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## ABSTRACT

**PURPOSE:** Dietary nitrate supplementation has been shown to reduce O<sub>2</sub> cost of submaximal exercise, improve exercise tolerance and enhance performance in moderately trained individuals. In contrast, data have been provided that elite athletes do not benefit from nitrate supplementation. The aim of this study was to evaluate the effects of short-term nitrate supplementation on endurance performance in subjects with different level of aerobic fitness.

**METHODS:** Twenty-one subjects (age 22.7±1.8 years) with different aerobic fitness level ( $\dot{V}O_{2peak}$  value ranging from 28.2 to 81.7 ml·kg<sup>-1</sup>·min<sup>-1</sup>) participated in a crossover double-blind placebo-controlled study. Subjects were tested after 6 days of supplementation with either 0.5 l per day of nitrate (5.5 mmol) containing water (NITR) or nitrate-free water (PLA). Participants performed an incremental running test until exhaustion and four repetitions of 6-min sub-maximal (about 80% of gas exchange threshold) constant load exercise on a motorized treadmill. Moreover, subjects performed a 3-km running Time Trial on the field. **RESULTS:** After NITR, a negative correlation between reduction of O<sub>2</sub> cost of submaximal exercise and individual aerobic fitness level was observed ( $r^2=0.80$ ;  $p<0.0001$ ). A significant inverse correlation was also found between aerobic fitness level and improvement in performance for 3-km Time Trial after NITR ( $r^2=0.76$ ;  $p<0.0001$ ). Additionally, subjects responded differently to dietary nitrate supplementation according to aerobic fitness level with higher fit subjects showing a lower increase in plasma [NO<sub>3</sub><sup>-</sup>] ( $r^2=0.86$ ;  $p<0.0001$ ) and [NO<sub>2</sub><sup>-</sup>] ( $r^2=0.75$ ;  $p<0.0001$ ). **CONCLUSIONS:** The results of the present study suggest that the individual aerobic fitness level affects the ergogenic benefits induced by dietary nitrate supplementation. The optimal nitrate loading regimen required to elevate plasma [NO<sub>2</sub><sup>-</sup>] and to enhance performance in elite athletes is different from that of low fit subjects and requires further studies.

**KEYWORDS:** Plasma nitrite, Oxygen cost, Performance,  $\dot{V}O_2$  kinetics

## INTRODUCTION

Nitrate ( $\text{NO}_3^-$ ) is a relatively inert anion that can be converted in vivo to bioactive nitrogen oxides including nitric oxide (NO) (30). The main dietary sources of inorganic nitrate are vegetables (e.g. spinach and beetroot), processed meats (where it is added as a preservative) and water (8). Circulating plasma  $\text{NO}_3^-$  can be actively taken up by salivary glands, excreted in saliva, and reduced to  $\text{NO}_2^-$  by commensal bacteria in the oral cavity (32). When swallowed in the acidic environment of the stomach,  $\text{NO}_2^-$  is further converted to NO, whilst the remainder is absorbed and increases circulating plasma  $[\text{NO}_2^-]$ . This  $\text{NO}_2^-$  can be further reduced to NO, particularly in tissues which are relatively hypoxic, such as contracting skeletal muscle (32), and play a significant role in a myriad of physiological processes, amongst which, at the skeletal muscle level, the increase in blood flow and a partial inhibition of mitochondrial respiration (40). This nitrate ( $\text{NO}_3^-$ ) – nitrite ( $\text{NO}_2^-$ ) – nitric oxide (NO) pathway represents an alternative (and differently regulated) system compared to the classic L-arginine NO synthesis pathway, and is involved in important physiological processes such as the regulation of blood flow (17) and blood pressure (29), cell signaling and glucose homeostasis (9), and tissue responses to hypoxia (32).

Recent studies have demonstrated that single or short-term (3-6 days) dietary nitrate supplementation in the form of sodium nitrate (30, 31) as well as beetroot juice (2, 27, 41): a) has a very limited effect on  $\dot{V}\text{O}_{2\text{max}}$ , maintaining or improving power output or speed at  $\dot{V}\text{O}_{2\text{max}}$  (23); b) can induce a significant reduction of the  $\text{O}_2$  cost during steady-state submaximal exercise, in association with no increase in blood lactate concentration (2, 28, 31), indicating that the lowered oxygen uptake following nitrate intake is not compensated by an enhanced anaerobic energy turnover; c) does not change the speed of the primary phase of the  $\dot{V}\text{O}_2$  response while

exercising at a moderate intensity (2, 28, 41) although it results in a small but significant speeding of  $\dot{V}O_2$  kinetics in situations where muscle  $O_2$  supply is more likely to be compromised, for example in older adults (26) and in young adults performing high-intensity exercise (7); d) may enhance performance across events where there is a significant oxidative contribution to energy turnover (23).

The precise mechanisms responsible for these effects have yet to be elucidated but are thought to be attributable to NO-mediated enhancements of muscle contractile function (1, 18) and/or mitochondrial efficiency (30).

Despite the considerable evidence of the positive ergogenic effects of nitrate supplementation in recreationally active and moderately trained individuals (with a maximal oxygen uptake around  $50\text{-}55\text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ ), recent studies have failed to show improved exercise performance following dietary nitrate supplementation in elite cyclists (4, 11, 13, 43), cross-country skiers (36) and runners (6). The reason(s) for the lack of effects observed in elite athletes is(are) not understood. It has been hypothesized that adaptations elicited by endurance training may play a role in nitrate supplementation response (4). Indeed, trained subjects appear characterized by a greater activity and presence of eNOS (endothelial nitric oxide synthase), the enzyme responsible for endogenous generation of nitric oxide (20, 33). An increase in eNOS activity may decrease the production of NO from nitrate and thus attenuate the benefits from nitrate supplementation (20). Moreover, highly trained athletes have higher plasma levels of nitrate and nitrite than sedentary subjects (42), such that the response to a standard dose of nitrate may be diminished. Interestingly, a limited number of “responders” were identified both in elite cyclist (13, 43) and in elite runners (6), indicating that aerobic fitness level may not be the only factor affecting the ergogenic effects of nitrate supplementation. In addition, different exercise

modality (i.e. running, cycling, etc), exercise testing protocol and supplementation regime (acute/chronic, different nitrate concentration) have been utilized in aforementioned studies, limiting the extent to which inferences on the effects of fitness on the efficacy of nitrate can be made.

