Reproducibility of NIRS-derived mitochondrial oxidative capacity in highly active older adults

Original Investigation

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HIGHLIGHTS

- NIRS is a reliable method for deriving a measure of mitochondrial oxidative capacity in lean, active older adults.
- NIRS is a practical and non-invasive approach of routinely assessing skeletal muscle mitochondrial oxidative capacity.
- NIRS-derived mitochondrial capacity shows a significant relationship with measures of aerobic fitness in older adults.

ABSTRACT

Introduction

In-vivo techniques using near-infrared spectroscopy (NIRS) have been developed to assess skeletal muscle mitochondrial oxidative capacity. However, the test-retest and day-to-day reliability of NIRS-derived mitochondrial oxidative capacity has yet to be established in older individuals. Therefore, the primary aim of this study was to determine the day-to-day and test-retest reliability of NIRS-derived mitochondrial oxidative capacity in older adults. The secondary aim was to examine the relationship between NIRS-derived mitochondrial capacity and whole-body aerobic fitness.

Material and Methods

Twenty-four healthy individuals (19M, 5F; aged 60 ± 4 years; maximal oxygen uptake ($\dot{V}O_{2peak}$) = 41.2 ± 6.8 $ml.kg^{-1}.min^{-1}$) completed three visits to the laboratory. Visit one assessed isometric maximal voluntary contractions of the knee extensors and aerobic capacity through an incremental exercise test. In visits two and three participants completed two measurements of NIRS-derived mitochondrial oxidative capacity in the vastus lateralis (VL).

Results

NIRS-derived mitochondrial oxidative capacity was found to have good to excellent day-to-day reliability (Day 1 vs Day 2; coefficient of variation (CV) = 7.0%; standard error of measurement (SEM) = 5.2; intra-class correlation coefficient (ICC) = 0.94) and test re-test reliability (Day 1 [Test 1 vs Test 2]; CV = 5.0%; SEM = 3.7; ICC 0.97 and Day 2 [Test 1 vs Test 2]; CV = 6.3%; SEM = 4.9; ICC = 0.93). NIRS-derived mitochondrial oxidative capacity was found to be significantly correlated with $\dot{V}O_{2peak}$ (r = -0.61; R^2 = 0.37; *P* = 0.002), oxygen uptake at the gas exchange threshold (r = -0.49; R^2 = 0.24; *P* = 0.02), and oxygen uptake at the respiratory compensation point (r = -0.57; R^2 = 0.32; *P* = 0.004).

Conclusion

NIRS provides a reliable method for deriving a measure of VL mitochondrial oxidative capacity in highly active older adults and demonstrates a significant relationship with measures of whole-body aerobic fitness.

KEYWORDS. reproducibility; ageing; near-infrared spectroscopy; aerobic fitness.

ABBREVIATIONS

^{31}P-MRS	Phosphorus magnetic resonance spectroscopy
CV	Coefficient of variation
HHb	Deoxygenated haemoglobin
HHbmax	HHb signal at 0% oxygenation
HRR	High-resolution respirometry
ICC	Intra-class correlation coefficient
IET	Incremental exercise test
MVC	Maximal voluntary contraction
$m\dot{V}O_2$	Muscle oxygen consumption
NIRS	Near-infrared spectroscopy
O_2Hb	Oxygenated haermoglobin
O_2Hbmax	O_2Hb at 100% oxygenation
O_2Hbmin	O_2Hb at 0% oxygenation
SEM	Standard error of measurement
tHb	Total haemoglobin
VL	Vastus lateralis
$\dot{V}O_{2peak}$	Maximal oxygen uptake
$\dot{V}E/\dot{V}O_2$	Ventilatory equivalent of oxygen
$\dot{V}E/\dot{V}CO_2$	Ventilatory equivalent of carbon dioxide

1. INTRODUCTION

Ageing has widely been accepted to result in reduced mitochondrial oxidative capacity in human skeletal muscle (Conley et al. 2000; Short et al. 2005; Conley et al. 2007; Johannsen et al. 2012). However, this decline is also likely attributable to reduced physical activity levels (Rasmussen et al. 2003; Grevendonk et al. 2021). Reduced mitochondrial oxidative capacity has been linked to age-related declines in muscle function and physical performance (Conley et al. 2007; Coen et al. 2013; Choi et al. 2016), in addition to the potential development of conditions including cardiovascular disease, diabetes and neurodegenerative diseases (Ballinger 2005; Szendroedi et al. 2012; Breuer et al. 2013; Wallace 2013). Given the crucial role of mitochondria in maintaining physiological function throughout life and into older age, it is important clinicians and researchers have validated techniques which are able to reliably assess mitochondrial function.

The current gold standard for measuring mitochondrial oxidative capacity is the *in vitro* measurement of high-resolution respirometry (HRR) performed in permeabilized muscle fibres (Lanza & Nair 2009). *In vitro* techniques allow for tight control of experimental conditions, thus providing important mechanistic insights into mitochondrial function (Kuznetsov et al. 2008; Lanza & Nair 2009). However, *in vitro* techniques have their limitations, namely the collection of invasive muscle biopsies and their translational significance with protocols subjecting collected tissues to non-physiological conditions. Phosphorus magnetic resonance spectroscopy (^{31}P-MRS) has been utilised to study mitochondrial oxidative capacity in living tissue, without the need for invasive procedures (Layec et al. 2011; Ryan et al. 2013). Unfortunately, the ^{31}P-MRS technique is difficult to apply widely, due to the high cost and limited availability of multinuclear MR scanners.

To overcome the limitations of the HRR and ^{31}P-MRS techniques, researchers have developed *in-vivo* techniques for assessing skeletal muscle mitochondrial oxidative capacity using NIRS (Motobe et al. 2004; Hamaoka et al. 2007; Ryan et al. 2012, 2013, 2014). NIRS devices continuously measure the absorption of near-infrared light at different wavelengths by the tissue of interest, allowing for the calculation of changes in deoxygenated haemoglobin (HHb) and oxygenated haemoglobin (O_2Hb; Scholkmann et al. 2014). By combining an exercise stimulus to increase muscle oxygen consumption ($m\dot{V}O_2$) with a repeated arterial occlusion protocol, it is possible with NIRS to measure $m\dot{V}O_2$ recovery, from which a time constant can be derived being a measure of mitochondrial oxidative capacity (Ryan et al. 2012, 2013, 2014). A faster time constant is indicative of a greater mitochondrial oxidative capacity (Motobe et al. 2004).

Importantly, NIRS-derived indices of skeletal muscle mitochondrial oxidative capacity have been shown to correlate well with HRR and ^{31}P-MRS measurements of skeletal muscle mitochondrial oxidative capacity (Ryan et al. 2013, 2014). Researchers have also found good reliability within and across days of NIRS-derived measurements of skeletal muscle mitochondrial oxidative capacity in young healthy individuals (Ryan et al. 2012; Southern et al. 2013; La Mantia et al. 2018; Beever et al. 2020; Hovorka et al. 2021; Hanna et al. 2021). NIRS therefore provides a cost effective, practical, and non-invasive reliable approach of routinely assessing skeletal muscle mitochondrial oxidative capacity *in-vivo*. However, the test-retest and day-to-day reliability has yet to be established in older individuals. Establishing the reliability of a cost effective and non-invasive measurement of mitochondrial function in older adults is of importance, given the potentially deleterious effect of ageing on mitochondrial function (Conley et al. 2000).

