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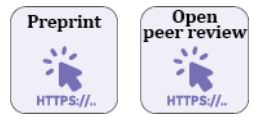


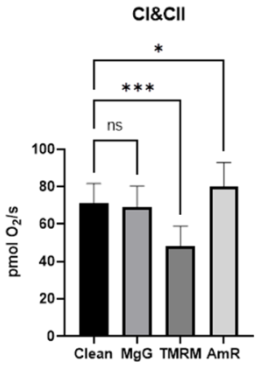
Effect of selected fluorophores on equine skeletal muscle mitochondrial respiration

Michael S. Davis[1], Montana R. Barrett[1], Warwick M. Bayly[2], Amanda Bolinger[1]

[1]Department of Physiological Sciences, College of Veterinary Medicine, Oklahoma State University
[2]Department of Veterinary Clinical Sciences, Washington State University, Pullman, WA, USA

* Corresponding author: michael.davis@okstate.edu

Summary



Equine skeletal muscle provides a rich source of mitochondria, suitable for both basic and applied studies of cellular energetics. Inclusion of fluorophores and the associated endpoints provides valuable information to complement measurements of mitochondrial oxygen flux, but investigators must be aware of the possible artifacts created by those fluorophores. Our studies demonstrate that Magnesium Green has no significant effect on respiration of isolated mitochondria, whereas tetramethylrhodamine inhibits phosphorylating respiration and increases LEAK respiration. Amplex UltraRed increases phosphorylating and non-phosphorylating respiration through Complex I. The effect of Amplex UltraRed is a novel finding, and further study is necessary to determine the mechanism underlying this artifact.

Keywords – horse, Amplex UltraRed, Magnesium Green, Tetramethylrhodamine, LEAK respiration

1. Introduction

Fluorespirometry is the analytical technique of using a combination of oxygen sensors and fluorescent dyes to quantify the metabolic activity of living cells or cellular organelles. Although the use of both techniques has been well-established in the scientific literature for many decades, recent advances in the precision of the analytical equipment has allowed for growing adoption of these tools in scientific laboratories around the world. Concurrent with this expansion has been the recognition of subtle differences in cellular metabolic properties between species and tissues (Doerrier et al 2018), highlighting the need for species- and tissue-specific analytical protocols for fluorespirometry.

Extrapolation of data obtained *ex vivo* to the relevant *in vivo* condition is best achieved when there is minimal analytical artifact, and in instances in which analytical artifact is unavoidable, it is important to know the precise nature of the analytical artifact. The use of fluorescent dyes in fluorespirometry provides a clear example of this challenge – fluorescent dyes, while absolutely necessary for measuring the endpoints of interest, may alter the cellular metabolism responsible for those endpoints (Krumschnabel et al 2014; Makrecka-Kuka et al 2015). To address this aspect of fluorespirometry, we compared the effects of 3 different common dyes used in fluorespirometry – Amplex UltraRed (used to quantify production of hydrogen peroxide), Magnesium Green (used to quantify ATP synthesis) and tetramethylrhodamine methylester (TMRM, used to quantify mitochondrial membrane potential) on oxygen flux in freshly-isolated equine skeletal muscle mitochondria.

2. Experimental methods

Study #1 was approved by the Washington State University Institutional Animal Care and Use Committee, and Study #2 was approved by the Oklahoma State University Institutional Animal Care and Use Committee.

2.1. Study population and sample preparation

Study #1 used 7 healthy Thoroughbred horses (2 mares, 5 geldings, 8.3±3.5 yr, range 4-13 yr). Biopsies were obtained before and after a 9-week aerobic conditioning program intended to increase fitness (see (Davis et al 2023) for full description of the study design). Study #2 used 6 healthy adult Thoroughbred geldings (15±5.8 yr, range 7-25 yr). Horses were at minimal aerobic fitness at the time of the study, having been housed together in pasture with no compulsory exercise for at least 24 months. None of the horses in either study had received any medications for at least 2 weeks prior to any biopsy procedure.