No study to date has directly compared whether highly trained subjects respond to nitrate supplementation differently from sedentary or recreationally active individuals in the same experimental conditions. Thus, the aim of this study was to investigate whether the individual aerobic fitness level influences the ergogenic effect of nitrate supplementation on: i) typical parameters of oxidative metabolism such as the maximal oxygen uptake ( $\dot{V}O_2$ ), the  $O_2$  cost of exercise and kinetics of pulmonary  $\dot{V}O_2$  following the onset of moderate-intensity exercise; and ii) exercise performance. The enhancing aspects of  $NO_3^-$  supplementation on the physiological response to exercise and performance will be correlated with resting plasma  $[NO_3^-]$  and  $[NO_2^-]$  levels and/or their changes following supplementation. We hypothesize that: 1) the ergogenic effects of nitrate supplementation are correlated with aerobic fitness level; and 2) the different responses amongst subjects with a wide range of physical fitness may be explained on the basis of plasma  $[NO_3^-]$  and  $[NO_2^-]$  changes.

## **METHODS**

**Subjects.** Twenty-one young male individuals (age,  $22.7 \pm 1.8$  yr; body mass,  $62.2 \pm 10.5$  kg; height,  $1.71 \pm 0.07$  m; body mass index,  $21.1 \pm 2.3$   $kg \cdot m^{-2}$ ) were selected, from a larger population, with respect to their fitness levels by means of a questionnaire on physical activity (IPAQ-SF) and volunteered to participate in this study. Six subjects were actively involved in

structured training programs and, although endurance training formed a large part of their training routine, they also undertook regular resistance conditioning. Three of these athletes were participants in national and international competitions. Four subjects trained 3 to 4 times per week and were participating in regional-level official competitions at the time of data collection. Seven participants were recreationally active individuals who engaged in a variety of activities (e.g., weightlifting, running, team sports) less than 2 times per week. Four subjects were young university students sedentary or engaging in exercise for <90 min per week. All participants were non-smokers, normotensive and were not assuming any drugs. The procedures used in this study were approved by the local ethics committee. All subjects gave their written informed consent after an explanation of the experimental procedures and before commencement of the study.

**Experimental design.** Each participant visited the laboratory on five separate occasions. On their first visit, anthropometric measurements and an incremental exercise (pre-examination) test to determine maximal oxygen uptake ( $\dot{V}O_{2\text{peak}}$ ) and the gas exchange threshold (GET) were performed. The protocol began with subjects running at 7-10 km·h<sup>-1</sup> for 6 min, according with their presumed fitness level estimated by IPAQ-SF; then the belt speed was increased by 1 km·h<sup>-1</sup> every minute until volitional exhaustion. The GET was determined as previously described (3) in order to calculate the treadmill speed that would require 80% of the individual GET (moderate-intensity exercise). The peak values of the main cardiovascular, respiratory and metabolic parameters were taken as the highest 30-s mean value attained prior the subject's volitional exhaustion (see Table 1). According to Rowell (39), subjects were divided into three groups on the basis of their  $\dot{V}O_{2\text{peak}}$  value (Low Aerobic Fitness - LOW n=8,  $\dot{V}O_{2\text{peak}}$  range 28.2-44.1 mL·kg<sup>-1</sup>·min<sup>-1</sup>; Moderate Aerobic Fitness - MOD n=7,  $\dot{V}O_{2\text{peak}}$  range 45.5-57.1 mL·kg<sup>-1</sup>·min<sup>-1</sup>;



High Aerobic Fitness - HIGH n=6,  $\dot{V}O_{2\text{peak}}$  range 63.9-81.1 mL·kg<sup>-1</sup>·min<sup>-1</sup>). Two days after they carried out a 3-km running test on a 400-m outdoor track with no dietary supplementation (pre-intervention control) in order to gain familiarity with this test.

Subsequently each subject was randomly assigned in a double-blind, crossover design to follow 6 days of supplementation with either sodium nitrate (about 5.5 mmol·day<sup>-1</sup>, Sigma, Italy) or the placebo (sodium chloride, 8.0 mmol·day<sup>-1</sup>, Sigma, Italy) dissolved in water. The daily dose was ingested once before breakfast. The different solutions could not be distinguished by taste or appearance. On day 5 of both supplementation periods, subjects underwent incremental exercise testing and two repetitions of a constant load moderate-intensity exercise. On day 6 subjects repeated two constant load moderate-intensity exercises and performed a 3-km Time Trial (see below for further details). The last ingestion (nitrate or placebo) was 3.5±0.5 h before the exercise tests. Athletes received nutritional guidelines in order to eat the same amount of moderate-high nitrate content foods (green vegetables, beetroot, strawberries, grapes, and tea). Subjects were also required to abstain from using antibacterial mouthwash and chewing gum, as there are known to destroy the oral bacteria responsible for the reduction of NO<sub>3</sub><sup>-</sup> to NO<sub>2</sub><sup>-</sup> (15). A 14-day washout separated the supplementation periods.

**Exercise tests.** Subjects were instructed to arrive at the laboratory in a rested and fully hydrated state and to avoid strenuous exercise in the 24 h preceding each testing session. In addition, they were told to avoid alcohol and caffeine intake 48 h before the exercise test. All laboratory exercise tests were carried out in a well-ventilated laboratory at 19–21°C on a motorized treadmill (Jaeger, Germany) set at a 1% gradient.

Subjects initially performed a ramp incremental exercise test for the determination of  $\dot{V}O_{2peak}$  and GET. The protocol began with subjects running at the speed requiring 80% of the individual GET for 6 min; then the belt speed was increased by  $1 \text{ km}\cdot\text{h}^{-1}$  every minute until volitional exhaustion. The peak values of the main cardiovascular, respiratory and metabolic parameters were taken as the highest 30-s mean value attained prior to the subject's volitional exhaustion. On days 5 and 6 of both supplementation periods, subjects completed two 6-min bouts of moderate intensity running (80% GET) for the determination of pulmonary  $\dot{V}O_{2peak}$  kinetics.

As for running performance, participants completed a 3-km running Time Trial on a 400-m outdoor track. To minimize the variability in pacing strategy (i.e., suboptimal or inconsistent pacing strategies) reported to occur at the start of a self-paced Time Trial independent of any intervention, participants were encouraged to begin Time Trials based on their experiences in preliminary trials. For this reason, participants were provided with feedback on their lap times for the first three laps but were blinded to exercise time thereafter. Performance times were recorded by using two synchronized stopwatches.