The mitochondria's oxidative capacity has been shown to be closely associated with whole-body aerobic capacity and exercise performance (Holloszy 1967; Gollnick et al. 1972; Adelnia et al. 2019). NIRS-derived measurements of mitochondrial oxidative capacity have also been correlated with whole body aerobic capacity in young adults (Brizendine et al. 2013; Beever et al. 2020; Guzman et al. 2020; Hovorka et al. 2021), but to the authors' knowledge such a comparison has not been made with older adults.

The primary aim of the current study was to assess the test-retest and day-to-day reliability of skeletal muscle mitochondrial oxidative capacity using NIRS in older individuals. It was hypothesised that NIRS-derived mitochondrial oxidative capacity would demonstrate good reproducibility within participant, and within and between days. The secondary aim of the study was to determine the relationship between NIRS-derived mitochondrial oxidative capacity and whole-body measures of aerobic fitness. It was hypothesised that NIRS-derived mitochondrial oxidative capacity would be correlated with measures of aerobic fitness.

2. METHODS

2.1. Participants

Twenty-eight healthy individuals (21 male, 7 female) between the ages of 50 and 70 years were recruited to participate in the study. All participants were regular exercisers, performing above the World Health Organisation guidelines (i.e., 2.5 to 5 hours of moderate exercise per week; Bull et al. 2020). Participants were required to be

non-obese, non-smokers, not have previous or current circulatory disorders, have no known or signs/symptoms of cardiovascular, neuromuscular, renal, or metabolic conditions, not be physically impaired (i.e., able to perform maximal exercise) and not have blood pressure greater than 140/90 mmHg. The study was completed with full ethical approval of the local Research Ethics Committee, according to Declaration of Helsinki standards. All participants provided written informed consent prior to testing.

2.2. Experimental design

Each participant completed three visits to the laboratory at the same time of day (±1 hour). Visit one being participant screening, laboratory familiarisation, measurement of isometric maximal voluntary contractions (MVC) of the knee extensors, and an incremental exercise test (IET) to determine aerobic capacity. At visits two and three, participants completed the measurements of NIRS-derived mitochondrial oxidative capacity.

Visits were conducted on non-concurrent days (with a maximum gap of 7 days between visits) and participants were instructed to refrain from any exercise in the day prior to testing and intense exercise in the two days prior. Participants were instructed to arrive euhydrated and in a post-prandial state, having eaten at least 4-hours prior to testing. Participants were told to not consume caffeine within 4-hours and alcohol within 24-hours of testing.

2.3. Preliminary measurements and incremental exercise testing

At visit one prior to exercise testing all participants provided written informed consent, completed a health questionnaire and the long form international physical activity questionnaire (Craig et al. 2003). Resting blood pressure, participant height, body mass and body composition were then measured.

Participants were seated on the Cybex isokinetic dynamometer (HUMAC Norm; CSMi, Stoughton, MA, USA), initialised and calibrated according to the manufacturer's instructions. The participants right leg was securely attached to the lever arm of the dynamometer, with their lateral epicondyle of the right femur in line with the axis of rotation of the lever arm. Participants knee angle were set at 90 degrees, with full extension being 0 degrees. Participants wore an over shoulder and waist seat belt to prevent unwanted movement and use of hip extensors during the contractions. Seating positions were recorded to ensure replication of setup in subsequent visits.

Once setup on the dynamometer, participants performed a warm-up of ten submaximal contractions of increasing effort after which a series of brief (6 s) MVCs were performed to establish maximum torque. MVCs were repeated (separated by 60 s rest) until a plateau in peak torque was reached (i.e., until three consecutive peak torques were within 5% of each other). The highest torque value was recorded as the MVC, which was then used to set the isometric exercise intensity (40% of MVC) for the NIRS-derived mitochondrial oxidative capacity protocol.

Participants then rested for 30 minutes before commencing the IET. The IET protocol was performed on a Lode Excalibur Sport (Groningen, The Netherlands). Participants completed a 10-minute warm-up at 50 W, after which the required cycling power output increased by 25 W every minute (i.e., 1 W every 2.4 s) until they reached volitional exhaustion (operationally defined as a cadence of < 60 revolutions/min for > 5 s, despite strong verbal encouragement).

During the IET respiratory gas exchange data were assessed using an Metalyzer 3B Cortex, online breath by breath gas analyser (Metalyzer 3B; CORTEX Biophysik GmbH, Leipzig, Germany). Prior to all testing the Cortex analyser was calibrated with ambient air and known concentrations of oxygen (17%) and carbon dioxide (5%). The bidirectional turbine (flow meter) was calibrated with a 3-litre calibration syringe.

2.4. NIRS-derived mitochondrial oxidative capacity protocol

Visits two and three involved the test re-test measurement of NIRS-derived mitochondrial oxidative capacity. To measure mitochondrial oxidative capacity NIRS was combined with a brief exercise stimulus and repeated transient arterial occlusions (Ryan et al. 2012).

NIRS data was collected using a portable continuous-wave NIRS device (Portamon, Artinis Medical Systems, The Netherlands), which simultaneously uses the Beer-Lambert and spatially resolved spectroscopy method. The three transmitters each emitted two-wavelengths of light (760 and 850 nm), and the optode distance was set at 35mm.

The NIRS optode was placed over the right VL muscle, 8 to 12 cm from the knee joint on the vertical axis. NIRS device location was outlined with an indelible marker to ensure reliable probe placement across visits. The NIRS device was covered with a soft black cloth to prevent signal contamination from external light sources and was affixed using kinesio tape (Kinesio Precut, Albuquerque, NM, USA) and a velcro strap to prevent movement.

Skinfold thickness at the site of application of the NIRS optode was determined before the testing sessions using Harpenden skinfold callipers (British indicators Ltd, Burgess Hill, UK).

A blood pressure cuff was placed at the top of the right thigh to obstruct the femoral artery, proximal to the NIRS device. To normalise the NIRS signal, a 5-minute arterial occlusion to deoxygenate the tissue under the NIRS optode (i.e., Ischemic physiological calibration) was applied using the blood pressure cuff (Hokanson SC12D; Bellevue, WA, USA) connected to a rapid-inflation system set to 300 mmHg (Hokanson E20; Bellevue, WA, USA), with the lowest O_2Hb taken as the measure of 0% oxygenation (O_2Hbmin and HHbmax). The peak hyperaemic response upon release of the blood pressure cuff indicated 100% oxygenation (O_2Hbmax). After which resting blood flow was measured as described by Southern et al. (2013) using the change in total haemoglobin (tHb) during three 90 s venous occlusions at 60 mmHg. Participants then rested for a 5-minute period to allow the NIRS signal to stabilise.

Prior to the commencement of exercise, a 10 s arterial occlusion (300 mmHg) was applied to measure resting $m\dot{V}O_2$. Participants then performed a set of 10 x 10 s isometric knee extension repetitions at 40% of MVC with 10 s rest periods between contractions, using the same dynamometer setup as visit 1.

Immediately following exercise, a series of 20 brief (10 s) arterial occlusions was applied. To minimise the discomfort to participants, the duration between arterial occlusions began at 10 s and extended to 20 s by the end of the repeated occlusions (i.e., 10 s for occlusions *1-10*, 15 s for occlusions *11-15*, 20 s for occlusions *16-20)* as recommended by Ryan et al. (2012). After 20-minutes rest, the exercise bout and arterial occlusions were repeated, providing two measures of mitochondrial oxidative capacity within a day. Figure 1A presents an example NIRS signal from one full test of mitochondrial oxidative capacity.

