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Mitochondrial oxygen affinity increases after sprint interval training and is related to the improvement in peak oxygen uptake

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Abstract

Aims: The body responds to exercise training by profound adaptations throughout the cardiorespiratory and muscular systems, which may result in improvements in maximal oxygen consumption (VO_2peak) and mitochondrial capacity. By convenience, mitochondrial respiration is often measured at supra-physiological oxygen levels, an approach that ignores any potential regulatory role of mitochondrial affinity for oxygen ($p50_{mito}$) at physiological oxygen levels.

Methods: In this study, we examined the $p50_{mito}$ of mitochondria isolated from the *Vastus lateralis* and *Triceps brachii* in 12 healthy volunteers before and after a training intervention with seven sessions of sprint interval training using both leg cycling and arm cranking. The changes in $p50_{mito}$ were compared to changes in whole-body VO_2peak.

Results: We here show that $p50_{mito}$ is similar in isolated mitochondria from the *Vastus* (40 ± 3.8 Pa) compared to *Triceps* (39 ± 3.3) but decreases (mitochondrial oxygen affinity increases) after seven sessions of sprint interval training (to 26 ± 2.2 Pa in *Vastus* and 22 ± 2.7 Pa in *Triceps*, both $P < .01$). The change in VO_2peak modelled from changes in $p50_{mito}$ was correlated to actual measured changes in VO_2peak ($R^2 = .41$, $P = .002$).

Conclusion: Together with mitochondrial respiratory capacity, $p50_{mito}$ is a critical factor when measuring mitochondrial function, it can decrease with sprint interval training and should be considered in the integrative analysis of the oxygen cascade from lung to mitochondria.

KEYWORDS

exercise, high intensity training, maximal oxygen consumption, mitochondria, oxygen affinity, sprint training

See Editorial Commentary: Burtscher M. 2020. A breath of fresh air for mitochondria in exercise physiology. *Acta Physiol*. 229, e13490.

1 | INTRODUCTION

It is now well recognized that oxygen delivery, primarily limited by maximal cardiac output, is a major constraining factor for whole-body maximal oxygen consumption. However, peripheral factors such as the mitochondrial oxidative capacity and the apparent mitochondrial oxygen affinity ($p50_{mito}$), have recently shown to interact with oxygen availability and play a smaller yet important regulatory role in determining the maximal oxygen consumption.[1-3] For a complete understanding of how $p50_{mito}$ regulates maximal oxygen consumption, it must be recognized that not only intrinsic mitochondrial characteristics such as cytochrome c oxidase activity and mitochondrial efficiency influence the $p50_{mito}$. Increases in mitochondrial density or number will also ultimately affect the in vivo $p50_{mito}$ since a higher mitochondrial density will lower the relative mitochondrial activation and therefore lower $p50_{mito}$[3] given that all other factors are unchanged.

$P50_{mito}$ has recently been shown to interact with peripheral oxygen delivery and the degree of mitochondrial activation and thereby constitutes an important regulating part of the oxygen cascade[3] that previously has been neglected. The $p50_{mito}$ is operationally defined as the oxygen pressure at the mitochondrial level where respiration is 50% of the maximal rate at saturating oxygen concentrations. Mitochondrial respiration (VO_2) is dependent on oxygen availability and $p50_{mito}$ according to Equation 1:

$$VO_2 = \left(V_{max} * pO_2\right) / \left(pO_2 + p50_{mito}\right), \quad (1)$$

where V_{max} is the oxygen saturated maximal mitochondrial respiration and pO_2 is the oxygen pressure at the mitochondrial level. Therefore, a reduction in $p50_{mito}$ should increase VO_2peak if other factors are held unchanged.

When measured ex vivo in isolated mitochondria, $p50_{mito}$ has been found to be around ~40 Pa in the presence of saturating ADP concentrations while mitochondria are respiring on complex I substrates and increases to 100-170 Pa using substrates for both complex I and II. Recent studies have found rather large variations in $p50_{mito}$ between individuals that are associated with the metabolic efficiency and cytochrome *c* oxidase composition of the subject.[2,4]

Since mitochondria in vivo rarely reach full ADP stimulation even during maximal whole body exercise because of central oxygen delivery limitation, it is reasonable to assume that the $p50_{mito}$ in vivo under physiological circumstances is lower than that with full ADP-stimulation measured ex vivo (11). This was confirmed by recent experimental findings varying the degree of oxygen delivery and thereby mitochondrial activation during exercise involving different muscle masses and by varying the oxygen concentration of the inspired air.[3] The increase in mitochondrial density often seen after exercise training could therefore be a response and mechanism to decrease mitochondrial activation and thereby lower the $p50_{mito.}$ At the physiological level, a decreased $p50_{mito}$ would improve peripheral oxygen extraction during maximal exercise and contribute to increase muscle VO_2.