**Measurements.** Pulmonary ventilation ( $\dot{V}E$ , in BTSP),  $O_2$  consumption ( $\dot{V}O_2$ ), and  $CO_2$  output ( $\dot{V}CO_2$ ), both in STPD, were determined breath-by-breath by a metabolic cart (Vmax29c; SensorMedics, Bilthoven, The Netherlands). Expiratory flow was determined by a mass flow sensor (hot wire anemometer).  $\dot{V}O_2$  and  $\dot{V}CO_2$  were determined by continuously monitoring  $PO_2$  and  $PCO_2$  at the mouth throughout the respiratory cycle and from established mass balance equations. Gas exchange ratio (RQ) was calculated as  $\dot{V}CO_2/\dot{V}O_2$ . HR was determined from the

ECG signal. At rest and at various times (1, 3, and 5 min) during recovery, 20  $\mu\text{L}$  of capillary blood was obtained from a preheated earlobe for the determination of blood lactate concentration ( $[\text{La}]_b$ ) by an enzymatic method (Biosen 5030; EKF, Cosmed, Italy) both for laboratory and field testing.

**Kinetics analysis.**  $\dot{V}\text{O}_2$  kinetics were evaluated during transitions from rest to constant load exercise. Breath-by-breath  $\dot{V}\text{O}_2$  values obtained during the four repetitions of the exercises were time aligned and then superimposed for each subject. Average  $\dot{V}\text{O}_2$  values every 10 s were calculated. Data obtained during the first 20 s of the transition (“cardiodynamic” phase) (37) were excluded from analysis. Thus,  $\dot{V}\text{O}_2$  kinetics analysis focused on the “phase 2” (or “fundamental” component) of the response, which more closely reflects gas exchange kinetics occurring in skeletal muscles (16).

To mathematically evaluate the  $\dot{V}\text{O}_2$  kinetics, data were first fitted by a monoexponential function of the type:

$$y(t) = y_{\text{BAS}} + A_f [1 - \exp - (x - \text{TD}_f) / \tau_f] \quad (\text{Eq. 1})$$

where  $y_{\text{BAS}}$  indicates the  $\dot{V}\text{O}_2$  value at baseline;  $A_f$  the amplitude of the  $\dot{V}\text{O}_2$  response calculated between the baseline value and the steady-state value for the fundamental component;  $\text{TD}_f$  is the time delay, and  $\tau_f$  the time constant of the function for the fundamental component.

To check the presence of a slow component of the kinetics (24), data were also fit by a double exponential function of the type:

$$y(t) = y_{\text{BAS}} + A_f [1 - \exp - (x - \text{TD}_f) / \tau_f] + A_s [1 - \exp - (x - \text{TD}_s) / \tau_s] \quad (\text{Eq. 2})$$

where  $A_s$ ,  $TDs$ , and  $\tau_s$  indicate, respectively, the amplitude, the time delay, and the time constant of the slow component of the kinetics. No slow component was observed in all the constant load exercises both for NITR and PLA.

**Blood sampling.** Resting blood sample was collected to determine plasma levels of nitrate and nitrite both on day 5 and 6. Venous blood was drawn from the antecubital vein into a 5-mL EDTA vacutainer tube (Vacutainer, Becton Dickinson, USA). Plasma was immediately separated by centrifuge (5702R, Eppendorf, Germany) at 1000 x g for 10 min at 4 °C. Plasma samples were then ultrafiltered through a 10 kDa molecular weight cut-off (AmiconUltra; Millipore, EMD Millipore Corporation, Billerica, USA) using an ultracentrifuge (4237R, ALC, Italy) at 14000 x g for 60 min at 4 °C to reduce background absorbance due to the presence of hemoglobin. The ultrafiltered was recovered and used to measure nitrite and nitrate concentration. We utilized a commercial colorimetric assay kit (Cayman Chemical, USA) which provides an accurate and convenient method for measurement of nitrate and nitrite concentration. Samples were read by the addition of Griess reagents at 545 nm by a microplate reader spectrophotometer (Infinite M200, Tecan, Austria). A linear calibration curve was computed from pure nitrite and nitrate standard. All samples were determined in duplicate and the inter-assay coefficient of variation was in the range indicated by the manufacturer.

**Statistical analysis.** Results are expressed as mean  $\pm$  SD. A two-way mixed-design ANOVA with repeated measures (independent measures on aerobic fitness level and repeated measures on supplementation) has been utilized to examine the effects of intervention, aerobic fitness level, and the interaction between the two. Post-hoc analysis was completed using Bonferroni multiple comparisons. Significance level was set at  $p < 0.05$ . When significant effects

of intervention were found, a student t-test for paired data was used to determine differences between PLA and NITR conditions. Pearson statistical test and the square of Pearson's correlation coefficient ( $r^2$ ) have been used to examine the relationship between variables. All statistical procedures were completed using Prism 6.0 (GraphPad Software, CA, USA).

## RESULTS

*Incremental Exercise (IE).* Average ( $\pm$ SD) peak values of the main cardiovascular, respiratory and metabolic parameters obtained in LOW, MOD and HIGH are shown in Table 2. All groups attained peak HR values around 95% of the age predicted maximum. Thus, taking into account also RQ,  $[La]_b$  peak and RPE peak values, it can be assumed that maximum exercise capacity had been reached in each group. As expected, speed and  $\dot{V}O_{2peak}$  values were lower in LOW than in MOD, and the latter achieved lower peak values than HIGH. GET occurred at  $76.8\pm 6.2\%$ ,  $83.5\pm 4.3\%$ ,  $86.9\pm 6.2\%$  of  $\dot{V}O_{2peak}$  in LOW, MOD and HIGH respectively. For all the variables, no differences were observed between PLA and NITR in the three groups.