2.2. Sample preparation and high-resolution respirometry

Skeletal muscle biopsies were obtained using sterile technique from the center of the semitendinosus muscle using a 12 ga UCH biopsy needle (Millenium Surgical Corp, Bella Wynn, PA) while under light sedation (xylazine 0.5 mg/kg IV) and using local anesthesia (2 mL mepivacaine 2 % solution infiltrated SQ) (Davis et al 2023). Biopsies were immediately transferred into vials with ice-cold BIOPS solution (2.77 mM CaK_2-

EGTA, 7.23 mM K_2-EGTA, 20 mM imidazole, 20 mM taurine, 50 mM K-MES, 0.5 mM dithiothreitol, 6.56 mM $MgCl_2$, 5.77 mM ATP, and 15 mM phosphocreatine, adjusted to pH 7.1) and transported with tubes in crushed ice to the laboratory (< 3 miles) for analysis. Mitochondria were isolated using a commercial kit (MITOISO1, Sigma Aldrich) according to the manufacturer's instructions, with the exception of using substrate-free media (225 mM mannitol, 75 mM sucrose, 1 mM EGTA) for suspension of the isolated mitochondria at the end of the procedure. The final pellet of isolated mitochondria was resuspended using 80 µL of suspension media per 100 mg of muscle used for mitochondria isolation. Study #1 was part of a larger study that investigated multiple different incubation conditions in random order using samples obtained from unfit rested horses, unfit horses after fatiguing exercise, fit rested horses, and fit horses after fatiguing exercise (Davis et al 2023), so multiple assays were performed on a single biopsy. In order to ensure that mitochondria were freshly isolated immediately prior to analysis, mitochondria were isolated in 2 batches approximately 4 hr apart, with mitochondria added to the respirometers immediately upon completion of the isolation procedure (biopsy material used in the second batch isolation remained in ice-cold BIOPS until processing) Thus, data from 3 horses in this report were obtained from mitochondria isolated approximately 30 min after biopsy, and data from the other 4 horses were obtained from mitochondria that were isolated from tissue that remained in ice-cold BIOPS for approximately 3.5-4 h prior to processing. In Study #2, biopsy material was processed immediately and maximum interval from the time of the biopsy procedure to the isolation of mitochondria was less than 30 minutes.

High-resolution respirometers (Oxygraph O2k, Oroboros Instruments, Innsbruck, Austria) were used to analyze the effects of different fluorophores on mitochondrial oxygen consumption as previously described (Davis, Barrett 2023). Respirometers were calibrated with room air prior to each assay. Samples were analyzed in duplicate for each tested condition. Respirometer chambers (2 mL) were filled with low-Mg MiR05 (0.5 mM EGTA, 1 mM $MgCl_2$, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM sucrose, 1 g/L BSA essentially fatty acid-free, adjusted to pH 7.1) and instruments were set at 38 °C to represent basal temperature of equine skeletal muscle. Instrument oxygen sensors were calibrated daily, and oxygen consumption was calculated as the negative slope of the oxygen measurement and reported as $pmol \times s^{-1} \times mL^{-1}$. In Study #1, samples were analyzed in duplicate with either Magnesium Green (1.1 µM) or Amplex UltraRed (10 µM), along with associated assay reagents (superoxide dismutase (5 U/mL) and horseradish peroxidase (1 U/mL)) in 2 separate high-resolution respirometers concurrently. In Study #2, the effects of the different fluorophores on mitochondrial respiration were measured using 4 high-resolution respirometers operated in parallel, with each sample analyzed in duplicate. The first machine did not contain any fluorophore and served as the control (Clean) for the remaining 3 machines containing Magnesium Green, TMRM (0.4 µM), or Amplex UltraRed. For both studies, 15 µL of isolated mitochondria suspension was added to each incubation chamber. In all cases, assays were performed using aliquots from the same mitochondrial suspension for a given biopsy, and the sample was vortexed between each titration to maintain uniform suspension of the sample. Residual oxygen consumption (*Rox*) was measured prior to the addition of any substrates (Gnaiger 2020), and this value was subtracted from the oxygen consumption values of each step in the Substrate-Uncoupler-Inhibitor Titration (SUIT) protocol. The SUIT consisted of sequential titrations of pyruvate (5 mM), glutamate (10 mM), and malate (2 mM) into each chamber to produce NADH and stimulate non-