To the best of our knowledge, only one study has measured ex vivo $p50_{mito}$ before and after a training intervention.[5] That study, which was designed to determine the effects of training in hypoxia and normoxia on mitochondrial function, found no robust effects of the intervention on mitochondrial respiration and $p50_{mito}$ was unchanged. A recent study has shown that mitochondria isolated from the liver of Atlantic killifish could change their $p50_{mito}$ as a functional adaptation to different ambient temperatures demonstrating the dynamic properties of this parameter.[6]

The present data are part of a larger study,[7,8] where we have shown that markers of mitochondrial density increased by 5%-15%, whereas intrinsic mitochondrial respiration (expressed per mg mitochondrial protein) was surprisingly reduced by 50% or more after training. This effect was related to oxidative inactivation of the citric acid cycle enzyme aconitase. As an extension of that work, the purpose of this study was therefore to determine the role of mitochondrial $p50_{mito}$ in determining peak oxygen consumption.[1-3] Mitochondrial p50 was measured ex vivo in *Vastus lateralis* and *Triceps brachii* muscles before and after a short training period of intense sprint interval leg cycling and arm cranking and relate potential changes to alterations in maximal oxygen consumption. Our hypothesis was that this type of intense training, that is an established model to initiate mitochondrial biogenesis,[9] decreases $p50_{mito}$ as a functional adaptation to improve oxygen uptake at the mitochondrial level.

2 | RESULTS

2.1 | Sprint interval training increases VO_2peak during both arm-cranking and leg cycling

The pre-training VO_2peak during arm-cranking was 38.4 ± 7.5 ml kg^{-1} min^{-1} and during leg cycling 47.0 ± 7.5 ml $kg^{-1}min^{-1}$. After the training period the VO_2peak during arm-cranking increased to 43.1 ± 6.5 ml kg^{-1} min^{-1} ($P < .001$) and during leg cycling to 49.9 ± 8.0 ml kg^{-1} min^{-1} ($P < .001$). These results have been reported in a previous publication but as absolute values (L min^{-1}).[7]

2.2 | $P50_{mito}$ shows large inter-individual variations, but is similar in the arms and legs

When isolated mitochondria from *Vastus* were fed electrons through complex I, using pyruvate and malate as substrates,

$p50_{mito}$ averaged 40 ± 3.8 Pa with a range between 27 and 71 Pa. In *Triceps* $p50_{mito}$ was similar to *Vastus* with an average value of 39 ± 3.3 Pa with a range between 27 and 60 Pa. There was no statistical difference in $p50_{mito}$ between *Vastus* and *Triceps*. Likewise, there was no difference in mitochondrial respiratory flux rate (V_{max}) between *Vastus* 5.8 ± 0.5 and *Triceps* 5.3 ± 0.7 pmol O_2 s^{-1} μg^{-1} protein ($P = .36$; Figure 1).

2.3 | Catalytic efficiency decreases as respiratory flux increases

To determine if $p50_{mito}$ was a direct function of the individual respiratory flux, we ran a correlation analysis between respiratory flux rate with complex I substrates against $p50_{mito}$ in the mitochondrial preparations from all our subjects. However, no such association was found, see Figure 2A. Next, we wanted to determine if the relative activation of mitochondrial respiration had any impact on $p50_{mito}$. We therefore added succinate, a respiratory substrate that enters the ETS through complex II and as expected found that the respiratory rate increased from 5.8 ± 1.8 to 8.8 ± 2.2 pmol s^{-1} μg^{-1} in *Vastus* and from 5.3 ± 2.5 to 7.8 ± 3.7 pmol s^{-1} μg^{-1} in *Triceps*, both $P < .001$, see Figure 2B. With this higher respiratory flux we found that $p50_{mito}$ increased to 77 ± 7.7 Pa in *Vastus* and to 77 ± 7.8 Pa in *Triceps* ($P < .001$ compared to complex I substrates for both), see Figure 2C.

Catalytic efficiency (calculated as $V_{max}/p50_{mito}$) was significantly lower in both *Triceps* and *Vastus* when mitochondria respired on complex I + II substrates (*Triceps* 110 ± 4 and *Vastus* 115 ± 4 pmol s^{-1} μg^{-1} Pa^{-1}) compared to complex I alone (*Triceps* 143 ± 6 and *Vastus* 156 ± 6 pmol s^{-1} μg^{-1} Pa^{-1}), see Figure 2D.

In agreement with previous studies,[3,10] this indicates that one of the factors that determines $p50_{mito}$ is the respiratory flux relative to the maximum capacity for the ETS, with higher relative flux rates yielding higher $p50_{mito}$ values. However, the decrease in catalytic efficiency with higher flux rates indicate that this relationship is non-linear.

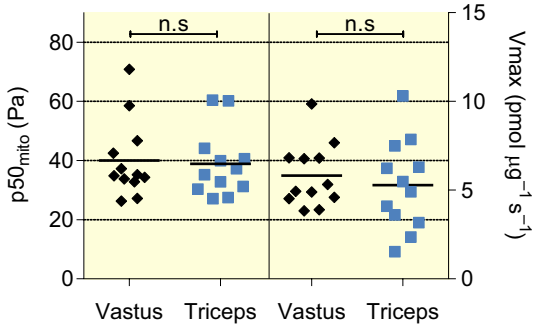


FIGURE 1 $p50_{mito}$ and V_{max} are similar in Vastus and Triceps. Left *y*-axis; the oxygen pressure (Pa) at $p50_{mito}$, and right *y*-axis; the V_{max} in isolated mitochondria from Vastus and Triceps respiring on pyruvate and malate with full ADP stimulation. n = 12 per group, individual values are shown, and the horizontal bar indicates the mean value. $P < .05$ is considered significant