*Constant Load Exercise (CLE).* Mean ( $\pm$ SD)  $\dot{V}O_2$  values over the final 30 s of moderate exercise are shown in Figure 1A. There was a progressive increase of  $O_2$  cost of exercise in LOW, MOD and HIGH according to the different absolute intensity (80% of GET) of exercise. A significantly lower  $\dot{V}O_2$  was observed in NITR vs. PLA both for LOW and MOD (9.8% and 7.3% respectively) whereas no change was found in HIGH. In Figure 1B individual percentage reduction of  $\dot{V}O_2$  at steady state of all subjects are plotted *versus* the corresponding  $\dot{V}O_{2peak}$  value. A highly significant linear relationship was observed with lower fit subjects showing a

greater reduction of oxygen cost of exercise. The observed  $r^2$  value indicates that 80% of  $\dot{V}O_{2ss}$  variability can be explained in terms of differences in aerobic fitness level. The same significant correlation ( $r^2=0.72$ ,  $p<0.0001$ ) was observed between GET and individual percentage reduction of  $\dot{V}O_2$  at steady state.  $\dot{V}CO_2$ , RQ,  $[La]_b$ ,  $\dot{V}E$  and HR values were not significantly different between two conditions in the three groups.

As for the kinetics of adjustment of  $\dot{V}O_2$  during CLE, in NITR a reduction of the amplitude of pulmonary  $\dot{V}O_2$  response, relative to PLA, was observed in LOW ( $1.23\pm 0.13$  vs.  $1.37\pm 0.12$  L $\cdot$ min $^{-1}$ , respectively), and MOD ( $1.54\pm 0.30$  vs.  $1.72\pm 0.24$  L $\cdot$ min $^{-1}$ , respectively), whereas no difference was observed in HIGH ( $2.09\pm 0.15$  vs.  $2.19\pm 0.19$  L $\cdot$ min $^{-1}$ , respectively). Resting  $\dot{V}O_2$ ,  $\tau_f$  and TDF were not affected by supplementation in the three groups.

*Running performance.* 3-km Time Trial results are shown in Figure 2A. There was a progressive reduction of finish time according to the aerobic fitness level of the subjects. In NITR the time to cover 3 km was statistically lower than in PLA both for LOW ( $886\pm 74$  vs.  $910\pm 82$  s,  $p=0.0002$ ) and MOD ( $723\pm 90$  vs.  $734\pm 93$  s,  $p=0.0002$ ). Performance did not change in HIGH ( $627\pm 30$  vs.  $629\pm 28$  s, in NITR and PLA respectively).  $[La]_b$  values were not statistically different in LOW ( $7.4\pm 2.3$  vs.  $7.3\pm 2.5$  mM), MOD ( $9.4\pm 1.6$  vs.  $10.6\pm 1.9$  mM) and HIGH ( $10.1\pm 3.5$  vs.  $8.4\pm 3.0$  mM) for conditions NITR and PLA, respectively. In Figure 2B individual 3-km Time Trial difference of all subjects are plotted *versus* the corresponding  $\dot{V}O_{2peak}$  value. A significant inverse linear relationship was observed with lower fit subjects showing a higher improvement in performance. The observed  $r^2$  value indicates that over 75% of 3-km Time Trial difference variability can be explained in terms of differences in aerobic fitness level. The same significant correlation ( $r^2=0.70$ ,  $p<0.0001$ ) was observed between GET and individual 3-km Time Trial difference.

*Plasma [NO<sub>3</sub><sup>-</sup>] and [NO<sub>2</sub><sup>-</sup>].* The group mean plasma [NO<sub>3</sub><sup>-</sup>] values of both supplementation periods are illustrated in Figure 3A. After PLA [NO<sub>3</sub><sup>-</sup>] values were significantly higher in HIGH (28.3±2.1 μM) relative to both MOD (23.2±3.8 μM) and LOW (21.3±3.4 μM). After NITR plasma [NO<sub>3</sub><sup>-</sup>] values significantly increased in the three groups (93.7±17.1, 140.4±16.8 and 181.7±32.7 μM in HIGH, MOD and LOW respectively, p<0.0001). Individual values of plasma [NO<sub>3</sub><sup>-</sup>] in relation to the corresponding  $\dot{V}O_{2peak}$  values are shown in Figure 3B both for PLA and NITR. As for PLA, a linear relation was observed between [NO<sub>3</sub><sup>-</sup>] and  $\dot{V}O_{2peak}$ , with lower fit subject showing a lower concentration of nitrate (r<sup>2</sup>=0.58, p<0.0001). After NITR an inverse linear relation was observed between the same variables (r<sup>2</sup>=0.73, p<0.0001).

The group mean plasma [NO<sub>2</sub><sup>-</sup>] values of both supplementation periods are illustrated in Figure 3C. After PLA no significant differences were observed among the three groups. After NITR plasma [NO<sub>2</sub><sup>-</sup>] values significantly increased in the three groups respect to PLA (462.3±249.5, 418.7±266.1 and 265.7±163.7 nM in LOW, MOD and HIGH respectively, p<0.0001) whereas no significant differences were observed among groups. No relationship was found between individual values of plasma [NO<sub>2</sub><sup>-</sup>] and the corresponding  $\dot{V}O_{2peak}$  value both for PLA and NITR. A negative linear relationship was observed between the individual increase of plasma [NO<sub>2</sub><sup>-</sup>] after NITR and the corresponding  $\dot{V}O_{2peak}$  values (Fig. 3D).

In addition, there was a significant negative correlation between the increase of plasma [NO<sub>2</sub><sup>-</sup>] after NITR and the difference in both O<sub>2</sub> cost of submaximal exercise (r<sup>2</sup>=0.49, p=0.0004; Fig. 4A) and 3-km running Time Trial performance (r<sup>2</sup>=0.49, p=0.0004; Fig. 4B).

## DISCUSSION

The main finding of this study is that the individual aerobic fitness level affects the ergogenic benefits induced by dietary nitrate supplementation. Indeed, our study shows that six days of sodium nitrate supplementation (about 5.5 mmol·day<sup>-1</sup>) result in a reduction of oxygen cost of exercise at moderate intensity and an improvement of 3-km running Time Trial only in subjects with a low-moderate level of aerobic fitness ( $\dot{V}O_{2\text{peak}} < 60 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ ) whereas no effects were observed in highly trained subject.