[Figure 1 here]

2.5. NIRS-derived mitochondrial oxidative capacity data analysis

NIRS data was acquisitioned via Bluetooth connection to a personal laptop and then exported at 10 Hz. NIRS data was then analysed using a custom written excel spreadsheet.

The method of blood volume correction as previously described by Ryan et al. (2012) was applied to the NIRS data. The application of the blood volume correction factor assumes that during an arterial occlusion, the changes in O_2Hb and HHb occur with a 1:1 ratio that represents mitochondrial oxygen consumption only, making the area under the NIRS optode a closed system (Ryan et al. 2012).

Equation. 1 below describes the calculation of the blood volume correction factor (β: which corrects the NIRS signal for changes in blood volume, proportioned into oxygenated and deoxygenated sources):

$$[1]\ \beta(t) = \frac{\mathrm{O2Hb(t)}}{(\mathrm{O2Hb(t)+HHb}(t))}$$

β = blood volume correction factor, *t* = time, O_2Hb = oxygenated haemoglobin/myoglobin signal, HHb = deoxygenated haemoglobin/myoglobin signal.

The β was calculated for each data point to account for small changes in the proportionality of the blood volume change throughout the cuff. Each data point was then corrected using the corresponding β according to equations 2 and 3 below:

$$[2]\ \mathrm{Corrected\ O2Hb} = \mathrm{O2Hb} - [\mathrm{tHb\ x}\ (1 - \beta)]$$

$$[3]\ \mathrm{Corrected\ HHb} = \mathrm{HHb} - (\mathrm{tHb\ x}\ \beta)$$

Using the corrected HHb signal for each occlusion, the slope of the HHb data (i.e., $m\dot{V}O_2$) was calculated using a linear regression over a 3-s span of data, selected to maximize slope fit (i.e., R^2 value). All slopes were required to have an $R^2 > 0.90$ (Figure 2A), to be used in further analysis. This resulted in all the data of $N = 3$ participants being removed from further analysis. The change in β was also calculated over the 3-s span of arterial occlusion data used to calculate $m\dot{V}O_2$ (Figure 2B).

The $m\dot{V}O_2$ values (expressed as a percentage of the ischemic calibration per unit time; %/s) were then plotted against time and fit with a monoexponential decay equation:

$y(t) = End - Delta \times e^{-k/t}$

Where y(t) = relative $m\dot{V}O_2$ during arterial occlusions (i.e., ΔHHb) at time t; t = time; Delta = the difference in $m\dot{V}O_2$ between end of exercise and at rest; End = the $m\dot{V}O_2$ immediately after exercise ended; and k = the time constant, taken as the measure of mitochondrial oxidative capacity. The rate constant was calculated as (1/k)x60, with a higher value indicating greater mitochondrial oxidative capacity.

All R^2 values of the fit of the monoexponential equation were required to be > 0.90, with the mean being 0.94 ± 0.03. This resulted in all the data of $N = 1$ participant being removed from further analysis. The sum of squares of the monoexponential curve fit was also calculated with values < 1.0 being accepted as 'good' model fit, the mean from all curve fits being 0.39 ± 0.24. Figure 1B presents an example of one participant within day test-retest $m\dot{V}O_2$ recovery data from the repeated arterial occlusions.

Reperfusion rate was measured using the corrected O_2Hb signal after the 5-min occlusion and defined as the half-life in seconds to reach maximal oxygenation i.e., O_2Hbmax (maximal oxygenation being defined as the plateau in the peak hyperemic response). Maximal physiological range was calculated as the difference between O_2Hbmin and O_2Hbmax NIRS values during the ischemic calibration and reported in optical density (i.e., arbitrary units).

[Figure 2 here]

2.6. Gas exchange data analysis

The participant's $\dot{V}O_{2peak}$ was assessed as the highest oxygen uptake that was attained during a 1-minute period in the test. Participants gas exchange threshold was determined as the breakpoint in carbon dioxide production and oxygen consumption (i.e., the point at which the carbon dioxide production begins to increase out of proportion to the oxygen consumption). This breakpoint also coincided with the increase in both ventilatory equivalent of oxygen ($\dot{V}E/\dot{V}O_2$) and end-tidal pressure of oxygen with no concomitant increase in ventilatory equivalent of carbon dioxide ($\dot{V}E/\dot{V}CO_2$; Beaver et al. 1986; Pallares et al. 2016). The respiratory compensation point was determined as an increase in both the $\dot{V}E/\dot{V}O_2$ and $\dot{V}E/\dot{V}CO_2$ and a decrease in partial pressure of end-tidal carbon dioxide (Whipp et al. 1989; Lucia et al. 1999).

2.7. Statistical analysis

Data are presented as individual values or mean ± SD (unless specified otherwise). Statistical analyses were conducted using IBM SPSS Statistics 26 (IBM, Armonk, New York, USA). Visual inspection of Q-Q plots and Shapiro-Wilk statistics were used to check whether data were normally distributed. Only data from $N = 24$ (19M; 5F) participants were analysed and presented herein. In total $N = 96$ NIRS-derived measurements of mitochondrial oxidative capacity were analysed, $N = 4$ from each participant.

Day-to-day and test-retest reliability (within each day) of NIRS-derived mitochondrial oxidative capacity was assessed through Bland-Altman plots, by plotting the mean of the two compared values against the difference, Day 1 vs. Day 2 and Test 1 vs. Test 2 for each day. The 95 % limits of agreement were calculated (1.96 * SD of the difference) and two-way random ICC for absolute agreement and the average CV and SEM were also calculated (for all NIRS-derived metrics). CVs for within participant (all four tests of mitochondrial capacity), between participants, within day and between day were also calculated for all NIRS-derived metrics collected during the measurement of mitochondrial oxidative capacity.

Two-way repeated measures analysis of variance (ANOVA) was performed for Days × Tests of the time constant, to check if a between or within day effect was present.

The relationships between whole-body measures of aerobic fitness and NIRS-derived mitochondrial oxidative capacity were assessed using Pearson's correlation coefficient. For the correlation analysis the mean time constant of the participants four NIRS-derived mitochondrial oxidative capacity tests was used.

The significance level was set at $P < 0.05$ in all cases.

3. RESULTS

Twenty-four participants data (19M; 5F) were included in the analysis. Table 1 presents participant characteristics, anthropometrics and IET data.

[Table 1 here]

3.1. Reproducibility results

All data from NIRS-derived mitochondrial oxidative capacity tests and test-retest reliability data are presented in table 2.

[Table 2 here]

ANOVA revealed no significant effect of day ($P = 0.25$), test ($P = 0.06$) or interaction effect between day and test ($P = 0.74$) for the time constant. Reproducibility data for all NIRS-derived metrics are visually presented in figure 3.

[Figure 3 here]

Bland-Altman plots of day-to-day and test-rest reliability of the time constants are presented in figure 4, including ICC, average CVs and SEM. Day-to-day CV for the time constant was 7.0% (range 0.3 to 17.8%) with an ICC of 0.94. Day 1 test re-test CV for the time constant was 5.0% (range 1.5 to 11.7%) with an ICC of 0.97 and day 2 test re-test CV for the time constant was 6.3% (range 0.4 to 16.3%) with an ICC of 0.93.

[Figure 4 here]

3.2. Correlation results

Correlations between NIRS-derived mitochondrial capacity with measures of aerobic fitness are presented in figure 5, with r, R^2 and P values. NIRS-derived mitochondrial oxidative capacity was significantly correlated with all aerobic fitness metrics assessed (Figure 5), including $\dot{V}O_{2peak}$ ($r = -0.61$; $R^2 = 0.37$; $P = 0.002$), oxygen uptake at the gas exchange threshold ($r = -0.49$; $R^2 = 0.24$; $P = 0.02$), and oxygen uptake at the respiratory compensation point ($r = -0.57$; $R^2 = 0.32$; $P = 0.004$).

