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The oral nitrate-reducing capacity correlates with peak power output and peak oxygen uptake in healthy humans

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ABSTRACT

Interest in inorganic nitrate and nitrite has grown substantially over the past decade as research has revealed the role of these anions in enhancing nitric oxide (NO) availability through an oral pathway. Nitrite synthesis in the mouth seems to be an important mechanism to feed the circulatory system with this anion. This is interesting since greater plasma nitrite concentration has been associated with better fitness levels in humans, but this question has not been investigated in relation to salivary nitrite concentration. Additionally, no previous study has investigated the oral nitrate-reducing capacity in regards to peak oxygen uptake (VO_{2peak}) or peak power output (W_{peak}) in humans. Thus, the main goal of this study was to investigate whether salivary nitrite and nitrate concentration and the oral nitrate-reducing capacity were associated with $VO2_{peak}$ and W_{peak} in healthy humans.

Fifty individuals (22 females and 28 males; 38.8 ± 14.3 years/old; BMI = 22.8 ± 3.9) performed a graded exercise test on a cycle ergometer to assess their VO_{2peak} and W_{peak}. Unstimulated salivary samples were taken before and 20 min after exercise to measure nitrate/nitrite, pH and lactate. The oral nitrate-reducing capacity was also assessed in 25 subjects before and after exercise.

Oral nitrate-reducing capacity was positively associated with W_{peak} (r_s = 0.64; P = 0.001) and the VO_{2peak} (r_s = 0.54; P = 0.005). Similar correlations were found when these variables were analysed after exercise. In addition, a significant decrease in salivary pH (pre: 7.28 ± 0.361; post-exercise: 7.16 ± 0.33; P = 0.003) accompanied by an increase of salivary lactate (pre: 0.17 ± 0.14 mmol/L; post-exercise: 0.48 ± 0.38; P < 0.001) was found after exercise. However, these changes did not have any impact on salivary nitrate/nitrite concentration and the oral nitrate-reducing capacity after exercise.

In conclusion, this is the first evidence showing a link between the oral nitrate-reducing capacity and markers of aerobic fitness levels in healthy humans.

1. Introduction

Nitrate and nitrite have emerged over the last decade as key regulators of nitric oxide (NO) metabolism in humans [1]. Nitrate is the main circulatory anion in healthy humans, and it has been estimated that about 25% of circulatory nitrate is absorbed in the salivary glands [2]. There, nitrate is excreted with saliva into the mouth where different species of oral bacteria can reduce it to nitrite [2]. Once nitrite is swallowed, a small part is rapidly absorbed into the circulation across the upper gastrointestinal tract [2]. Then, circulating nitrite can be reduced to NO through different pathways [3,4].

The main sources of nitrate in the body are diet (mainly green leafy vegetables and beetroot) and endogenous synthesis of NO through the enzymatic L-Arginine/NO Synthase (NOS) pathway [2,4]. Substantial research over the last decade has focused on dietary nitrate from two different perspectives: 1) as a potential ergogenic aid in sport, and 2) as a potential blood pressure-lowering dietary compound in health and disease [5,6]. From the first perspective, recent data suggest that supplementation of dietary inorganic nitrate may be useful to enhance aerobic performance, especially in low or moderately trained individuals, but not in well-trained athletes [7]. This has been attributed to exercise-induced adaptations at different levels [7]. For instance, chronic endurance training may lead to reconditioning responses on cardiovascular function, skeletal muscle vascularization, and enhanced mitochondrial efficiency [8]. Exercise can also enhance NO synthesis through the L-Arginine/NOS pathway, which in turn, increases endogenous nitrate and nitrite bioavailability [6].