Previous studies have shown that in recreationally active subjects ( $\dot{V}O_{2\text{peak}}$  around 50-55 ml·kg<sup>-1</sup>·min<sup>-1</sup>) short periods (3-6 days) of ingestion of either nitrate salts or nitrate rich beetroot juice reduces oxygen consumption by 3-14% during steady state submaximal cycling (2, 30, 31, 41, 44), running (28), and knee extensor exercise (1). In addition to improved exercise economy, nitrate supplementation enhanced exercise tolerance by 3–25% in cycling and running time to exhaustion tests in recreationally active men (2, 28, 44) and improved performance by 1.2–3% during cycling Time Trial tests (11, 27) and running (35) in moderately trained individuals. In our study nitrate supplementation induced a reduced oxygen consumption between 7 and 10% at moderate intensity (80% of GET) without affecting other pulmonary, cardiovascular and metabolic parameters in moderate fit subjects. The same effect, but with a larger reduction of oxygen consumption (7-13%), was observed in low aerobic fitness subjects ( $\dot{V}O_{2\text{peak}} < 45 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ ). As for 3-km running Time Trial (presumably carried out at about 85% of  $\dot{V}O_{2\text{peak}}$ ), LOW and MOD subjects completed the task faster (about 1-4%) following the sodium nitrate supplementation. Thus, our results in sedentary and moderately trained subjects confirm data present in literature.



As for highly trained individuals ( $\dot{V}O_{2\text{peak}} \geq 60 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ ), recent evidences show that athletes are less likely to positively respond to nitrate supplementation (4, 6, 12, 13, 36, 43). For example, Peacock et al. (36) found that the consumption (about 10 mM) of potassium nitrate 2.5 h before exercise neither reduced submaximal oxygen consumption during a low-intensity exercise nor enhanced 5-km running performance compared with placebo in ten highly trained cross-country skiers ( $\dot{V}O_{2\text{peak}} \sim 70 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ ). In addition, Boorsma et al. (6) showed that both acute and chronic (8-days) high-dose nitrate supplementation ( $\sim 13\text{-}19 \text{ mM}$ ) did not reduce running oxygen consumption or improved 1500 m Time Trial performance in a group of highly trained distance runners ( $\dot{V}O_{2\text{peak}} \sim 80 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ ). In our study we examined six subjects with a high level of aerobic fitness ( $\dot{V}O_{2\text{peak}} \sim 72 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ ). Six days of sodium nitrate supplementation did not change oxygen consumption, as well as other pulmonary, cardiovascular and metabolic parameters, in a constant load exercise at moderate intensity (80% of GET). Moreover, the performance on 3-km running Time Trial was not different from placebo. Thus, our results provide further evidence that elite athletes are unlikely to benefit from nitrate supplementation. Overall, when we take into account all the three groups, a strong correlation was observed between the individual  $\dot{V}O_{2\text{peak}}$  and the exercise responses, i.e., lower fit subjects showed both a higher reduction of  $O_2$  cost of submaximal exercise and a more relevant improvement in 3-km running Time Trial performance.

It remains unclear which could be the cause(s) responsible for the different ergogenic effects of the same amount of nitrate supplementation in relation to the individual aerobic fitness level. Some authors have suggested that the ergogenic effects of nitrate supplementation are related to enhanced nitric oxide bioavailability and it could be hypothesized that elite athletes already have optimal levels of NO due to: a) the reduction of  $\text{NO}_2^-$  to NO is potentiated in

hypoxic (10) and acidic (34) environments (in which the production of NO from the NOS pathway is impaired) whereas endurance training (22), at least by increasing capillary density in skeletal muscle, may preserve muscle oxygenation and thus diminish the likelihood of developing a hypoxic and/or acidic environment in the active muscle; b) the greater eNOS activity found in well-trained subjects (33), may reduce the importance of the  $\text{NO}_3^-$ - $\text{NO}_2^-$ -NO pathway in well-trained compared to moderately trained or sedentary individuals; c) due to the large daily energy expenditure and assuming the consumption of a mixed diet, it is likely that elite athletes have a greater dietary nitrate intake. Thus, the physiological adaptations induced by chronic endurance training and the possible great ingestion of vegetables rich in nitrate may reduce the effectiveness of dietary  $\text{NO}_3^-$  supplementation. Interestingly, the effects of nitrate supplementation appear to be more pronounced in type II muscle fibers (14, 18). Considering that there is evidence of a correlation between  $\dot{V}\text{O}_{2\text{max}}$  and percentage of type I fibers in the vastus lateralis muscle (e.g. 21) it could be hypothesized that the progressive blunting in the effects of nitrate as subjects'  $\dot{V}\text{O}_{2\text{peak}}$  increased might be linked to a lower proportion of type II fibers.

Regardless of greater endogenous production and/or greater dietary intake, well trained athletes exhibit higher baseline plasma  $[\text{NO}_3^-]$  and  $[\text{NO}_2^-]$  values than sedentary or lesser trained subjects (5, 42). Data of the current study are in agreement with these findings, since we have found that the greater the aerobic fitness level of the subject the higher was the absolute plasma  $[\text{NO}_3^-]$  level after placebo (i.e., baseline value). A novel observation in the present study is that after 6 days of ingestion of the same amount of sodium nitrate ( $5.5 \text{ mmol}\cdot\text{day}^{-1}$ ), the variation of plasma  $[\text{NO}_3^-]$  and  $[\text{NO}_2^-]$  is more pronounced in low fit subjects than in high fit subjects. We have not a clear explanation of the smaller increase of plasma  $[\text{NO}_3^-]$  and  $[\text{NO}_2^-]$  observed in

elite athletes after NITR supplementation. Subjects were asked to refrain from using antibacterial mouthwash, thus it seems rather unlikely that this feature could be related to modification in the microflora of the oral cavity. A further possibility is that urinary nitrate and nitrite excretion rose more substantially in elite athletes. Unfortunately, nitrate and nitrite urine concentration were not analyzed in the present study, so we cannot exclude that this occurred. Another possibility is that elite athletes have experienced a greater “consumption” of plasma nitrate and nitrite during daily training sessions, since previous studies have reported an effective consumption of these anions during exercise (5, 31). In this regard, it should also be considered that following non-exhaustive exercise, nitrate (25) and nitrite (38) levels have been reported to be higher than before exercise, probably due to the shear stress stimulating NO production in the arginine oxygen dependent pathway.