[Figure 5 here]

4. DISCUSSION

4.1. Reproducibility of NIRS-derived mitochondrial oxidative capacity

The main finding of the study was the good to excellent day-to-day (Figure 4A) and test re-test (Figures 4B & 4C) reliability of the time constant, being the NIRS-derived measure of mitochondrial oxidative capacity. The between day CV of the time constant in the current study is in line with previous research examining the day-to-day reliability of NIRS-derived mitochondrial oxidative capacity of the VL muscle in young individuals, which demonstrated CVs of 10.0% (Ryan et al. 2012), 8.9% (Beever et al. 2020), and 7.9% (Hovorka et al. 2021). The between day reproducibility results of the current study are also comparable to previous research reporting between day CV for the medial gastrocnemius muscle (10.0% to 11.2%; Southern et al. 2013).

As would be anticipated, within day CV was lower than between day CV, given there would be subtle differences in NIRS optode placement between days as well as inherent day-to-day biological variability. Notably, the test re-test reliability of the current study is comparable to the ^{31}P-MRS technique, which has been found to produce CVs of 7.6% (Ryan et al. 2013) and 3.5% (Lanza et al. 2011).

It is worth noting that reproducible NIRS-derived mitochondrial oxidative capacity measures can only be achieved through rigorously controlling NIRS setup and providing clear instructions to participants. The importance of methodological rigor is highlighted by the exclusion of four participants' NIRS data due to poor slope fit of HHb during one or more arterial occlusions ($R^2 < 0.90$), or a poor the fit of the monoexponential equation ($R^2 < 0.90$). From observation, the likely reason for the data not meeting the imposed criteria was the movement of the limb during the arterial occlusion, causing movement artifacts and incomplete occlusions. The potential limitations of NIRS-derived mitochondrial oxidative capacity have been discussed previously (Adami & Rossiter 2018). However, many of these limitations can be overcome to ensure 'technically acceptable' NIRS data is collected and analysed, through methodological choices (i.e., suitable exercise stimulus; NIRS location and setup; effective occlusion protocols) and data analysis practices (i.e., checking data is 'technically acceptable' prior to analysis; applying blood volume corrections), thus ensuring reproducible measurements.

The isometric knee extension exercise was utilised in the current study to ensure a repeatable metabolic stimulus was applied to the VL muscle and to minimise movement of the limb throughout the protocol. The effectiveness and repeatability of this exercise stimulus is demonstrated by the mean 6-fold increase in $m\dot{V}O_2$ from rest to immediately after exercise and the 10.5% within day CV and 8.3% between day CV for the $m\dot{V}O_2$ measured immediately after exercise (Table 2). Importantly, the exercise stimulus was not so intense that oxygen delivery

to the VL was limiting to $m\dot{V}O_2$ recovery, as shown by the tissue saturation index of 61.3 ± 9.2% at the end of exercise (Figure 2C). Based on current findings researchers may consider utilising fixed intensity isometric contractions as the exercise stimulus to measure NIRS-derived mitochondrial oxidative capacity.

In the current study all participants had low skin and adipose tissue thickness at the NIRS optode site (6.6 ± 2.6 mm; Table 1), allowing NIRS light to reach the muscle and be received at the NIRS detector more readily. Adipose tissue thickness has been shown to effect NIRS measurements (Van Beekvelt et al. 2001), thus current reproducibility results should be used with caution when assessing individuals with greater skin and adipose tissue thickness (i.e., > 10mm) at the muscle of interest. Additionally, mitochondrial oxidative capacity was only derived from the VL muscle, as such current reproducibility results should not be extrapolated to other muscles. Further research is required to extend the reproducibility of NIRS-derived oxidative capacity to other muscles in older adults.

The reliability statistics of all NIRS-derived metrics are reported in table 2 and individual participant data points visually presented in figure 3. The reproducibility of these metrics is important to deriving reliable measures of mitochondrial oxidative capacity with NIRS, while also being reference values for future research. Resting $m\dot{V}O_2$ (Figure 3A) and end $m\dot{V}O_2$ (Figure 3B), the main metrics in the determination of NIRS-derived mitochondrial oxidative capacity, were found to be highly reproducible (Table 2). Both metrics can also be used in isolation as a measure of muscle metabolic rate at rest and after exercise. The maximal physiological range was reproducible within participants and demonstrates the 5-minute ischemic physiological calibration occlusion to be a repeatable method for normalising the NIRS signal prior to the NIRS-derived mitochondrial oxidative capacity protocol (Table 2; Figure 3F).

The reperfusion rate of the muscle can also be ascertained from the 5-minute ischemic physiological calibration occlusion, with current data showing NIRS-derived reperfusion rate is reproducible in highly active older adults (Table 2; Figure 3E). Assessing reperfusion rate should be considered 'good' practice when using the NIRS-technique, as decreases in muscle reperfusion may impair muscle oxygen availability, and in turn negatively affect the measurement of NIRS-derived mitochondrial oxidative capacity. In addition, reperfusion rate can be used as a measure of muscle vasodilation and microvascular function. Ageing has been linked to declines in muscle vasodilation and microvascular function (Tonson et al. 2017), with researchers showing older adults to have a slower NIRS-derived reperfusion rate in comparison to younger adults (Lagerwaard et al. 2020).

4.2. Relationship between whole-body aerobic fitness and NIRS-derived mitochondrial oxidative capacity

The secondary findings of the study were the significant relationships between NIRS-derived mitochondrial oxidative capacity and measures whole-body aerobic fitness in highly active older adults (Figure 5). These findings corroborate previous research which demonstrated NIRS-derived mitochondrial oxidative capacity to be closely related to whole-body aerobic capacity in young adults (Brizendine et al. 2013; Beever et al. 2020; Guzman et al. 2020; Hovorka et al. 2021). Indeed, such a relationship between mitochondrial oxidative capacity and measures of whole-body aerobic fitness is expected, given the role of the mitochondria in producing most of the energy (adenosine triphosphate) required for aerobic work (Rolfe & Brown 1997; Rasmussen & Rasmussen 2000).

All participants of the current study were regularly undertaking forms of endurance exercise (self-reported exercise of 10.4 ± 5.0 hours per week; Table 1), which is likely to be beneficial for the maintenance and improvement of mitochondrial oxidative capacity in older adults (Chrøis et al. 2020; Fritzen et al. 2020). Indeed, Proctor et al. (1995) demonstrated endurance trained older adults (aged 50 to 65 years) to have similar oxidative capacity in type I fibres of the VL muscle, compared to endurance trained younger adults. In contrast, researchers using NIRS found older adults to have a higher time constant (i.e., lower VL muscle oxidative capacity), in comparison to younger adults matched for physical activity levels (Lagerwaard et al. 2020). Moreover, the measured time constant of the older adults in the current study is higher than time constants found for young adults in previous research, some of whom are reported to have similar or lower $\dot{V}O_{2peak}$ values (Brizendine et al. 2013; Beever et al. 2020; Hovorka et al. 2021). While direct comparisons cannot be made between studies due to methodological differences, continued physical activity into older age is likely to be important in offsetting an age-related decline in mitochondrial oxidative capacity and the deleterious effects on muscle function, physical performance, and general health (Chrøis et al. 2013; Distefano et al. 2018).