phosphorylating (LEAK) respiration supported by NADH oxidized through Complex I (L_N). ADP (5 mM) was then added to stimulate phosphorylating respiration (OXPHOS) through Complex I (P_N). The addition of succinate (10 mM) resulted in phosphorylating respiration through the combination of Complex I and Complex II (P_{NS}). Finally, rotenone (0.5 µM) was added to block Complex I, with the resulting oxygen flux representing the capacity of Complex II to support mitochondrial oxygen consumption through the oxidation of succinate alone (P_S). Nomenclature used for each state follows the paradigm of the designating the overall mitochondrial state (*L* or *P* for LEAK or phosphorylating, respectively), with subscripts denoting the specific electron feed (N, S, or NS for NADH, succinate, or both, respectively. Note that no explicit uncoupling titration was used due to past findings that at physiological incubation temperature (38 °C), OXPHOS capacity of equine mitochondria is not limited by the phosphorylation system. Therefore, oxygen consumption does not increase following titration of an uncoupler in the presence of kinetically-saturating ADP concentrations (Davis et al 2020). Thus, the results following ADP titration, while representing a state of phosphorylating respiration, also represent the maximum capacity of the electron transfer system. In Study #1, citrate synthase activity was determined on the remaining isolated mitochondrial suspension using a commercial kit (MAK193, Sigma Aldrich) and following manufacturer's instructions.

2.3. Data reduction and analysis

For all protocols, LEAK respiration was expressed as both *Rox*-corrected oxygen flux and as the percentage of the corresponding *Rox*-corrected phosphorylating respiration ($FCR_L = L_N/P_N \times 100$). The flux control ratio for NADH-supported and succinate-supported respiration (FCR_N and FCR_S, respectively) was calculated as the proportion of corrected maximal respiration supported by Complex I ($P_N/P_{NS} \times 100$) and Complex II ($P_S/P_{NS} \times 100$), respectively, and oxidative phosphorylation apparent control efficiency was calculated as $1\text{-}L_N/P_{NS}$. Results for each parameter of interest were calculated for individual respirometer chambers, then duplicates were averaged by subject for each experimental condition. In Study #1, data were analyzed using paired student's t-test. In all cases, the AmR and MgG assays for a given biopsy were performed on aliquots of a single mitochondrial suspension, eliminating the possibility of an effect of sample or isolation differences affecting the conclusions of this report. In Study #2, data were analyzed using repeated measures one-way ANOVA (Prism 9.5.1, GraphPad Software, San Diego, CA). If the overall ANOVA yielded $p<0.05$, then post-hoc pairwise comparisons were performed using Fisher's Least Squares Difference to compare the effects of the different fluorophores to the control. Similar to Study #1, all fluorophore assays for a given biopsy were performed on aliquots of a single mitochondrial suspension, eliminating the possibility of an effect of sample or isolation differences affecting the conclusions of this report. Finally, the AmR and MgG data from both studies were analyzed together for effects of study and fluorophore using 2-way repeated measures ANOVA, with subjects within a single study as the repeating factor. In all cases, the residual plots were examined to confirm that the presumption of normal distribution of data was valid.

3. Results

In Study #1, Amplex UltraRed was associated with higher L_N, P_N, and P_{NS} values, a lower FCR_S value, and a slightly higher value for calculated Efficiency (Table 1). In Study #2, there was no effect of MgG on any measured or calculated respirometry value. TMRM increased L_N by 52 % (Figure 1a) and decreased $P_{(NS)}$ by 32 % (Figure 1b), the latter due to inhibition of both P_N and P_S (Figure 1c and d). P_N was inhibited to a much greater degree than P_S (41 % and 11 %, respectively), resulting in a decrease in FCR_N but an increase in FCR_S (Figure 2a and b) despite decreased flux through CII. In addition, there was a modest decrease in OXPHOS efficiency. FCR_L tripled in the presence of TMRM due to the combination of increased L_N and decreased phosphorylating respiration through Complex I. AmR caused a 12 % increase in P_{NS} (Figure 1b) despite causing a 13 % decrease in CII-based respiration (Figure 1d) and corresponding decrease in FCR_S, and no significant change in CI-based respiration.