Two previous studies by Rassaf et al. [9] and Totzeck et al. [10] in

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healthy humans found a positive and significant correlation between circulatory nitrite, power output and heart rate, respectively. However, currently it is unknown whether the main source of circulatory nitrite originates from the endothelium or is derived from the reduction of nitrate by oral commensal bacteria. Several studies using antibacterial mouthwash to inhibit the activity of oral bacteria and nitrite synthesis in the mouth have found a reduction of plasma nitrite availability suggesting that this is a key source of circulatory nitrite [11–13]. On the other hand, a recent study by Burleigh et al. [14] suggests that circulatory nitrite concentration is independent of oral nitrite synthesis in healthy individuals. These authors argued that excess nitrite formation in the mouth could be excreted via the urinary system in order to maintain NO homeostasis [14]. The impact of exercise on the salivary concentration of nitrate and nitrite as well as the oral nitrate-reducing capacity is currently unknown. Recent research on the gut microbiome suggests that exercise is a key modulator of bacterial diversity [15–17], however, this has not been investigated with respect to the oral microbiome. This is interesting since exercise has been associated with lower prevalence of periodontal disease in humans and rats [18,19]. As periodontitis is commonly linked to bacterial dysbiosis [20], this suggests that exercise may be able to induce positive changes in the oral bacterial community lowering the risk of periodontal disease.

Another key question is the difference in NO homeostasis between men and women. A recent study by Kapil et al. [21] has shown that the oral nitrate-reducing capacity differs between males and females, however this has not been investigated with respect to aerobic fitness levels. Thus, the main aims of this study were to investigate whether salivary nitrate and nitrite concentrations were associated with the peak of oxygen uptake (VO_{2peak}) and peak power output (W_{peak}) in males and females. In the second part of this study, we investigated whether oral nitrate-reducing capacity was related to the VO_{2peak} and W_{peak} in a similar cohort of subjects. First, we hypothesised that higher VO_{2peak} and W_{peak} values would be associated with greater salivary nitrate/nitrite levels in both males and females. Then, we hypothesised that greater salivary nitrite would be related to higher oral nitrate-reducing capacity and, consequently, better aerobic fitness levels.

2. Materials and methods

2.1. Participants

Fifty-three participants were recruited for this study, two participants however could not complete the protocol due to physical problems, and another participant did not follow the full protocol. Thus, fifty participants were included in this study (Table 1). They were recruited from local cycling, triathlon and University sport clubs. A medical questionnaire was completed prior to the study, and individuals with any pathology that could affect the oral cavity such as gingivitis or periodontitis, or which could affect vascular function such as diabetes, hypertension, smoking or psychological conditions were excluded. Furthermore, individuals under medication or treatment potentially affecting oral bacteria (e.g antibacterial mouthwash) were excluded. All participants gave their written informed consent to participate in the study. The procedures carried out were approved by the human ethics committee of the Faculty of Health and Human Sciences (17/18–415) (University of Plymouth, UK) in accordance with the Declaration of Helsinki.

2.2. Protocol

Participants were required to report to the laboratory on one occasion in a rested state having not participated in strenuous exercise in the preceding 24 h. They were encouraged refrained from eating at least 3 h before the exercise test. Food rich in nitrate and caffeine were restricted 24 and 12 h before the trial, respectively. On their arrival at the laboratory, body fat percentage and body mass were measured using a single frequency (50 kHz) leg-to-leg bioelectrical impedance analyser (Tanita, TBF-300MA, Tokyo, Japan). Height was measured using a stadiometer (Seca, Hamburg, Germany). Then, a whole non-stimulated saliva sample (~ 3 mL) was collected spitting into a sterile Falcon tube to analyse nitrate, nitrite, pH and lactate. Oral nitrate-reducing capacity was then measured in 25 participants (Table 1).

After biological samples were taken, subjects performed a graded exercise test until volitional exhaustion on a bicycle ergometer (Lode Corival, Groningen, Netherlands) to assess their VO_{peak} and power output W_{peak}. The test commenced with a 5-min warm-up with the power output set at 70 W for females and 100 W for males. Pedalling cadence was kept within the range of 80–100 rpm. Upon completion of the warm-up, exercise intensity was increased by 20 W every minute for females and by 25 W for males. The exercise test ended when the subject either reached physical exhaustion or was unable to maintain a minimum pedalling cadence of 70 rpm. Oxygen uptake (VO_2) and carbon dioxide production (VCO_2) were measured at 10-s intervals by a computerized gas analyser (Cortex Metalyzer 3B, Leipzig, Germany), which was calibrated in accordance with the manufacturer's instructions prior to each test. Heart rate was measured via a wireless transmitter (Polar, Kempele, Finland). VO_2 and power output (W) averaged over the last 30-s period of the test, was defined as VO_{2peak} and W_{peak}, respectively.