Now, even if it remains unclear why the same amount of nitrate ingested causes a different change in  $[\text{NO}_3^-]$  and  $[\text{NO}_2^-]$  according to the aerobic fitness level of the subjects, the relevant result of this study is that the changes in plasma  $[\text{NO}_3^-]$  and  $[\text{NO}_2^-]$  appeared to be linked to the improvements in exercise performance. In particular, we found a strong correlation between the change in plasma  $[\text{NO}_2^-]$ , a biomarker of NO production and availability, and the change in exercise performance following NITR. Our data confirm and extend previous observations obtained in homogeneous groups of well-trained cyclists (43) and rowers (19). Taken together these findings underline the importance of consuming an appropriate nitrate dose in order to produce a meaningful increase in plasma  $[\text{NO}_2^-]$  and, consequently, to improve exercise performance. It could be hypothesized that elite athletes may require a greater nitrate exposure than others to elicit any positive effects from dietary nitrate and thus, our results could be explained by an inadequate dose of nitrate supplementation. Nevertheless, even if we did not

test the effects of different dose of sodium nitrate on exercise performance, some literature data help us to exclude this possibility. In particular, whereas in recreationally active subjects the existence of a dose-response relationship between the acute amount of nitrate ingested and plasma  $[\text{NO}_2^-]$  and reduction of the  $\text{O}_2$  cost of moderate-intensity exercise has been reported (44), in elite runners acute and chronic supplementation with high doses of nitrate-rich beetroot juice failed to improve submaximal treadmill running economy or 1500-m Time Trial performance despite a dramatic increase in plasma  $[\text{NO}_3^-]$ . Interestingly, it has also been demonstrated that among elite athletes there are “responders” and “non responders” to same amount of nitrate. For example, despite no statistical effects on the whole group, Wilkerson et al. (43), Christensen et al. (13) and Boorsma et al. (6) identified some subjects in which nitrate supplementation enhanced performance (“responders”). Even if habitual nitrate intake, activity of oral nitrate reductases and other factors have been considered to be responsible, the physiological basis for the apparent existence of responders and non-responders to nitrate supplementation is presently unclear and requires further study.

Finally, moving our attention from the oxygen cost of constant load submaximal exercise to other typical parameters of oxidative metabolism, we did not observe any significant changes of them after dietary nitrate supplementation independently of the level of the aerobic fitness of the subjects. Indeed, peak oxygen consumption and gas exchange threshold were similar between PLA and NITR in the three groups. This finding is in accordance with several previous studies that have reported no change on maximal oxygen uptake following single or short-term (3-6 days) nitrate supplementation either in the form of sodium nitrate (31) or beetroot juice (2, 41, 44). Moreover, no alterations in  $\dot{V}\text{O}_2$  kinetics were observed in the three groups according to previous observation both in moderate trained (2, 41) and highly trained subjects (13).

In conclusion, our study shows that the ergogenic effects of nitrate supplementation are significantly related to the individual aerobic fitness level, with no benefits observed on highly trained subjects ( $\dot{V}O_{2\text{peak}} > 60 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ ). These different effects on  $O_2$  cost of exercise and performance seem related to the relative changes in plasma  $[\text{NO}_3^-]$  and  $[\text{NO}_2^-]$ . The clear negative relationship between the difference in plasma  $[\text{NO}_2^-]$  with NITR compared to PLA and the difference in exercise performance, provides further evidence to the notion that nitrate supplementation might be ergogenic if it results in an appreciable increase of plasma  $[\text{NO}_2^-]$ . The optimal nitrate loading regimen required to elevate plasma  $[\text{NO}_2^-]$  and to enhance performance in elite athletes is different from that of low fit subjects and requires further attention.

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#### **CONFLICT OF INTEREST**

There is no conflict of interest with any financial organization regarding the material discussed in the manuscript. The results of the present study do not constitute endorsement by the American College of Sports Medicine.

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## FIGURE CAPTIONS

Figure 1. A) Mean ( $\pm$ SD) oxygen consumption during steady state running at 80% of GET in all groups (LOW, MOD and HIGH) after placebo (PLA) and nitrate (NITR) supplementation, white and black columns respectively. \*Statistical difference between PLA and NITR ( $p < 0.05$ ). B) Percentage reduction of oxygen consumption during moderate constant load exercise after NITR versus individual  $\dot{V}O_{2peak}$  value. Vertical dotted lines define the three groups: LOW, MOD and HIGH.

Figure 2. A) Mean ( $\pm$ SD) time to complete the 3-km Time Trial in all groups (LOW, MOD and HIGH) after placebo (PLA) and nitrate (NITR) supplementation, white and black columns respectively. \*Statistical difference between PLA and NITR ( $p < 0.001$ ). B) Percentage change in finish time after NITR versus individual  $\dot{V}O_{2peak}$  value. Vertical dotted lines define the three groups: LOW, MOD and HIGH.

Figure 3. A) Plasma nitrate concentration in all groups (LOW, MOD and HIGH) after placebo (PLA) and nitrate (NITR) supplementation. \*Statistical difference between PLA and NITR ( $p < 0.0001$ ). #Statistical difference from HIGH ( $p < 0.01$ ) §Statistical difference from MOD ( $p < 0.001$ ). B) Individual plasma nitrate concentration after PLA (empty circles) and NITR (filled circles) versus  $\dot{V}O_{2peak}$  values. Vertical dotted lines define the three groups: LOW, MOD and HIGH. C) Plasma Nitrite concentration in all groups (LOW, MOD and HIGH) after placebo (PLA) and nitrate (NITR) supplementation. \*Statistical difference between PLA and NITR

( $p < 0.001$ ). D) Changes (express as fold of PLA) in plasma Nitrite concentration after NITR versus individual  $\dot{V}O_{2peak}$  value. Vertical dotted lines define the three groups: LOW, MOD and HIGH.

Figure 4. A) Individual change of plasma nitrite concentration after NITR (express as fold of PLA) versus individual percentage change of oxygen consumption during moderate exercise. B) Individual change (express as fold of PLA) of plasma nitrite concentration after NITR versus time percentage change to complete 3-km Time Trial.