2.4 | Short-term high intensity sprint training decreases both $p50_{mito}$ and the catalytic efficiency

We have previously shown that seven sessions of high-intensity sprint training over 11 days can inhibit mitochondrial respiration through ROS-mediated aconitase inhibition in these subjects.[7] The finding that the relative mitochondrial flux rate and $p50_{mito}$ increased in parallel allowed us to hypothesize that the inhibited mitochondrial respiration after the training period also could have affected $p50_{mito}$. Indeed, we found that $p50_{mito}$ was substantially reduced post training in *Vastus* to 26 ± 2.2 Pa ($P < .01$ compared to pre) when respiring on complex I substrates but not significantly ($P = .11$) when $p50_{mito}$ was assessed using both complex I and II substrates (see Figure 3A). In *Triceps* $p50_{mito}$ was reduced post training to 22 ± 2.7 Pa ($P < .01$ compared to pre) on complex I substrates and to 48 ± 6.8 Pa ($P < .05$ compared to pre) on complex I + II substrates (see Figure 3B).

Since both V_{max} and $p50_{mito}$ decreased after the intervention, we also determined catalytic efficiency and found it to be reduced after training both in *Vastus* (complex I substrates from pre 0.16 ± 0.06, post 0.08 ± 0.003 pmol s^{-1} μg^{-1} Pa^{-1}, $P < .01$, on complex I + II substrates from 0.11 ± 0.04 to 0.07 ± 0.01 pmol s^{-1} μg^{-1} Pa^{-1}, $P < .01$ see Figure 3C) and in *Triceps* (complex I substrates pre 0.14 ± 0.06, post 0.06 pmol s^{-1} μg^{-1} Pa^{-1} ± 0.01, $P < .01$, on complex II substrates pre 0.11 ± 0.03, post 0.05 ± 0.01 pmol s^{-1} μg^{-1} Pa^{-1}, $P < .01$ see Figure 3D). These results indicate that the magnitude of reduction in V_{max} was more substantial than the decrease in $p50_{mito}$, negatively influencing the catalytic efficiency.

2.5 | Reductions in $p50_{mito}$ and increases in VO_2peak are interconnected

A correlation analysis revealed a significant association between the increase in VO_2peak and the decrease in $p50_{mito}$ in *Triceps* and *Vastus* together (see Figure 4A, $R^2 = .28$, $P = .016$).

The assumptions made here are that (a) changes in $p50_{mito}$ explain a substantial part of the changes in VO_2peak, (b) oxygen delivery limits VO_2peak during cycling exercise, and thereby the relative activation of mitochondria, and (c) oxygen delivery, mitochondrial oxygen availability and mitochondrial activation are relatively unchanged pre- to post-training.