At the cessation of the exercise test the subject was seated and allowed to rest for 20 min. During this period water was permitted however food and other drinks were restricted. After rest, another

Table 1
Main characteristics of the participants (mean ± SD).

	Males			Females			All
	Without ONRC	With ONRC	Overall	Without ONRC	With ONRC	Overall	
Participants (*n*)	13	15	28	12	10	22	50
Age (y/o)	43.9 ± 14.2	40.6 ± 15.4	38.9 ± 14.8	38.8 ± 13.2	29.3 ± 10.7	38.1 ± 13.9*	38.8 ± 14.3
Body mass (kg)	76.0 ± 15.1	76.6 ± 9.3	72.1 ± 13.4	62.0 ± 9.5	59.6 ± 7.8	66.8 ± 13.6*	69.7 ± 13.2
BMI	23.3 ± 2.7	23.7 ± 3.5	23.0 ± 3.0	22.3 ± 2.7	21.6 ± 2.4	22.5 ± 2.6	22.8 ± 2.9
Body fat (%)	16.1 ± 4.8	16.4 ± 5.4	17.9 ± 5.8	25.7 ± 6.0	22.5 ± 5.5	21.5 ± 6.3*	19.8 ± 6.7
VO_{2peak} (mL/kg/min)	50.7 ± 10.7	52.7 ± 12.7	49.3 ± 11.5	42.2 ± 9.2	42.2 ± 8.1	42.7 ± 9.0*	47.6 ± 11.4
W_{peak} (W/kg)	4.5 ± 1.0	4.4 ± 1.1	4.3 ± 1.1	3.8 ± 0.9	3.6 ± 0.8	4.0 ± 0.9*	4.2 ± 1.0
Salivary NO_{3^-} (μM/L)	189 ± 255	225 ± 234	225 ± 278	360 ± 355	154 ± 106	223 ± 281	225 ± 264
Salivary NO_{2^-} (μM/L)	142 ± 110	74 ± 63	100 ± 88	154 ± 106	78 ± 69	128 ± 101	113 ± 95
ONRC (nitrite μM)	–	275 ± 138		–	231 ± 143		257 ± 138
Salivary glucose (mM/L)	0.04 ± 0.03	0.04 ± 0.03	0.03 ± 0.03	0.03 ± 0.02	0.02 ± 0.01	0.03 ± 0.02	0.03 ± 0.03
Salivary lactate (mM/L)	0.21 ± 0.20	0.18 ± 0.13	0.17 ± 0.15	0.13 ± 0.09	0.11 ± 0.09	0.16 ± 0.15	0.17 ± 0.14
Salivary pH	7.41 ± 0.37	7.25 ± 0.31	7.26 ± 0.33	7.34 ± 0.46	7.07 ± 0.23	7.29 ± 0.39	7.28 ± 0.36

VO_{2peak}: peak oxygen uptake; W_{peak}: peak power output; NO^{3-}: nitrate; NO^{2-}: nitrite; ONRC: Oral-nitrate-reducing capacity; * Statistical differences ($P < 0.05$) between males and females.

Table 2
Spearman's correlations between salivary nitrite (NO_{2-}) and nitrate (NO_{3-}) pre and post-exercise test.

	Males (n = 28)		Females (n = 22)		All (n = 50)	
	Pre-saliva NO_{2-} r_s (p)	Post-saliva NO_{2-} r_s (p)	Pre-saliva NO_{2-} r_s (p)	Post-Saliva NO_{2-} r_s (p)	Pre-Saliva NO_{2-} r_s (p)	Post-saliva NO_{2-} r_s (p)
Pre-saliva NO_{3-}	0.28 (0.15)	0.16 (0.42)	0.58 (< 0.01)*	0.55 (< 0.01)*	0.34 (0.02)*	0.33 (0.02)*
Post-saliva NO_{3-}	0.49 (< 0.01)*	0.49 (< 0.01)*	0.40 (0.06)	0.48 (0.02)*	0.38 (< 0.01)*	0.65 (< 0.001)*

* Significant correlation ($P < 0.05$).

salivary sample (~ 3 mL) was taken to measure nitrate and nitrite. Finally, the oral nitrate-reducing rate was measured using the same procedure as before exercise.