4.3. Limitations

A notable limitation of the current study design is the lack of an *in vitro* measure of mitochondrial oxidative capacity. While NIRS-derived mitochondrial oxidative capacity has been shown to be well-correlated with both HHR and ^{31}P-MRS (Ryan et al. 2013, 2014), the addition of an *in vitro* measure of mitochondrial oxidative

capacity in the current study would have provided further cross-validation of NIRS-derived measures of mitochondrial oxidative capacity. In addition, current findings are limited to lean and highly active older adults, and therefore should not be extrapolated to less active older adults with higher adipose tissue thickness.

4.4. Conclusion

The current study provides evidence demonstrating NIRS to be a reliable method for deriving a measure of mitochondrial oxidative capacity in lean, highly active older adults. The results of this study therefore offer additional evidence for the use of NIRS as a practical and non-invasive approach of routinely assessing skeletal muscle mitochondrial oxidative capacity. Current data can be used as reference for researchers in future work when measuring mitochondrial oxidative capacity with NIRS. In addition, NIRS-derived mitochondrial oxidative capacity is significantly correlated with measures of whole-body aerobic fitness and could therefore potentially be used in combination for monitoring exercise interventions in active older adults.

AUTHOR CONTRIBUTION STATEMENT

CF and JH designed research. CF conducted experiments, data collection and data analysis. CF, JH and AM wrote the manuscript. All authors read and approved the manuscript.

DECLARATIONS

Funding

None

Conflicts of interest/Competing interests

None

Ethics approval

The study was completed with full ethical approval, according to the Declaration of Helsinki standards.

Consent to participate

All participants provided signed informed consent prior to testing,

Consent to publication

All participants consented to having research findings published. All authors consented to publication of manuscript.

Code availability

Data analysis software application used (SPSS and Excel) openly available.

ACKNOWLEDGEMETS

Not Applicable

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FIGURE CAPTIONS

Fig. 1. (A) Example of one participant's NIRS signal from the measurement of mitochondrial oxidative capacity protocol **(B)** Example of one participant's test-retest $m\dot{V}O_2$ recovery data from the repeated arterial occlusions (Solid line = Test 1 data; Dashed line = Test 2 data; Triangles = $m\dot{V}O_2$ from corrected HHb signal; Circles = $m\dot{V}O_2$ from corrected O_2Hb signal).

Fig. 2. Data from all repeated arterial occlusion protocols (N = 96 protocols) used to measure the participant's NIRS-derived mitochondrial oxidative capacity **(A)** Mean of all R^2 values from slope of the HHb signal during the 3-s of arterial occlusion used to calculate the $m\dot{V}O_2$ value, **(B)** Change in β (blood volume correction factor) per second during the 3-s of arterial occlusion used to calculate the mVO_2 value, **(C)** Tissue saturation index during the isometric knee extension exercise protocol (Data are Means ± SD).

Fig. 3. Reproducibility results **(A)** Time constant, **(B)** Resting $m\dot{V}O_2$, **(C)** End $m\dot{V}O_2$, **(D)** Resting blood flow, **(E)** Reperfusion rate, **(F)** Max physiological range (Data are presented for each participant; Closed circles = Male data points; Open circles = Female data points).

Fig. 4. Bland-Altman plots showing day-to-day reliability **(Panel A)** and within day test-retest reliability **(Panels B and C)** for NIRS-derived skeletal muscle mitochondrial oxidative capacity of the vastus lateralis (Closed circles = Male data points; Open circles = Female data points; Solid line = Mean difference; Dotted lines = 95% limits of agreement; ICC = Intraclass correlation coefficient; SEM = Standard error of measurement; CV = Mean coefficient of variation).

Fig. 5. Correlation analysis of NIRS-derived mitochondrial oxidative capacity with measures of aerobic fitness **(A)** Relative $\dot{V}O_{2peak}$, **(B)** Power at $\dot{V}O_{2peak}$, **(C)** Relative oxygen uptake at gas exchange threshold, **(D)** Power at gas exchange threshold, **(E)** Relative oxygen uptake at respiratory compensation point, **(F)** Power at respiratory compensation point (Closed circles = Male data points; Open circles = Female data points; Solid line = Linear regression; Dashed lines = 95% confidence intervals; $\dot{V}O_2$ = oxygen uptake; GET = gas exchange threshold; RCP = respiratory compensation point).

Table 1 Participant characteristics, anthropometrics and IET data (mean ± SD)

	All Data	Female	Male
N	24	5	19
Age (years)	60 ± 4	62 ± 3	60 ± 4
Height (cm)	174.3 ± 8.7	162.3 ± 5.3	177.4 ± 6.3
Mass (kg)	71.3 ± 8.8	60.2 ± 7.1	74.2 ± 6.6
VL Skinfold (mm)	6.6 ± 2.6	8.0 ± 3.4	6.2 ± 2.3
Fat Mass (%)	21.0 ± 7.6	27.1 ± 11.1	19.4 ± 5.8
Lean Body Mass (%)	79.0 ± 7.6	72.9 ± 11.1	80.6 ± 5.8
Lean Body Mass (kg)	56.2 ± 8.2	43.4 ± 4.8	59.6 ± 4.7
Lean Body Mass Index ($kg.m^2$)	18.4 ± 1.6	16.4 ± 0.9	19.0 ± 1.3
Systolic BP (mmHg)	133 ± 6	135 ± 4	133 ± 6
Diastolic BP (mmHg)	81 ± 5	77 ± 5	82 ± 5
MVC (N.m)	151.1 ± 39.4	107.7 ± 25.0	162.5 ± 34.4
40% MVC (N.m)	60.4 ± 15.8	43.1 ± 10.0	65.0 ± 13.8
Relative $\dot{V}O_{2peak}$ ($ml.kg^{-1}.min^{-1}$)	41.2 ± 6.8	34.0 ± 6.0	43.1 ± 5.8
Power at $\dot{V}O_{2peak}$ (W)	275 ± 51	203 ± 24	295 ± 37
Relative $\dot{V}O_2$ at GET ($ml.kg^{-1}.min^{-1}$)	27.0 ± 5.7	22.3 ± 5.2	28.2 ± 5.3
Power at GET (W)	157 ± 40	108 ± 25	169 ± 33
Relative $\dot{V}O_2$ at RCP ($ml.kg^{-1}.min^{-1}$)	34.0 ± 6.6	27.0 ± 4.5	35.8 ± 5.8
Power at RCP (W)	208 ± 42	150 ± 19	224 ± 31
Exercise time per week (hours)	10.4 ± 5.0	10.5 ± 5.3	10.3 ± 5.1
MET hours per week	82.3 ± 46.6	87.8 ± 51.9	80.8 ± 46.5

Abbreviations: BP = blood pressure; VL = vastus lateralis; MVC = maximal voluntary contraction; $\dot{V}O_{2peak}$ = maximal oxygen uptake; $\dot{V}O_2$ = oxygen uptake; GET = gas exchange threshold; RCP = respiratory compensation point; MET = metabolic equivalents.