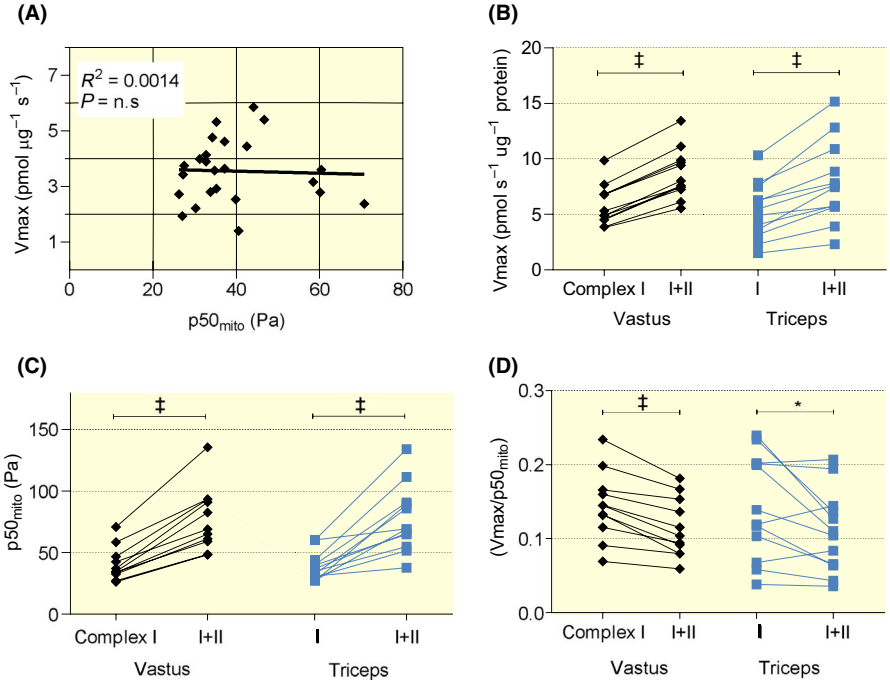


FIGURE 2 p50$_{mito}$ is not related to differences in V_{max} between individuals but is increased as respiration rate increases by simultaneous stimulation of complex I and II. (A) No association was found between V_{max} and p50$_{mito}$ in isolated mitochondria from both Vastus and Triceps from 12 subjects respiring on the complex I substrates pyruvate and malate in the presence of ADP (R^2 = .0014, n = 24, p = n.s). (B) V_{max} increases as the complex II substrate succinate is added to mitochondria already respiring on the complex I substrates pyruvate and malate both in Vastus and Triceps. Data are presented as individual values, n = 12, $P < .0001$. (C) Comparing the p50$_{mito}$ using the same experimental approach as in (B) we found that a higher respiratory flux was related to significant increases in p50$_{mito}$ (n = 12, $P \leq 0.0001$ compared to with complex I substrates in both Vastus and Triceps). (D) Catalytic efficiency was calculated as V_{max}/p50$_{mito}$ and found to be lower in both Vastus (n = 11, $P < .0001$) and in Triceps (n = 12, $P < .05$) when mitochondria respired on both complex I and II substrates compared to complex I substrates alone

If those assumptions hold true, changes in VO_2peak can be calculated from the changes in p50$_{mito}$ by integrating them into Equation 1. The change in VO_2peak is then described in Equation 2:

$$\Delta VO_2peak = \left(VO_2peak_{pre} / \left(PO_{2pre} / \left(PO_{2pre} + p50_{mitopre}\right)\right)\right) * \left(PO_{2post} / \left(PO_{2post} + p50_{mitopost}\right)\right) - VO_2peak_{pre} \quad (2)$$

By assuming a constant mitochondrial PO_2 of 0.3 kPa both pre- and post-training and inserting our measured VO_2peak_{pre} values from both leg and arm exercise together with the ex vivo assessed p50$_{mitopre}$ and p50$_{mitopost}$ values from both *Vastus* and *Triceps* mitochondria, we generated 20 calculated changes in VO_2peak and compared those values to the actual measured changes in VO_2peak. A significant association was found between the measured and calculated changes in VO_2peak (P = .002, R^2 = .41, see Figure 4A) indicating that the change in p50$_{mito}$ explained a large part of the variance in the VO_2peak response to this training programme. Also shown in Figure 4B is that the measured VO_2peak is ~100 ml min^{-1} higher than the value calculated from Equation 1 above. This difference is likely attributed to a higher oxygen delivery because of cardiac or haematological adaptations after training.

3 | DISCUSSION

In this study, we show that p50$_{mito}$ should be considered as an important regulatory step in the oxygen transport system. Furthermore, p50$_{mito}$ can change with training and the decreased p50$_{mito}$ after short-term sprint training is directly related to the increase in VO_2peak.

As shown in the present and earlier reports[3-4,10] p50$_{mito}$ is dictated by a number of factors. First and most obvious is the activity of cytochrome c oxidase, the enzyme where oxygen is trapped and reduced to water. At a fixed cytochrome *c* oxidase activity, a higher flux through the electron transport system would yield a higher p50$_{mito}$. This fact is illustrated by our experimental findings, showing that when substrates for complex II is added to ADP stimulated respiration with complex I substrates, V_{max} (Figure 2B) and p50$_{mito}$ (Figure 2C) increases in parallel. The mitochondrial flux rate is in turn dictated by several factors, the most important are activation by ADP and substrate availability.

It is important to consider the difference between p50$_{mito}$ as assessed ex vivo using isolated mitochondria with saturating substrate and ADP concentrations during an aerobic-anoxic transition and the actual physiological