3. Analyses

3.1. Salivary nitrate and nitrite

For the measurement of nitrate and nitrite in saliva a standard curve was produced by injecting 10 µL of nitrate and nitrite solutions (1.9 µM, 3.9 µM, 7.8 µM, 15.6 µM, 31.2 µM, 62.5 µM, 125 µM and 250 µM) and a control sample containing ultrapure water (Purelab OptionQ, Elga Veolia, UK) and carrier solution. The area under the curve for the latter was subtracted from the nitrate and nitrite solutions to account for nitrate and nitrite in saliva. Samples were thawed and centrifuged 10 min at 14,000 rpm. Then, 100 µL of supernatant was collected and diluted 10 times with carrier solution (containing 10% methanol, 0.15 M NaCl/NH_4Cl, and 0.5 g/L 4Na-EDTA). Then, 10 µL was directly injected in a HPLC system (ENO-30, Eicom, Japan) to measure nitrate and nitrite. The same procedure was followed in samples collected to assess the oral nitrate-reducing capacity. However, only the absolute nitrite concentration was measured from these samples as the solution used to assess the nitrate-reducing capacity contained 80 µmol of sodium nitrate that could interfere with the natural content of this anion in saliva. The reduction column of the HPLC system (ENO-30, Eicom, Japan) was removed for these analyses, which allowed us to reduce the duration of each chromatogram up to 7 min.

3.2. Oral nitrate-reducing capacity

Participants rinsed their mouth with a 10 mL solution of ultrapure water (Purelab OptionQ, Elga Veolia, UK) containing 80 µmol of sodium nitrate (Sodium Nitrate, Fisher Chemical, UK) for 5 min reflecting a 10-fold higher salivary nitrate concentration that corresponds to levels achieved after a nitrate rich meal. The solution was collected into sterile Falcon tubes and centrifuged rapidly at 4000 rpm for 10 min at 4 °C. The supernatant were collected and stored at −80 °C until absolute nitrite determination was performed using a HPLC system (ENO-30, Eicom, Japan).

3.3. Salivary pH, lactate and glucose

One mL of saliva was used to measure the pH (25 °C) using an electronic pH meter (PH-208, Lutron, Taiwan) calibrated in accordance with the manufacturer's instructions. Salivary lactate and glucose were analysed by a biochemistry analyser (YSI 2300 Stat Plus, YSI Life Sciences, US).

3.4. Dietary nitrate intake

Participants completed a 3-day dietary record before the visit to the laboratory, which was used to validate dietary nitrate consumption prior to the exercise test. Dietary nitrate intake from vegetables was also estimated in forty-six individuals using data obtained from the European Food Safety Authority [22]. The dietary records of four participants were not fully completed by four participants.

3.5. Statistical analyses

All data are presented as mean ± SD and were analysed to determine the normal distribution using the Shapiro Wilk's test. Comparison between groups (males/females) were performed using the Mann-Whitney U test as data were not normally distributed. Comparison between pre and post exercise measurements in saliva parameters were carried out using a Wilcoxon Signed Ranks Test for all the variables except for salivary pH as this was normally distributed, where a paired t-test was used instead. The association between salivary markers, W_{peak} and VO_{2peak} as well as salivary nitrate and nitrite before and after exercise was analysed using two-tailed Spearman's rank correlation analyses (95% confidence).

4. Results

4.1. Baseline characteristics of study subjects

Table 1 shows the baseline characteristics of the subjects. Males were older (age, $P = 0.048$), heavier (body mass, $P < 0.001$) and had lower body fat levels than females ($P < 0.001$). Additionally, males had higher VO_{2peak} values ($P = 0.002$) and W_{peak} ($P = 0.008$) as expected, but no differences were observed ($P > 0.05$) in any salivary parameters between males and females (Table 1). Estimated dietary intake of nitrate from vegetables was 43.1 ± 48.4 mg/day.