Table 2 NIRS-derived mitochondrial oxidative capacity data and test-retest reliability data

	Mean ± SD	Within Participant CV	Between Participant CV	Within Day CV	Between Day CV	Day-to-Day	
						ICC	SEM
Time Constant (s)	66.6 ± 19.7	8.1%	30.4%	5.7%	7.0%	0.94	5.2
Rate Constant (min^{-1})	1.01 ± 0.37	8.1%	37.7%	5.7%	6.9%	0.93	0.1
Resting m$\dot{V}O_2$ (%/s)	0.50 ± 0.32	23.1%	64.7%	19.4%	15.8%	0.93	0.1
End m$\dot{V}O_2$ (%/s)	3.05 ± 1.36	12.3%	45.2%	10.5%	8.3%	0.96	0.3
Last m$\dot{V}O_2$ (%/s)	0.47 ± 0.22	15.0%	48.1%	10.9%	12.5%	0.84	0.1
Plateau m$\dot{V}O_2$ (%/s)	0.46 ± 0.22	14.3%	47.8%	10.5%	11.9%	0.85	0.1
Delta Rest-End	2.54 ± 1.34	15.4%	53.3%	12.2%	12.1%	0.95	0.3
Delta End-Plateau	2.59 ± 1.30	13.9%	50.7%	11.2%	10.0%	0.95	0.2
Reperfusion Rate (s)	11.1 ± 4.0	14.9%	28.0%		14.9%	0.67	2.1
Resting Blood Flow (mL/(min.100.mL))	1.87 ± 0.58	16.7%	31.3%		16.7%	0.58	0.4
Maximal Physiological Range (AU)	41.7 ± 15.9	8.7%	38.6%		8.7%	0.91	5.3

Abbreviations: m$\dot{V}O_2$ = muscle oxygen consumption; End m$\dot{V}O_2$ = m$\dot{V}O_2$ immediately after exercise; Last m$\dot{V}O_2$ = m$\dot{V}O_2$ from the final repeated arterial occlusion; Plateau m$\dot{V}O_2$ = m$\dot{V}O_2$ at the point the recovery curve no longer changes; CV = coefficient of variation; ICC = intraclass correlation coefficient; SEM = standard error of measurement.

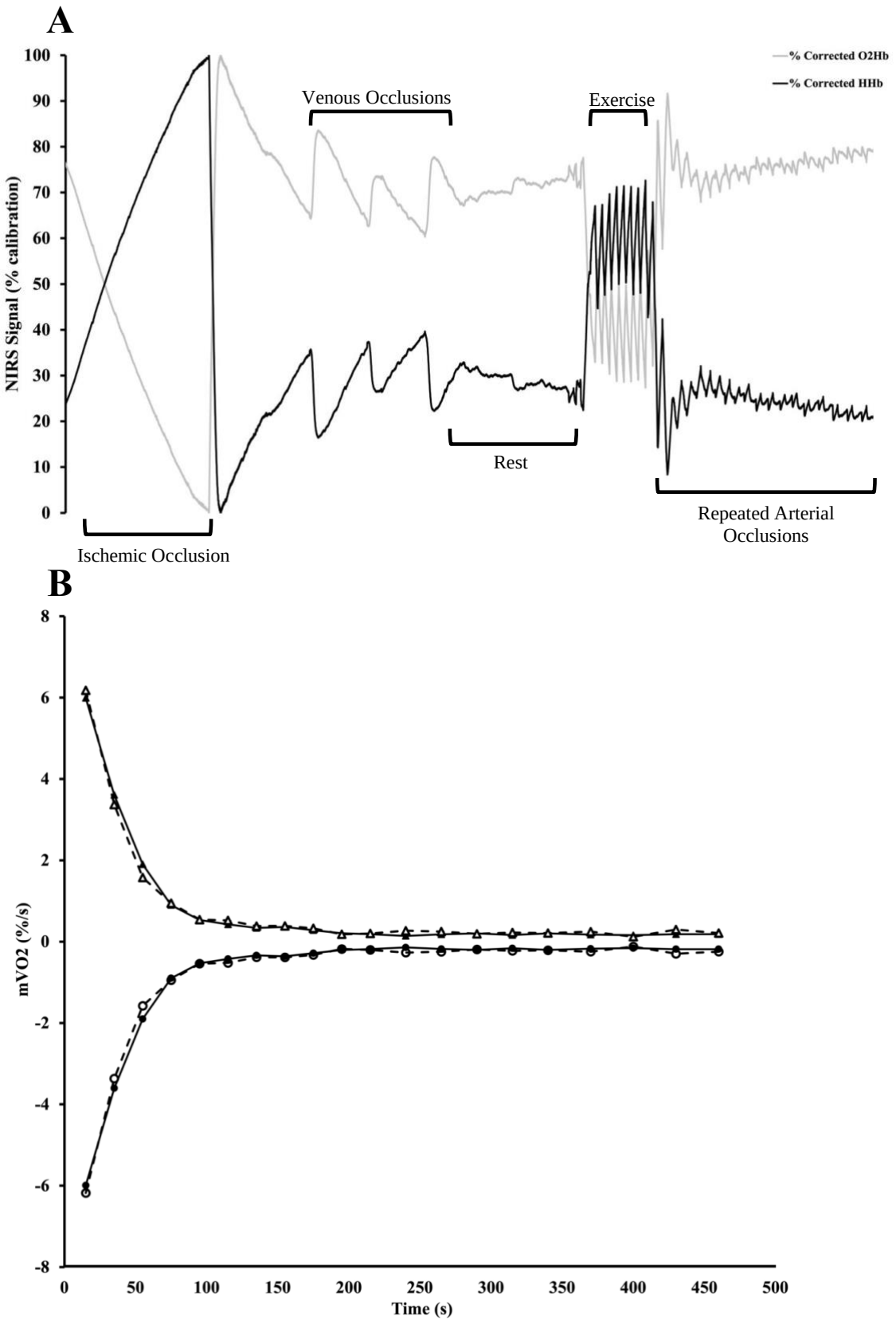
A
% Corrected O2Hb
% Corrected HHb
NIRS Signal (% calibration)
Venous Occlusions
Exercise
Rest
Ischemic Occlusion
Repeated Arterial Occlusions
B
mVO2 (%/s)
Time (s)

Fig1.