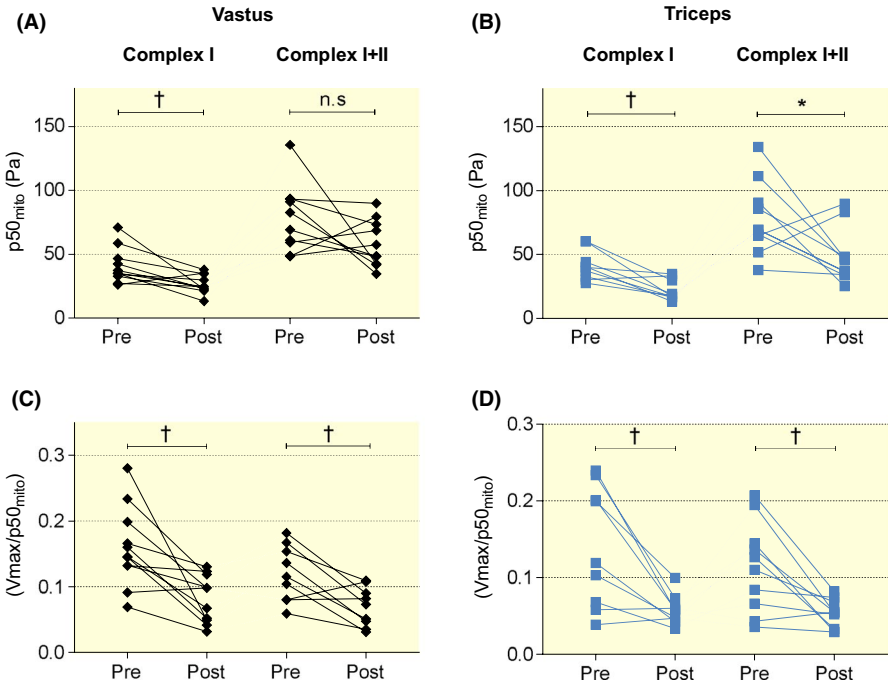


FIGURE 3 Both $p50_{mito}$ and catalytic efficiency are decreased after sprint training. $p50_{mito}$ was found to be lower after compared to before training in Vastus when respiring on complex I substrates ($P < .01$, n = 11) but not on complex I and II substrates ($P = .11$, n = 10, [A]). In Triceps $p50_{mito}$ was lower after compared to before training both when respiring on complex I ($P < .01$, n = 9) and complex I and II substrates ($P < .05$, n = 10, [B]). Catalytic efficiency was similarly reduced after training both in Vastus and Triceps and regardless of substrate combination ($P < .001$ for all, n = 9-12, [C,D])

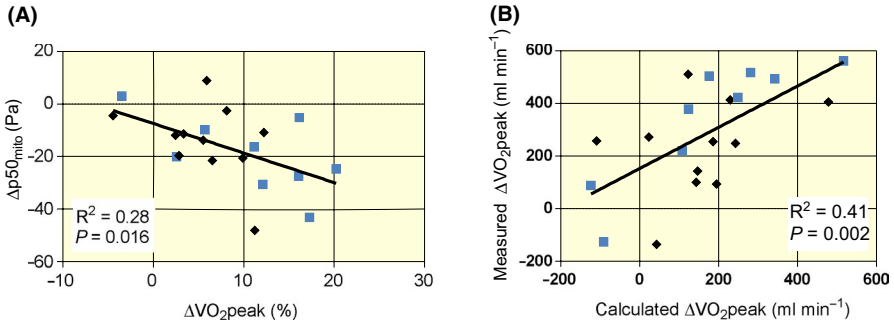


FIGURE 4 Changes in $p50_{mito}$ are associated with changes in whole body oxygen uptake. Squares are data from Triceps/arm exercise, diamonds are Vastus/leg exercise. (A) A significant correlation was found between the pre- to post-training-induced changes in $p50_{mito}$ and change in VO_2peak ($R^2 = .28$, $P = .016$, n = 20). (B) Using the equation $\Delta VO_2peak = (VO_2peak_{pre}/(PO_{2pre}/(PO_{2pre} + p50_{mitopre})))*(PO_{2post}/(PO_{2post} + p50_{mitopost}))-VO_2peak_{pre}$ and assuming a constant pO_2 of 0.3 kPa, we calculated the change in VO_2peak from the change in $p50_{mito}$ and correlated those values to the measured VO_2peak after the training period. A significant association was found between these two parameters ($R^2 = .41$, $P = .002$, n = 20)

$p50_{mito}$ when the mitochondrial population is exposed to less than saturating concentrations of both substrates and ADP. During intense exercise when large muscle groups are activated, mitochondria possess a large overcapacity relative to the oxygen supply rate and thus mitochondrial flux rate is a direct function of oxygen supply rate, that is, oxygen availability limits mitochondrial respiration.[11] As shown in this paper, the ratio of mitochondrial flux rate to mitochondrial V_{max} is one of the factors that determines the actual $p50_{mito}$ and a high mitochondrial V_{max} is necessary to keep $p50_{mito}$ at a low level, thereby allowing for a greater pressure gradient and more efficient diffusion of oxygen from the capillary supply head to the mitochondria. The above relationship between mitochondrial V_{max} and $p50_{mito}$ with oxygen extraction and VO_2peak provides the mechanistic basis for the physiological significance of a mitochondrial capacity that surpasses the capacity to deliver oxygen to the tissue. In this study, we used both arm-cranking and leg-cycling as exercise modes. The size of the muscle mass involved in exercise is rather different between the two exercise modes and it can be assumed that the regulation of VO_2peak is also dependent on different factors between the two modes.[12] The arm muscle mass is smaller and thus total mitochondrial capacity is lower during maximal arm exercise. Despite a lower cardiac output, arm blood flow per unit muscle mass is higher compared with leg exercise as are the diffusion distances, and the mean transit time is faster. All these factors impose a

higher mitochondrial activation compared to leg exercise and for an efficient oxygen extraction $p50_{mito}$ needs to be kept at a low level. It is therefore somewhat surprising that both V_{max} and $p50_{mito}$ were similar in Vastus and Triceps pre-training. This could perhaps be explained by the lower fitness level of the muscles of the upper body in this relatively untrained population. However, both exercise-modes are oxygen supply limited and thus p50mito should play a critical regulatory role.

It has recently been reported that trained individuals have a higher mitochondrial capacity compared to oxygen supply rate than untrained individuals[13] and higher arterio-venous oxygen extraction at maximal work rates. Applying our theoretical framework to those findings indicate that the higher mitochondrial capacity allows a lower $p50_{mito}$ and therefore a higher oxygen extraction and oxygen consumption. This finding is also in agreement with the high oxygen extraction capacity reported in elite athletes.[14] We have previously measured ex vivo $p50_{mito}$ in untrained men, trained men and trained women and found that women have substantially higher $p50_{mito}$ than men.[15] Future studies including haemodynamic measures are needed to resolve whether a true sex difference exist in the mitochondrial regulation of oxygen uptake during maximal exercise.