4.2. Correlations between salivary nitrate and nitrite before and after exercise

Table 2 shows correlations between salivary nitrate and nitrite before and after exercise in males and females. Interestingly, while males did not show a correlation between concentration of salivary nitrate and nitrite before exercise ($r_s = 0.28$, $P = 0.15$), a significant association was found in females between both anions ($r_s = 0.58$, $P = 0.005$). After exercise, both groups showed a similar response as a positive and significant correlation was found between salivary nitrate and nitrite concentrations (males $r_s = 0.49$, $P = 0.008$; females $r_s = 0.48$, $P = 0.022$). This association was even stronger when both groups were analysed together ($r_s = 0.65$, $P = 0.001$).

4.3. Correlations between salivary nitrate and nitrite and VO_{2peak} and W_{peak}

No correlations were found between salivary nitrate and VO_{2peak} and W_{peak} in either males or females before exercise (Table 3). A moderate and significant correlation ($r_s = 0.44$, $P = 0.04$) between salivary nitrite and W_{peak} was found only in females after exercise, however, salivary nitrite did not correlate with VO_{2peak} ($r_s = 0.31$, $P = 0.16$).

Changes in salivary nitrate before and after exercise were negatively and significantly associated with the W_{peak} in males (Fig. 1), but not with nitrite (W_{peak}, $r_s = < 0.1$, $P = 0.8$; VO_{2peak}, $r_s = -0.1$, $P = 0.7$). No correlations were found between changes in salivary nitrate-W_{peak} ($r_s = -0.13$, $P = 0.6$) or nitrate-VO_{2peak} ($r_s = -0.17$, $P = 0.4$) and nitrite-W_{peak} ($r_s = -0.48$, $P = 0.10$) or nitrite-VO_{2peak} ($r_s = -0.48$, $P = 0.10$) in females.

Table 3
Spearman's correlations between salivary markers (nitrate [NO_3-]; nitrite [NO_2-]; lactate; pH; and the oral nitrate-reducing rate [ONRC]) and peak power output (W_{peak}) and peak oxygen uptake (VO_{2peak}) in males and females (mean ± SD).

	Males (n = 28)				Females (n = 22)				All (n = 50)			
	Pre-Exercise		Post-Exercise		Pre-Exercise		Post-Exercise		Pre-Exercise		Post-Exercise	
	W_{peak} (W/kg) r_s (p)	VO_{2peak} (mL/Kg/min) r_s (p)	W_{peak} (W/kg) r_s (p)	VO_{2peak} (mL/Kg/min) r_s (p)	W_{peak} (W/kg) r_s (p)	VO_{2peak} (mL/Kg/min) r_s (p)	W_{peak} (W/kg) r_s (p)	VO_{2peak} (mL/Kg/min) r_s (p)	W_{peak} (W/kg) r_s (p)	VO_{2peak} (mL/Kg/min) r_s (p)	W_{peak} (W/kg) r_s (p)	VO_{2peak} (mL/Kg/min) r_s (p)
Salivary NO_3-	0.16 (0.41)	0.15 (0.45)	0.07 (0.78)	0.05 (0.81)	0.38 (0.08)	0.19 (0.40)	0.37 (0.09)	0.19 (0.41)	0.18 (0.21)	0.11 (0.46)	0.09 (0.56)	0.01 (0.95)
Salivary NO_2-	0.22 (0.27)	0.21 (0.28)	0.09 (0.65)	0.12 (0.55)	0.26 (0.24)	0.16 (0.49)	0.44 (0.04)*	0.31 (0.16)	0.20 (0.17)	0.16 (0.28)	0.19 (0.18)	0.18 (0.22)
Salivary lactate	0.19 (0.34)	0.14 (0.48)	0.02 (0.93)	−0.06 (0.75)	0.40 (0.07)	0.21 (0.35)	0.41 (0.06)	0.33 (0.14)	0.33 (0.02)*	0.26 (0.07)	0.19 (0.20)	0.14 (0.34)
Salivary pH	0.03 (0.86) (n = 15)	−0.90 (0.66)	0.21 (0.29)	0.15 (0.46)	0.12 (0.61) (n = 10)	0.19 (0.41)	0.11 (0.64)	0.14 (0.52)	0.04 (0.81) (n = 25)	−0.01 (0.99)	0.04 (0.80)	−0.01 (0.99)
ONRC	0.64 (0.01)*	0.54 (0.04)*	0.64 (0.01)*	0.47 (0.08)	0.50 (0.14)	0.43 (0.22)	0.41 (0.24)	0.51 (0.13)	0.64 (0.001)*	0.54 (0.005)*	0.63 (0.001)*	0.54 (0.003)*