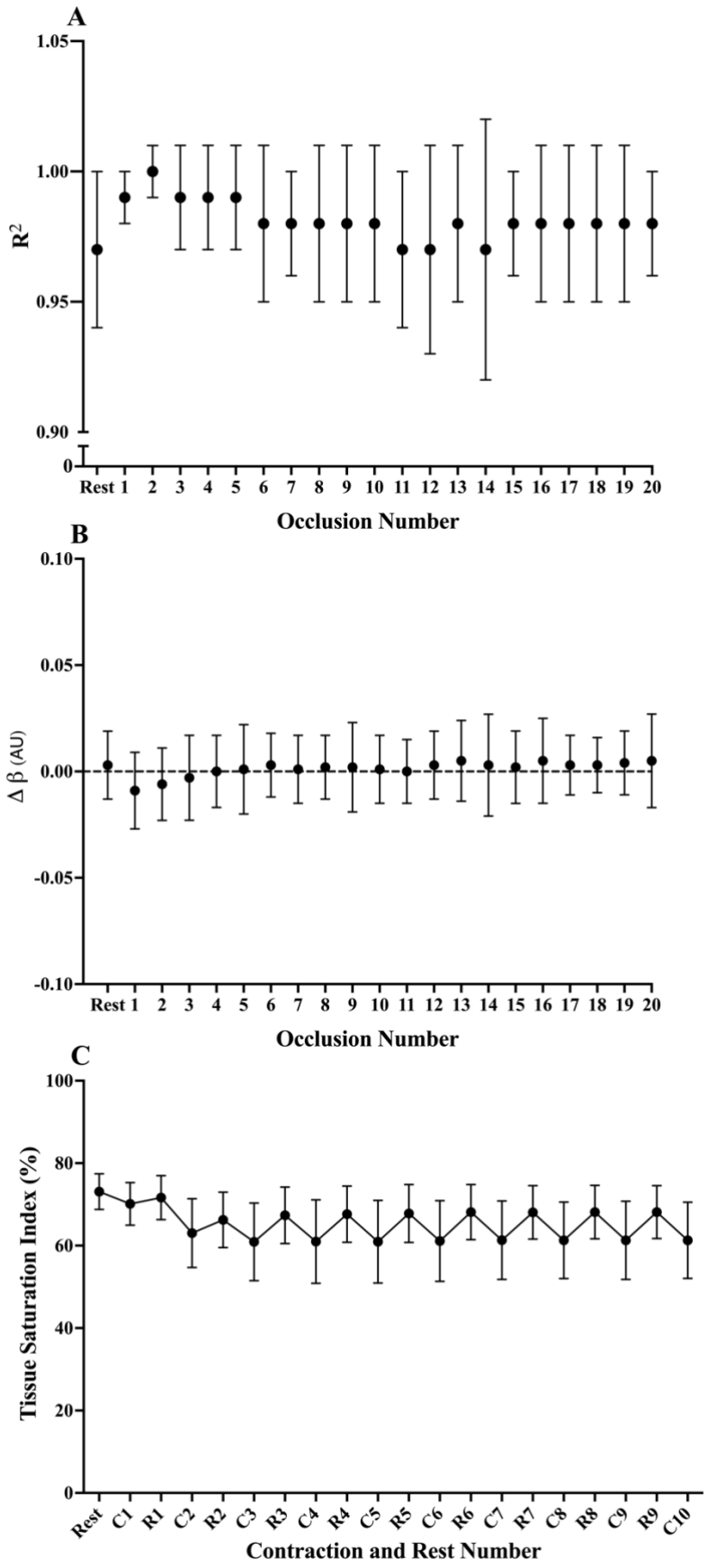
A
1.05
1.00
0.95
0.90
0
R2
Rest 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Occlusion Number
B
0.10
0.05
0.00
-0.05
-0.10
Δ β (AU)
Rest 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Occlusion Number
C
100
80
60
40
20
0
Tissue Saturation Index (%)
Rest C1 R1 C2 R2 C3 R3 C4 R4 C5 R5 C6 R6 C7 R7 C8 R8 C9 R9 C10
Contraction and Rest Number

Fig2.

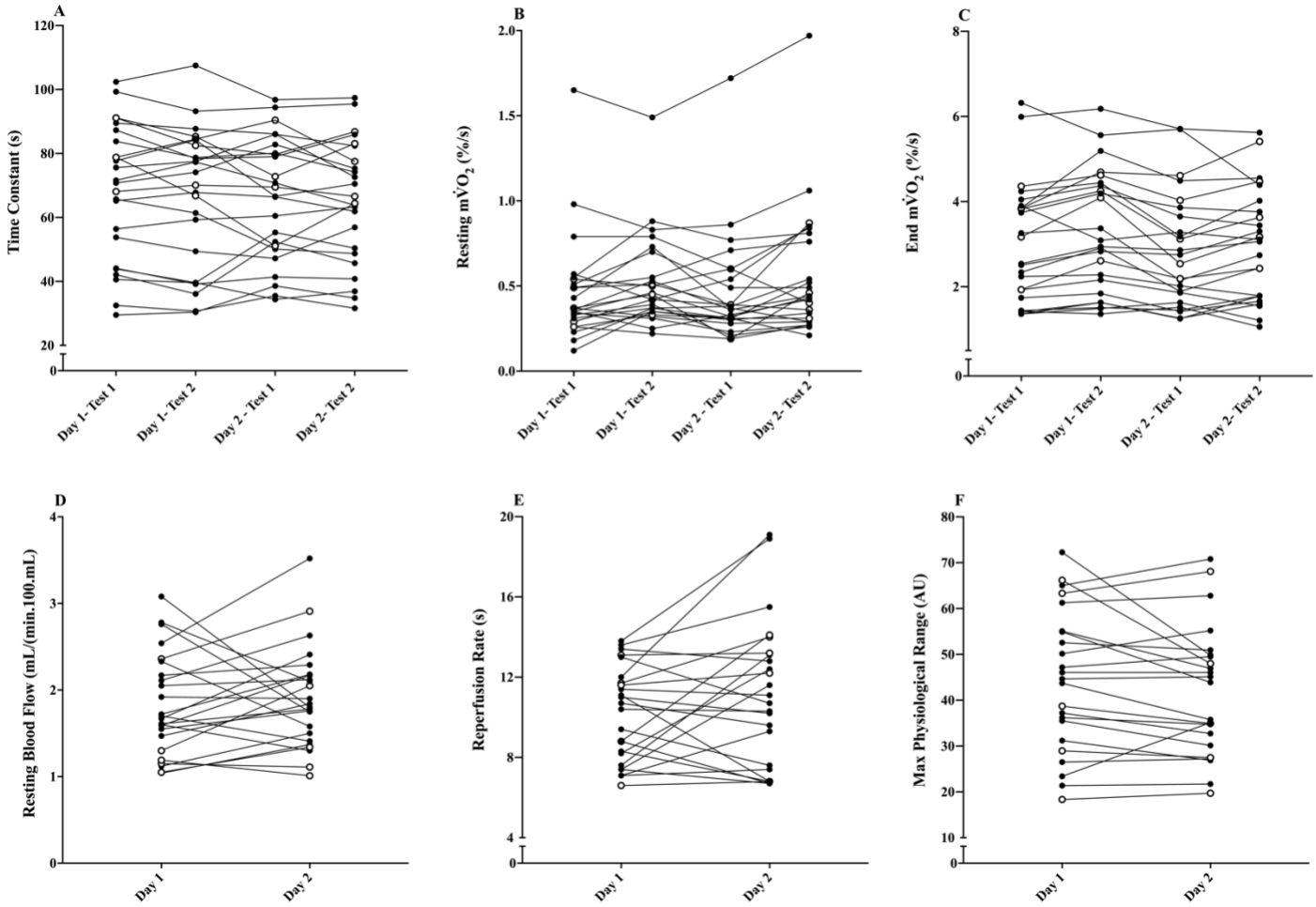
A
Time Constant (s)
120
100
80
60
40
20
0
Day 1- Test 1
Day 1- Test 2
Day 2 - Test 1
Day 2- Test 2
B
Resting mV̇O2 (%/s)
2.0
1.5
1.0
0.5
0.0
Day 1- Test 1
Day 1- Test 2
Day 2 - Test 1
Day 2- Test 2
C
End mV̇O2 (%/s)
8
6
4
2
0
Day 1- Test 1
Day 1- Test 2
Day 2 - Test 1
Day 2- Test 2
D
Resting Blood Flow (mL/(min.100.mL)
4
3
2
1
0
Day 1
Day 2
E
Reperfusion Rate (s)
20
16
12
8
4
0
Day 1
Day 2
F
Max Physiological Range (AU)
80
70
60
50
40
30
20
10
0
Day 1
Day 2

Fig3.