The phenomenon of a high mitochondrial capacity allowing for a lower $p50_{mito}$ during exercise also explains why acute changes in oxygen delivery are not fully mirrored by changes in VO_2peak. For example, when oxygen delivery was increased by blood-transfusions, systemic oxygen delivery was increased by approximately 30% but VO_2peak was only increased by 7%, explained by a reduction in oxygen extraction.[16] This finding has been interpreted to support the "symmorphosis" theory; that each step in the oxygen cascade is aligned for optimal function.[17] Our present findings modify this theory by indicating that a mitochondrial capacity in excess of O_2 delivery maintains low $p50_{mito}$ and thereby enhances oxygen extraction.

In this study, a very demanding training protocol was used in active but untrained subjects. A fundamental adaptation to exercise training is an increase in mitochondrial content with a similar increase in the oxidative capacity of skeletal muscle.[18-20] However, intrinsic mitochondrial respiration, that is, respiration expressed per unit of mitochondria, does not increase in the same reliable fashion as mitochondrial content and can in some cases even decrease after training.[21] The lowering of $p50_{mito}$ that accompanies the reduction in mitochondrial respiration and aconitase inactivation reported previously in the present population could possibly be an acute mechanism to increase VO_2peak before longer term adaptations that increase mitochondrial biogenesis occur and therefore increase mitochondrial capacity.

The present study is the first to report changes in $p50_{mito}$ in humans after any sort of intervention and future studies will reveal if decreases in $p50_{mito}$ is a fundamental adaptation to exercise training. If that is the case, characteristics of mitochondria from well-trained subjects with low $p50_{mito}$ could be a high cytochrome *c* oxidase capacity compared to the rest of the electron transport system or mitochondria with a relatively high degree of uncoupling that has been shown to lower $p50_{mito}$ effectively.[2] However, if the lower $p50_{mito}$ was related to mitochondrial uncoupling a decreased whole-body efficiency would be expected. In this study, this was not the case as efficiency was similar pre- to post-training during leg cycling and improved during arm cranking.[8]

Despite the fact that exercise with the arms compared to the legs induces a markedly different haemodynamic response,[22] both exercise modes are limited by oxygen delivery to the active limbs.[12] The limited oxygen supply during maximal exercise situates $p50_{mito}$ in a regulatory role in the oxygen transport system and it is reasonable to assume that the effect of $p50_{mito}$ on maximal oxygen consumption is similar in leg and arm exercise. It is noteworthy that we find similar $p50_{mito}$ values in *Triceps* and *Vastus* despite the fact that arm muscles have been found to extract less oxygen than leg muscles for a given oxygen delivery.[14] This phenomenon is explained by the higher mitochondrial density in *Vastus*, as assessed by citrate synthase activity, previously shown in these subjects.[8] At a given oxygen delivery, a higher mitochondrial density will result in a lower mitochondrial activation and thereby lower $p50_{mito}$ as shown recently.[3]

An interesting finding is that the significant association between the calculated and measured change in VO_2peak does not intersect the origin in Figure 4B, instead the measured VO_2peak is 100 ml min^{-1} higher than the calculated value. This indicates that also other factors, most likely a higher oxygen delivery, contributes to the increase in VO_2peak after training in this study.

3.1 | Study limitations

This study enrolled a relatively small number of subjects (n = 12) and employed a specific training protocol involving seven sessions of intense sprint training over a short time period. We did not include other haemodynamic measures or changes in cardiac structure or function employed previously (1, Boushel et al Acta 2014) that could have provided important information on the adaptations to this training regime. Future studies will reveal how $p50_{mito}$ adapts to longer-term training, training protocols with different intensities and if changes in $p50_{mito}$ are involved in the mitochondrial adaptations to hypoxia or different pharmacological treatments.

In conclusion, this study shows that mitochondrial oxygen affinity can be increased ($p50_{mito}$ reduced) by an intensive

exercise programme and that the decrease in $p50_{mito}$ is related to the increase in VO_2peak.

4 | MATERIALS AND METHODS

The experimental protocol, training intervention, pre- and post-testing have all been described previously in more detail.[8] See Scheme 1 for an overview of the experimental setup.