Table 1. Comparison of respirometry variables obtained with either Magnesium Green or Amplex UltraRed.

	MgG	AmR
L_N	2.78±0.96	3.25±1.13***
P_N	59.99±20.40	75.03±32.02***
P_{NS}	89.27±29.54	113.70±40.12***
P_S	49.82±15.70	52.47±17.24
FCR_L	4.71±0.66	4.69±1.64
FCR_N	66.88±3.86	64.87±9.70
FCR_S	64.87±9.70	46.62±4.53***
Efficiency	0.969±0.004	0.971±0.006*

Data from Study #1, N=7 subjects, 27 biopsies (one horse was biopsied 3 times, all other horses biopsied 4 times). Data shown as mean±SD *p<0.05, ***p<0.001 using paired student's t-test.

The combined analysis of the Study #1 and Study #2 data found an effect of fluorophore on L_N, P_N, P_{NS}, and FCR_S (p=0.0015, p=0.0415, p=0.0002, and p<0.0001, respectively), and a significant interaction between study and fluorophore on P_S (p=0.0432). Furthermore, there were trends (p<0.1) towards an independent effect of Study on P_{NS}, P_S, FCR_L, FCR_S, and Efficiency.

Citrate synthase activity in samples obtained in Study #1 was 0.063±0.014 nmol·min^{-1}·µL^{-1} of isolated mitochondria suspension for biopsies obtained from unfit rested horses, 0.092±0.009 nmol·min^{-1}·µL^{-1} for biopsies obtained from unfit horses after exercise, 0.073±0.014 nmol·min^{-1}·µL^{-1} for biopsies obtained from fit rested horses, and 0.101±0.016 nmol·min^{-1}·µL^{-1} for biopsies obtained from fit horses after exercise. The use of paired statistical analysis controlled for sample-to-sample variability in mitochondria content.