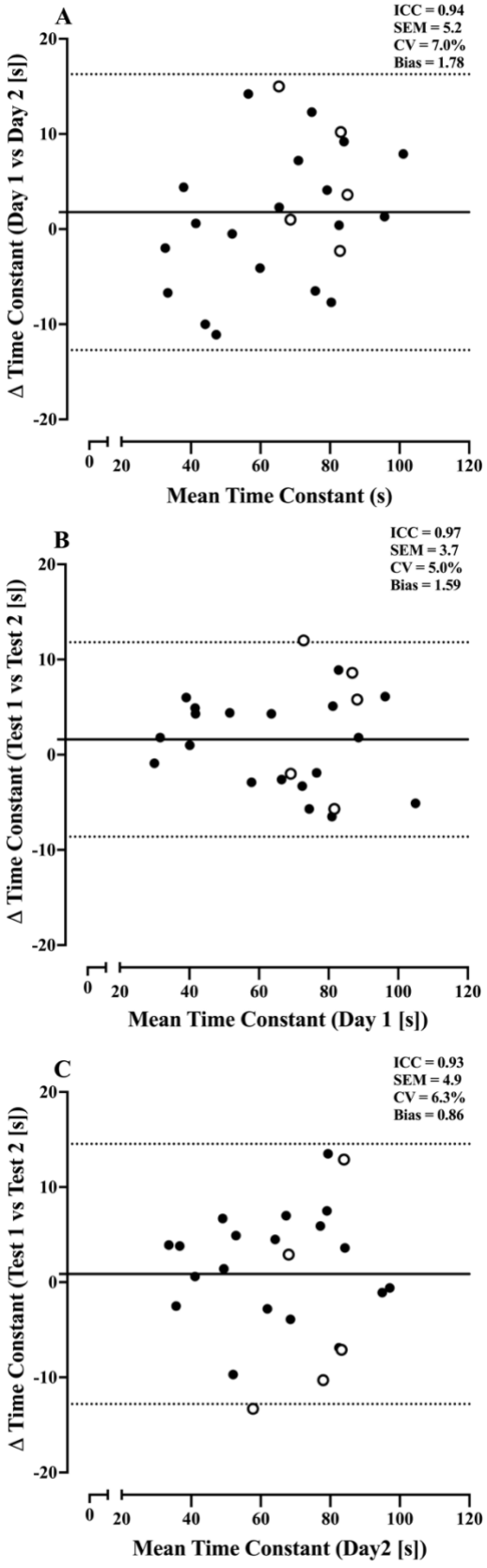
A
ICC = 0.94
SEM = 5.2
CV = 7.0%
Bias = 1.78
Δ Time Constant (Day 1 vs Day 2 [s])
Mean Time Constant (s)
B
ICC = 0.97
SEM = 3.7
CV = 5.0%
Bias = 1.59
Δ Time Constant (Test 1 vs Test 2 [s])
Mean Time Constant (Day 1 [s])
C
ICC = 0.93
SEM = 4.9
CV = 6.3%
Bias = 0.86
Δ Time Constant (Test 1 vs Test 2 [s])
Mean Time Constant (Day2 [s])

Fig4.

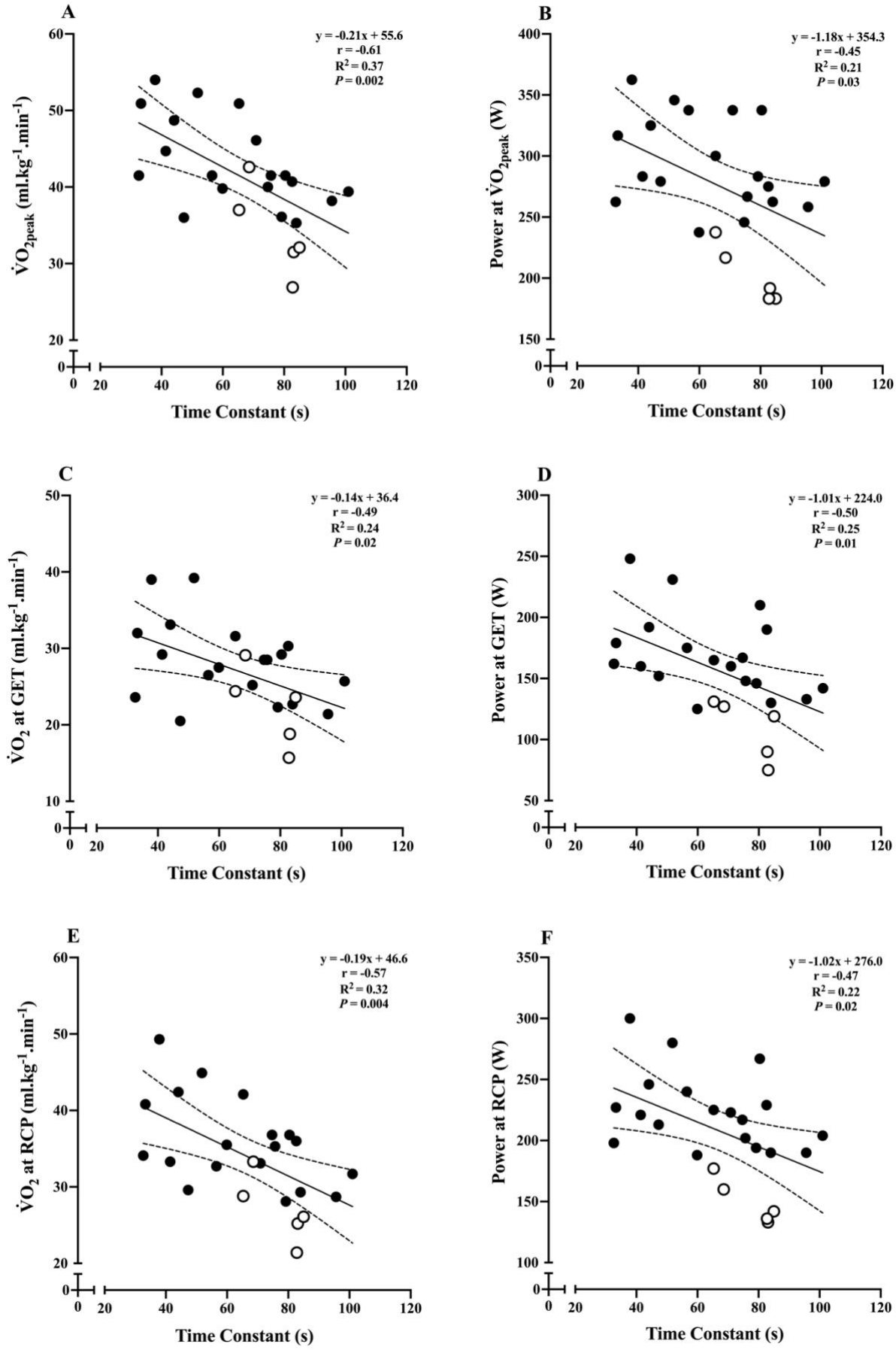
A
y = -0.21x + 55.6
r = -0.61
R² = 0.37
P = 0.002
V̇O2peak (ml.kg-1.min-1)
Time Constant (s)
B
y = -1.18x + 354.3
r = -0.45
R² = 0.21
P = 0.03
Power at V̇O2peak (W)
Time Constant (s)
C
y = -0.14x + 36.4
r = -0.49
R² = 0.24
P = 0.02
V̇O2 at GET (ml.kg-1.min-1)
Time Constant (s)
D
y = -1.01x + 224.0
r = -0.50
R² = 0.25
P = 0.01
Power at GET (W)
Time Constant (s)
E
y = -0.19x + 46.6
r = -0.57
R² = 0.32
P = 0.004
V̇O2 at RCP (ml.kg-1.min-1)
Time Constant (s)
F
y = -1.02x + 276.0
r = -0.47
R² = 0.22
P = 0.02
Power at RCP (W)
Time Constant (s)

Fig5.