Figure 1

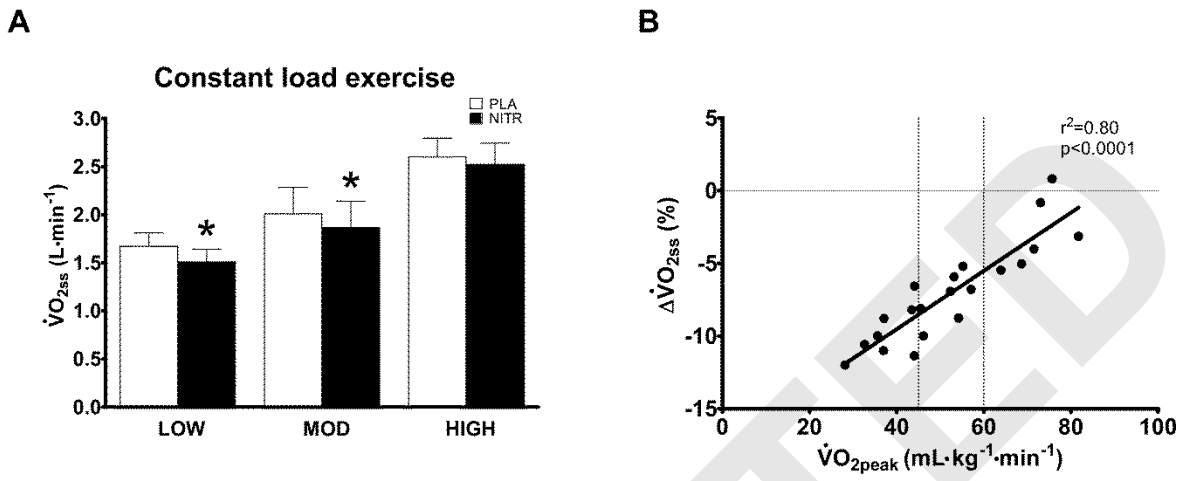


Figure 2

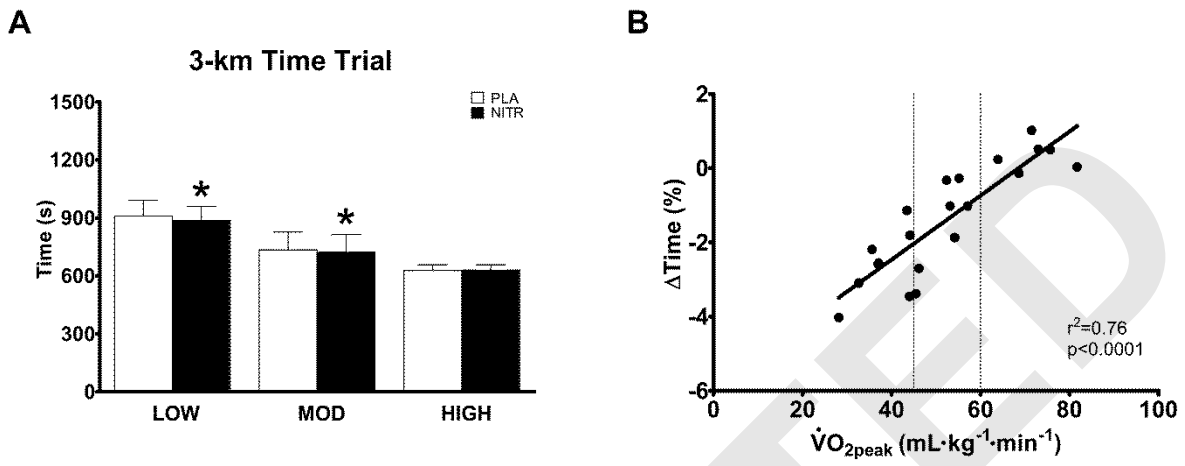


Figure 3

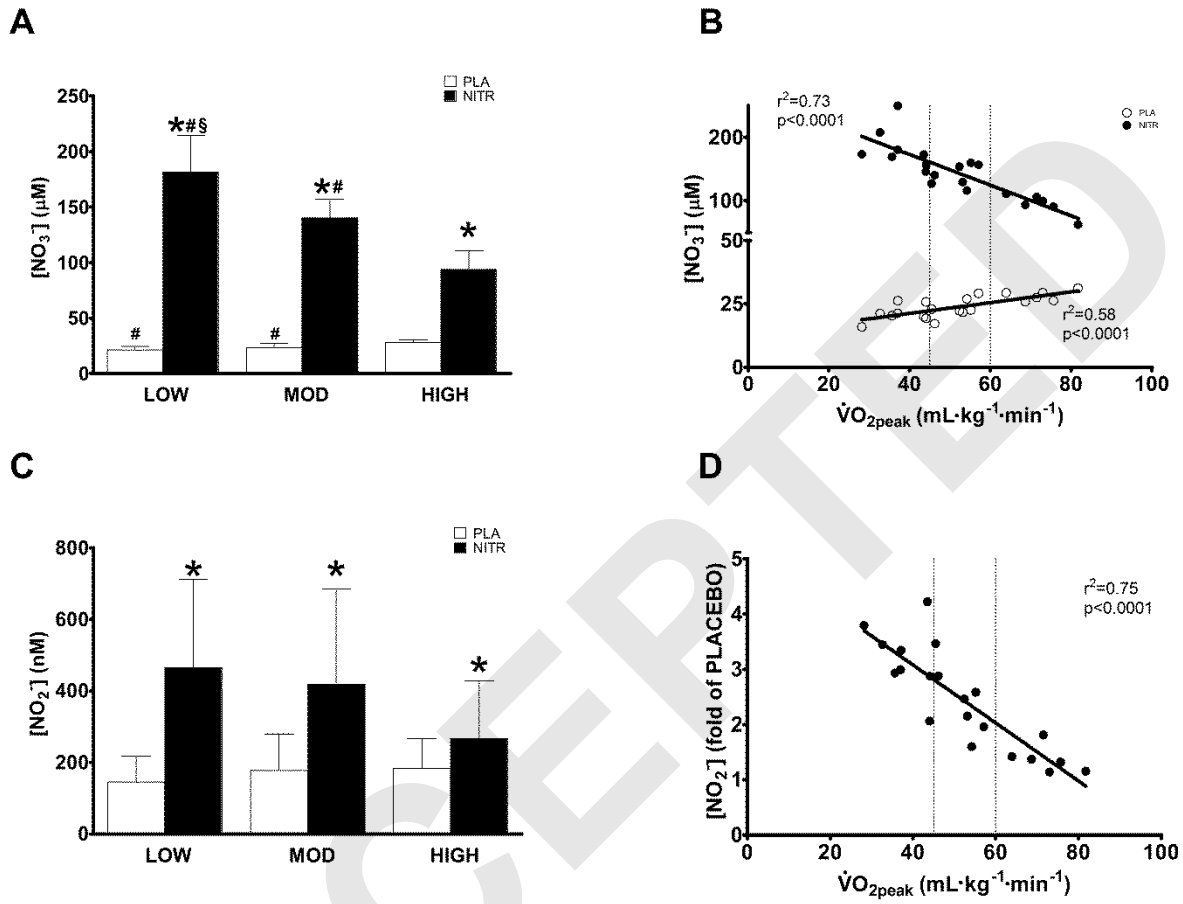


Figure 4

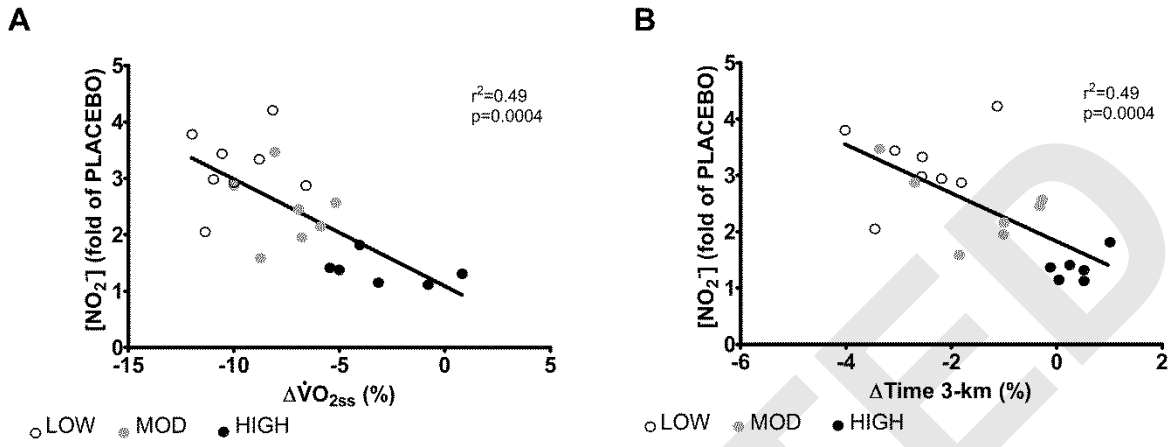




Table 1. Peak values of the main cardiovascular, respiratory and metabolic parameters at the end of the pre-examination incremental test in the whole investigated sample.

	<b>Mean (<math>\pm</math>SD)</b>	<b>Range</b>
speed (km·h <sup>-1</sup> )	17 $\pm$ 2.8	10 – 22
$\dot{V}O_2$ (L·min <sup>-1</sup> )	3.40 $\pm$ 1.55	1.45 – 6.66
$\dot{V}O_2$ (mL·kg <sup>-1</sup> ·min <sup>-1</sup> )	52.4 $\pm$ 15.3	28.2 – 81.7
$\dot{V}CO_2$ (L·min <sup>-1</sup> )	3.71 $\pm$ 1.70	1.47 – 7.39
RQ	1.1 $\pm$ 0.1	1.0 – 1.3
$\dot{V}E$ (L·min <sup>-1</sup> )	106.7 $\pm$ 25.5	57.1 – 150.5
HR (bmin <sup>-1</sup> )	188.0 $\pm$ 7	177 – 197
[La] <sub>b</sub> (mM)	9.1 $\pm$ 2.7	6.0 – 15.1
RPE	17.3 $\pm$ 1.6	13 – 19

Values are mean $\pm$ SD. Speed, treadmill speed;  $\dot{V}O_2$ , O<sub>2</sub> uptake;  $\dot{V}CO_2$ , CO<sub>2</sub> output; RQ, gas exchange ratio;  $\dot{V}E$ , pulmonary ventilation; HR, heart rate; [La]<sub>b</sub>, blood lactate concentration; RPE, rated of perceived exertion