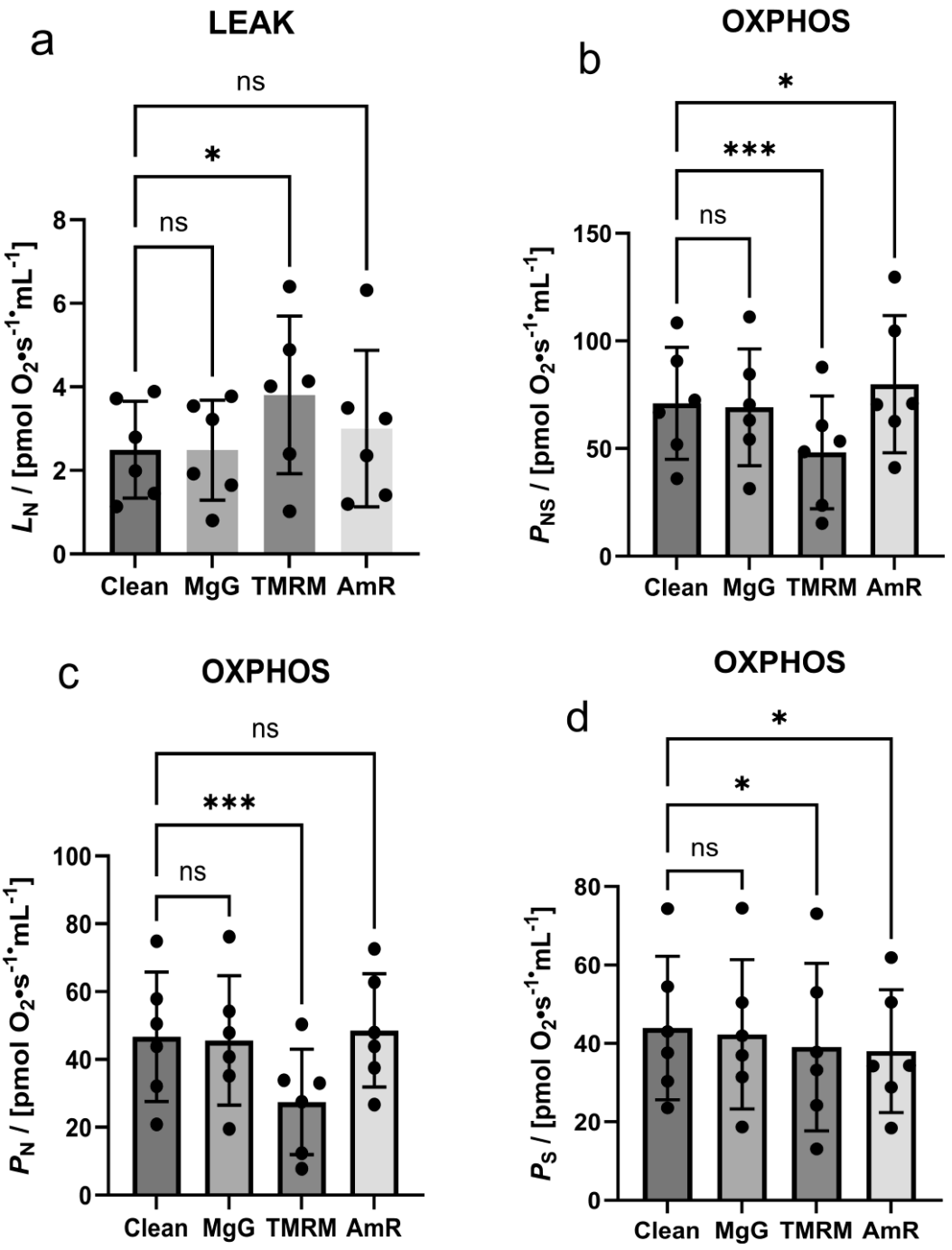


Figure 1. Effect of fluorophores on LEAK and OXPHOS respiration. a: LEAK; **b:** OXPHOS through Complex I & II; **c:** OXPHOS through Complex I; **d:** OXPHOS through Complex II. N=6 subjects, ***=significantly different from control assay, p<0.001 *=significantly different from control assay, p<0.05 on one-way repeated measures ANOVA and post-hoc Fisher's Least Squares Differences. Data shown as mean±SD.