4.1 | Subject characteristics

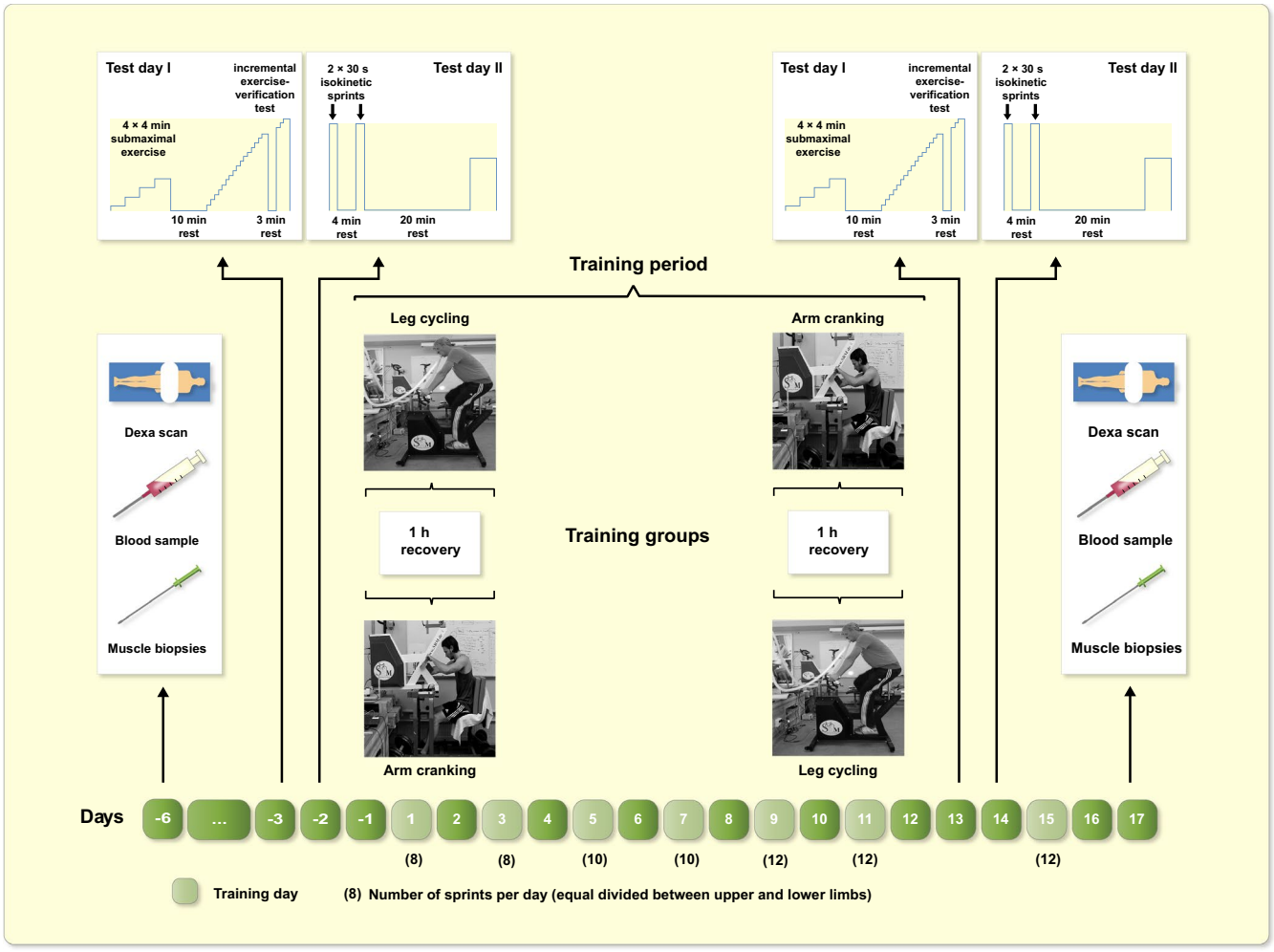
Twelve untrained or only moderately active, male subjects (24.2 ± 3.8 years; 183.8 ± 6.8 cm; 80.0 ± 14.7 kg, BMI 23.6 ± 0.9 kg m^{-2}) volunteered for this study. Subjects gave their written consent after being informed of the study procedure. The study was approved by the Regional Ethical Review Board in Umeå, Sweden (2014/91-31) and performed according to the principles of the declaration of Helsinki.

4.2 | Blood sampling, muscle biopsies and body composition

Briefly, 6 days before the start of the training period, the subjects reported to the laboratory early in the morning following an overnight fast. Thereafter, a venous blood sample and muscle biopsies (from the *m. Vastus lateralis* and *m. Triceps brachii*) were obtained. Local anaesthesia (2-3 mL 2% carbocaine) was applied; and a biopsy was taken with a modified Bergström needle with suction through an incision in the skin and fascia. These same procedures were repeated at least 48 hours after the last training session (7th).

4.3 | Isolation of mitochondria

The isolation of mitochondria was performed in an isolation solution (100 mmol L^{-1} sucrose, 100 mmol L^{-1} KCl, 50 mmol L^{-1} Tris-HCl, 1 mmol L^{-1} KH2PO4, 100 μmol L^{-1} EGTA and 0.1% BSA, pH 7.4) using differential centrifugation. In the first



SCHEME 1 Overview of the experimental protocol. Training sessions, pre- and post-tests were performed using both arm cranking and traditional cycling with the legs. The number of intervals per session increased throughout the training period. Biopsies were taken as denoted above

isolation step, bacterial protease (0.2 mg mL^{-1}) was added to the medium to liberate intra-myofibrillar mitochondria. Differential centrifugation was carried out at 700 g for 10 minutes where after the supernatant was recovered and centrifuged at 10 000 g for 10 minutes. After careful washing, the pellet was resuspended in isolation medium and again centrifuged at 7000 g for 5 minutes. The final pellet was resuspended in preservation solution (0.5 mmol L^{-1} EGTA, 6 3 mmol L^{-1} MgCl, 60 mmol L^{-1} K-lactobionate, 20 mmol L^{-1} taurine, 10 mmol L^{-1} KH2PO4, 20 mmol L^{-1} HEPES, 110 mmol L^{-1} sucrose, 1 mg mL^{-1} BSA, 20 mmol L^{-1} histidine, 3 mmol L^{-1} glutathion, 1 mmol L^{-1} leupeptine, 2 mmol L^{-1} glutamate, 2 mmol L^{-1} malate and 2 mmol L^{-1} Mg-ATP) and allowed to stabilize for at least 30 minutes before the respiratory experiments were initiated.