Table 2. Peak values of the main cardiovascular, respiratory and metabolic parameters at the end of the incremental test after placebo (PLA) and nitrate (NITR) supplementation in the three groups (LOW, MOD and HIGH).

	LOW		MOD		HIGH	
	PLA	NITR	PLA	NITR	PLA	NITR
speed (km h <sup>-1</sup> )	14.4 ± 1.2	14.5 ± 0.8	17.4 ± 1.9	17.7 ± 1.9	20.0 ± 1.4	20.0 ± 0.9
$\dot{V}O_2$ (L·min <sup>-1</sup> )	2.04 ± 0.38	2.13 ± 0.40	3.10 ± 0.49	3.07 ± 0.47	5.57 ± 0.64	5.51 ± 0.64
$\dot{V}O_2$ (mL·kg <sup>-1</sup> ·min <sup>-1</sup> )	37.8 ± 5.8	39.3 ± 5.9	52.0 ± 4.5	51.5 ± 4.7	72.4 ± 6.1	71.7 ± 6.1
$\dot{V}CO_2$ (L·min <sup>-1</sup> )	2.21 ± 0.42	2.35 ± 0.51	3.41 ± 0.47	3.60 ± 0.61	6.05 ± 0.90	6.19 ± 0.94
RQ	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.2 ± 0.1	1.1 ± 0.1	1.1 ± 0.1
$\dot{V}E$ (L·min <sup>-1</sup> )	85.1 ± 13.5	88.0 ± 8.3	104.5 ± 15.5	109.2 ± 16.6	138.1 ± 10.9	130.9 ± 13.9
HR (b·min <sup>-1</sup> )	188 ± 6	188 ± 6	187 ± 7	189 ± 8	189 ± 8	188 ± 7
[La] <sub>b</sub> (mM)	7.6 ± 2.4	7.8 ± 1.8	10.4 ± 2.9	11.1 ± 3.0	8.7 ± 2.3	10.6 ± 2.4
RPE	16.8 ± 2.1	17.9 ± 1.1	17.1 ± 1.6	17.0 ± 1.5	18.2 ± 0.8	18.5 ± 1.0

Values are mean±SD. Speed, treadmill speed;  $\dot{V}O_2$ , O<sub>2</sub> uptake;  $\dot{V}CO_2$ , CO<sub>2</sub> output; RQ, gas exchange ratio;  $\dot{V}E$ , pulmonary ventilation; HR, heart rate; [La]<sub>b</sub>, blood lactate concentration; RPE, rated of perceived exertion