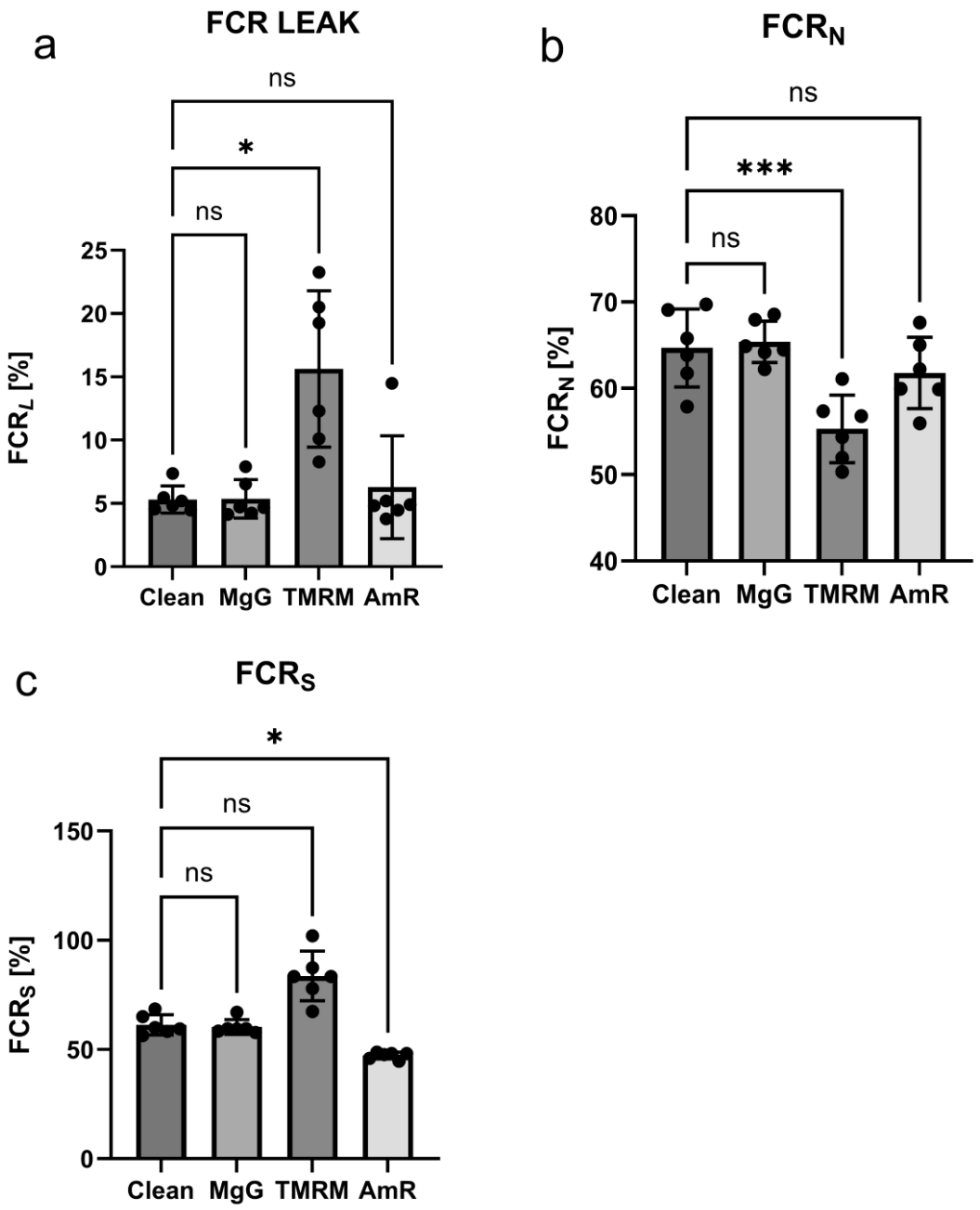


Figure 2. Effect of fluorophores on Flux Control Ratios. **a:** LEAK Flux Control Ratio; **b:** Complex I Flux Control Ratio; **c:** Complex II Flux Control Ratio. N=6 subjects, ***=significantly different from control assay, $p<0.001$ *=significantly different from control assay, $p<0.05$ on one-way repeated measures ANOVA and post-hoc Fisher's Least Squares Differences. Data shown as mean±SD.

4. Discussion

An ideal *ex vivo* assay accurately replicates the corresponding *in vivo* phenomenon. In that regard, the use of Magnesium Green (MgG) is a useful fluorophore for studies of mitochondrial physiology in that its presence in the assay media did not have any detected

effects on respiration of equine skeletal muscle mitochondria compared to the fluorophore-free control. This finding is consistent with what has been reported by Cardoso et al for mitochondria isolated from mouse cardiac muscle (Cardoso et al 2021). It is important to note that the assay using MgG to quantify the rate of ATP synthesis (Chinopoulos et al 2014) employs a lower concentration of magnesium in the incubation media than is typically recommended. Although this is 1/3rd the concentration typically used for high-resolution respirometry, previous studies have demonstrated that the addition of 1 M total Mg^{2+} results in concentrations of free Mg^{2+} that are comparable to that found in living cells.