4.4 | Analysis of mitochondrial oxygen affinity

High-resolution respirometry (O2k, Oroboros, Austria) was used to assess respiration in isolated mitochondria. The apparent km ($p50_{mito}$) for oxygen was determined using the software Datlab 2 (Oroboros, Austria) by integrating a hyperbolic function of mitochondrial oxygen consumption and oxygen pressure during and transition from aerobic respiration to anoxia. Experiments were performed in a respiration medium containing (EGTA 0.5 mmol L^{-1}, $MgCl_2$ 3 mmol L^{-1}, K-lactobionate 60 mmol L^{-1}, taurine 20 mmol L^{-1}, KH_2PO_4 10 mmol L^{-1}, HEPES 20 mmol L^{-1}, sucrose 110 mmol L^{-1} and BSA 1 g L^{-1}). The respiratory substrates used were pyruvate 5 mmol L^{-1}, malate 1 mmol L^{-1}, in the presence of ADP 2.5 mmol L^{-1} for the determination of complex I-mediated respiration, and after addition of succinate 10 mmol L^{-1} complex I + II-mediated respiration was assessed. The transition between the aerobic to the anoxic state occurs within seconds and electrical interference (noise) in this critical period can disturb the signal so the mathematical curve-fitting becomes invalid. See the corresponding n-values at each figure for how many $p50_{mito}$-values that were included in the analysis.

4.5 | Test day I

Three days before starting the training programme, the volunteers performed an incremental exercise test of arm cranking and leg pedalling (in random order), separated by a 2-h rest. Initially, four consecutive bouts (each 4 minutes long) at intensities between 40% and 90% of VO_2peak (100 rpm, respiratory exchange ratio [RER] <1.0) were performed, with one of the submaximal intensities set at 80 W. After 10 minutes rest, the incremental exercise test to determine maximal oxygen uptake (VO_2peak) began. The maximal arm-cranking test started at 20 W and the intensity increased by 10 W every 30 seconds. During leg-cycling the initial intensity was 60 W and the increased 25 W every 30 seconds. At least three of the four criteria for VO_2peak for the arms and legs were met in all subjects. The criteria were defined as a plateau in oxygen uptake (ie an elevation of <2.1 mL min^{-1} kg^{-1} with increasing resistance), a RER >1.10, a heart rate within 2.5% of that expected according to age, and a capillary blood lactate concentration >6 mmol L^{-1}. Three minutes after termination of this test, a verification test was performed starting at the highest load attained during the incremental exercise. The oxygen uptake (VO_2), carbon dioxide production (VCO_2) and pulmonary ventilation (V_E) were monitored continuously employing an open-circuit metabolic cart (AMIS 2001 model C; Innovision A/S, Odense, Denmark) as described previously[8] and averaged every 10 seconds. VO_2peak was defined as the highest average 20-s VO2 value during either the incremental exercise, the verification test or the TT (not described here, see[8]). The same absolute intensities were used for the pre- and post-testing.

4.6 | The training protocol

On each training day, 4-6 30-s isokinetic Wingate sprints of arm cranking and leg cycling training were performed, separated by one hour. Half of the subjects trained their arms first and then their legs, and the other half vice-versa (in randomized order). On the first and the second days of training, the subjects performed four sprints with each limb; on the 3rd and 4th day five sprints; on the 5th, 6th and 7th day six sprints were performed, with 4 minutes of recovery between consecutive sprints with the same limbs. The seventh training session was performed the day after post- testing days and 2 days before post-sampling of blood and muscle. At least one day always elapsed between the training sessions.

4.7 | Muscle biopsies treatment

Muscle pieces were cleaned on filter paper to remove any visible blood, fat or connective tissue. Then a portion of muscle was immediately frozen in liquid nitrogen and stored at −80°C for later analysis. Another piece of muscle was fast immersed in ice-cold isolation medium (Sucrose 100 mmol L^{-1}; KCl 100 mmol L^{-1}; Tris-HCl 50 mmol L^{-1}; KH2PO4 1 mmol L^{-1}; EDTA 100 μmol L^{-1}; BSA essential fatty acid free 0.1% pH 7.4).

4.8 | Statistical analysis

GraphPad Prism 8.0 was used for statistical analysis. Initially a two-way ANOVA was used to determine if the changes were significantly different between *Vastus* and *Triceps*. No such differences were found and instead paired students

t-tests were used to determine difference between the pre- and post-session. Kolmogorov-Smirnov test was used to assess normality distribution. Data are presented as individual values with connecting lines. For correlation analysis, Pearson *r* linear regression analysis was used on normally distributed data. $P < .05$ was considered statistically significant.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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