$) rates were corrected for instrumental background and residual oxygen consumption (ROX) and normalized per µg mitochondrial protein measured using the BCA Assay.

Mitochondrial $J_{O2}$ and NADH autofluorescence were also simultaneously measured using the NextGen-O2k in MiRK03 without BSA at 37°C at an $O_2$ concentration between 50-190 µM. Measurements used a modified SUIT protocol adapted from SUIT-032. After adding the mitochondria, ADP (50 µM) was titrated to oxidize endogenous substrates, followed by sequential additions of pyruvate (5 mM), glutamate (10 mM), and malate (2 mM) to stimulate NADH-linked LEAK. ADP was increased to a final concentration of 2.5 mM to stimulate NADH-linked OXPHOS. Finally, myxothiazol (0.5 µM) was added to inhibit mitochondrial complex III and establish a maximally reduced NADH fluorescence reference under anoxia.

### *Statistical Analysis*

Mean differences in $J_{O2}$ from the mitochondrial preparations using the commercially available and modified PTFE pestles were compared using paired t-tests within each substrate state. Mean differences in LV and gastroc $J_{O2}$ were determined using two-way ANOVA followed by Šídák's multiple comparisons. A minimum of $n = 6$ biological replicates were included per tissue or experimental condition unless otherwise indicated. Statistical significance was defined as $p<0.05$.

## RESULTS AND DISCUSSION

### *Optimization of Mitochondrial Isolation in Left Ventricle and Gastrocnemius*

The initial objective of this study was to optimize a robust protocol for the isolation of mitochondria for high-resolution respirometry, with particular emphasis on preserving mitochondrial outer membrane integrity, respiratory coupling, and oxidative capacity. A significant reduction in the cytochrome c control factor (FCFc) with the modified pestle (~7% vs. ~9% p = 0.0048) was observed, indicating improved preservation of mitochondrial outer membrane integrity. Importantly, the coupling-control efficiencies were similar between the two isolation approaches.

To assess whether the improvement in outer membrane integrity was accompanied by changes in mitochondrial respiratory performance, respiratory capacities were compared across sequential respiratory states. N-linked and NS-linked OXPHOS capacity, as well as NS-linked and S-linked ET capacities, remained comparable between preparations from the two pestles. LEAK respiration also did not differ. Together, these findings demonstrate that modification of pestle clearance significantly improved the preservation of LV mitochondrial outer membrane integrity without compromising respiratory coupling or oxidative capacity.

To determine whether the effects of pestle modification on mitochondrial isolation were consistent across striated muscles, the commercially available and modified PTFE pestles were evaluated using the gastroc harvested from the same mice used for LV mitochondria isolation. The gastroc preparations exhibited no significant difference in the FCFc between the pestles, indicating comparable preservation of outer mitochondrial membrane integrity. Similarly, the coupling-control efficiency remained unchanged. Like LV tissues, the assessment of mitochondrial respiratory capacities revealed no significant differences between the two isolation approaches across the evaluated respiratory states, including N-linked and NS-linked OXPHOS, as well as NS-linked and S-linked ET capacities.

### *Tissue-Specific Differences in Mitochondrial Respiratory Capacity*

LV mitochondria exhibited significantly higher OXPHOS and ET capacities than gastroc mitochondria. These differences were evident across all ADP-stimulated respiratory states following sequential addition of pyruvate and malate, glutamate, and succinate, as well as under maximally uncoupled conditions following the titration of Carbonyl cyanide m-chlorophenyl hydrazone, and subsequent addition of rotenone, isolating complex II associated ET capacity. The differences $J_{O2}$ ranged from ~34% to ~144% higher in LV than gastroc mitochondria (all $p<0.05$). LEAK respiration did not differ significantly between tissues. Despite these differences in respiratory capacity, the coupling-control efficiency was similar between LV and gastroc mitochondria.

### *Tissue-Specific Differences in Mitochondrial NADH Redox State*

To further investigate the relationship between mitochondrial respiratory function and redox regulation, $J_{O2}$ and NADH autofluorescence were simultaneously measured in mitochondria isolated from the left ventricle and gastroc using the NextGen- O2k. The NADH-linked LEAK $J_{O2}$ was comparable between LV and gastroc mitochondria. Similarly, the reduced NADH fraction during LEAK respiration did not differ between tissues. However, during ADP-stimulated NADH-linked OXPHOS, LV mitochondria exhibited ~116% higher $J_{O2}$ than gastroc mitochondria ($p<0.05$). This greater respiratory capacity was accompanied by a ~18% lower reduced NADH

fraction ($p<0.05$) in LV mitochondria, showing that LV mitochondria maintain a more oxidized NADH pool during phosphorylating respiration, likely supporting higher $J_{O2}$.

## CONCLUSIONS

The functional quality of isolated mitochondria is a critical determinant of the reliability and interpretation of mitochondrial respiratory measurements. Mechanical homogenization is an important step in mitochondrial isolation, because tissue disruption is necessary to release mitochondria, whereas excessive mechanical stress may compromise membrane integrity and respiratory function [1, 13-15]. Optimization of homogenization conditions is particularly important when isolated mitochondria are intended for functional characterization using high-resolution respirometry.

Our findings demonstrate distinct mitochondrial respiratory and redox phenotypes in murine left ventricle and gastrocnemius muscles. LV mitochondria exhibited significantly greater OXPHOS and ET capacities despite comparable respiratory coupling efficiency. Importantly, the higher ADP-stimulated respiratory flux in LV mitochondria was associated with a lower reduced NAD(P)H fraction, suggesting tissue-specific differences in the balance between NADH generation and oxidation during active oxidative phosphorylation. Optimization of mitochondrial isolation improved outer membrane integrity in LV preparations without compromising respiratory capacity. Together, these findings highlight the complementary value of simultaneous respiratory and redox measurements for characterizing tissue-specific mitochondrial bioenergetics.

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**KEYWORDS**
Isolated Mitochondria; Bioenergetics; OXPHOS; LEAK; Redox Balance; Coupling, $NAD^+$; NADH; TCA Cycle; Multisensor

**AUTHOR CONTRIBUTIONS**
AS and BAI conceived and designed the study. AS and AP performed experiments. TDA and JF provided cardiac and skeletal muscle tissues. AS and BAI analyzed the data. TDA, HP, SAK, and BAI provided expertise in cardiac and skeletal muscle mitochondrial bioenergetics. AS prepared first draft of manuscript. All authors reviewed the manuscript.

**Conflicts of Interest**
BAI is an extended Board Member of the Mitochondrial Physiology Society (MIP) and on the MIP 2026 planning committee. All other authors declare no conflicts of interest.

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