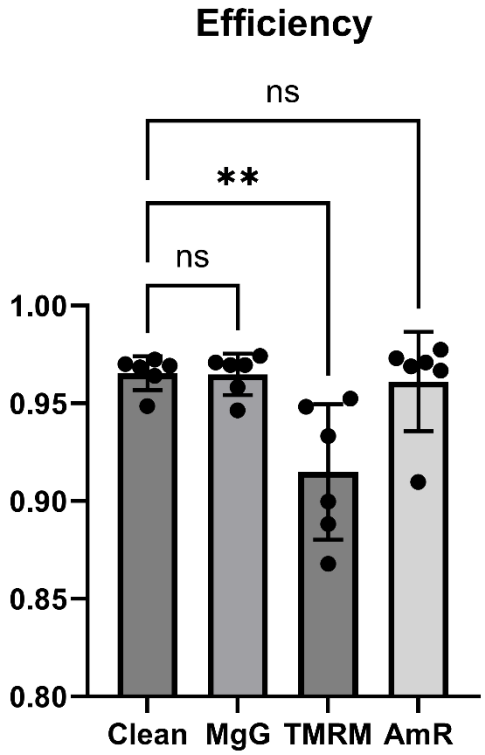


Figure 3. Effect of fluorophores on calculated efficiency. N=6 subjects, **=significantly different from control assay, $p<0.01$ on one-way repeated measures ANOVA and post-hoc Fisher's Least Squares Differences. Data shown as mean±SD.

TMRM inhibited phosphorylating respiration through both Complex I and Complex II, although not equally: respiration through Complex I was affected to a greater extent than respiration through Complex II. This is similar to the pattern reported for safranin, which like TMRM is used to measure mitochondrial membrane potential (Krumschnabel et al 2014). In the case of safranin, the inhibition of phosphorylating respiration is believed to be due primarily to inhibition of phosphorylation and, to a lesser extent, inhibition of respiration because the addition of uncoupler results in partial recovery of respiratory capacity. However, our previous work has demonstrated that when incubating at 38 °C, uncoupler titrations do not increase respiration above OXPHOS capacity (Davis et al 2020), showing no limitation of the electron transfer capacity by the components of the phosphorylation system. This lack of ETS limitation by the phosphorylation system at 38°C, as well as the increase in proton leak, may have prevented the detection of phosphorylation inhibition by TMRM. In contrast to safranin, TMRM caused a 3-fold increase in LEAK respiration, comparable in magnitude to that caused by physiological hyperthermia (Davis et al 2020). The mechanism for TMRM-induced proton leak is unknown but is mechanistically consistent with the progressive uncoupling of phosphorylating respiration that has been previously reported (Sumbalova, Gnaiger 2015). The fluorescent signal from TMRM displayed the predicted changes in relative membrane potential, at least at a qualitative level, but due to the fluorophore-associated changes in mitochondrial respiration, TMRM may not be the best tool for measurement of membrane potential. Inhibition of respiration by fluorophores is typically dose-dependent; therefore, additional testing may be needed to minimize the concentrations used while maintaining sufficient fluorescent signal for quantification of mitochondrial membrane potential.

The reagents that make up the Amplex UltraRed assay for mitochondrial ROS production were associated with an increase in respiration (both non-phosphorylating and phosphorylating) supported by NADH oxidation through Complex I in younger horses that were in an exercise conditioning program; however, the AmR assay was associated with inhibition of phosphorylating respiration through Complex II in older, unfit horses. The difference in fitness history is a logical explanation for the trends towards greater mitochondrial respiration in the subjects of Study #1, and may also be responsible for the difference in sensitivity to the AmR assay reagents. A 2015 study by Makrecka-Kuka et al reported that, at concentrations greater than 10 µM, Amplex Red had an inhibitory effect on respiration through a combination of Complex I and II in HEK 293T cells (Makrecka-Kuka et al 2015). The stimulatory effect reported here is a novel and unexpected finding. Differences in sample type and preparation, and the additional presence of superoxide dismutase and horseradish peroxidase in this study compared to the previously published study, may be responsible for the different results. The specific mechanism for the Amplex UltraRed stimulation of respiration is unknown and requires further research, but the artifact created by this assay on mitochondrial respiration should be considered when assessing experimental results of similar or smaller magnitude.

Abbreviations

AmR	Amplex UltraRed	FCR_S	Complex II flux control ratio
BIOPS	biopsy preservation medium	MgG	Magnesium Green
FCR_L	LEAK flux control ratio	TMRM	Tetramethylrhodamine
FCR_N	Complex I flux control ratio	*Rox*	Residual oxygen consumption rate

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