* Significant correlation ($P < 0.05$).

4.4. Correlations between the oral nitrate-reducing capacity and VO_{2peak} and W_{peak}

The oral nitrate-reducing capacity was significantly associated with W_{peak} ($r_s = 0.64$, $P = 0.01$) and VO_{2peak} ($r_s = 0.54$, $P = 0.04$) in males before exercise (Table 3). While the association between the nitrate-reducing capacity and W_{peak} did not change after exercise ($r_s = 0.64$, $P = 0.01$), a lower and non-significant correlation was observed with the VO_{2peak} ($r_s = 0.47$, $P = 0.08$). In females, the oral nitrate-reducing capacity was not associated with W_{peak} or VO_{2peak} before and after exercise (Table 3). When males and females were analysed together (Fig. 2), a positive and significant association was found between the oral nitrate-reducing capacity and W_{peak} and VO_{2peak} both before and after exercise.

4.5. Changes in saliva parameters between pre and post-exercise measurements

A significant increase in salivary lactate was observed in females and males after exercise. This response coincided with a significant reduction of salivary pH in males (Fig. 3). Although a similar trend was observed in females, the reduction in salivary pH was not statistically significant ($P = 0.64$). When males and females were analysed together salivary pH was significantly lower after exercise compared to pre-exercise values ($P = 0.003$) (Fig. 3).

The concentration of salivary nitrate, nitrite and the oral nitrate-reducing capacity did not differ ($P > 0.05$) between pre and post-exercise measurements in either males or females (Fig. 4). No correlations were found ($P > 0.05$) when comparing changes in salivary pH and lactate with changes of salivary nitrate and nitrite concentrations before and after exercise in males and females. Additionally, there were no correlations ($P > 0.05$) between changes in the oral nitrate-reducing capacity and changes in pH and lactate before and after exercise.

5. Discussion

The main finding of this study was the positive and significant correlation between the oral nitrate-reducing capacity and W_{peak} and VO_{2peak}. In males, a negative correlation between changes in salivary nitrate concentration and W_{peak} and VO_{2peak} was also found which aligns with the first finding however the same correlation was not found in females, although a similar trend was demonstrated. Using both gender data, we found that the correlation between the oral nitrate-reducing capacity and W_{peak} and VO_{2peak} was even stronger.

Regarding the lack of correlation between the oral nitrate-reducing capacity and W_{peak} and VO_{2peak} in females, this may be due to the lower number of participants as well as the lack of highly trained females ($VO_{2peak} > 60$ mL/kg/min) compared to males as both groups showed similar levels of salivary nitrate/nitrite concentrations, oral nitrate-reducing capacity and salivary pH, lactate and glucose before exercise. Interestingly, the correlation between aerobic fitness markers and nitrate-reducing capacity improved when males and females were analysed together. This may have been due to a large sample size and an increased range of fitness levels. However, currently we cannot discard that potential differences may exist between genders regarding the composition and activity of the oral microbiome. A recent study by Kapil et al. [21] reported higher salivary nitrite concentrations in females which coincided with higher oral nitrate-reducing capacity, but not to a different composition of the oral microbiome. Whilst this is contrary to our results, further studies are needed to investigate this question in more detail.

Additionally, and opposite to our original hypothesis, higher salivary nitrite concentrations were not correlated with greater values of VO_{2peak} and W_{peak}. We found a significant and positive correlation between salivary nitrate and nitrite concentrations especially after exercise, but this was not associated with changes on the oral nitrate-

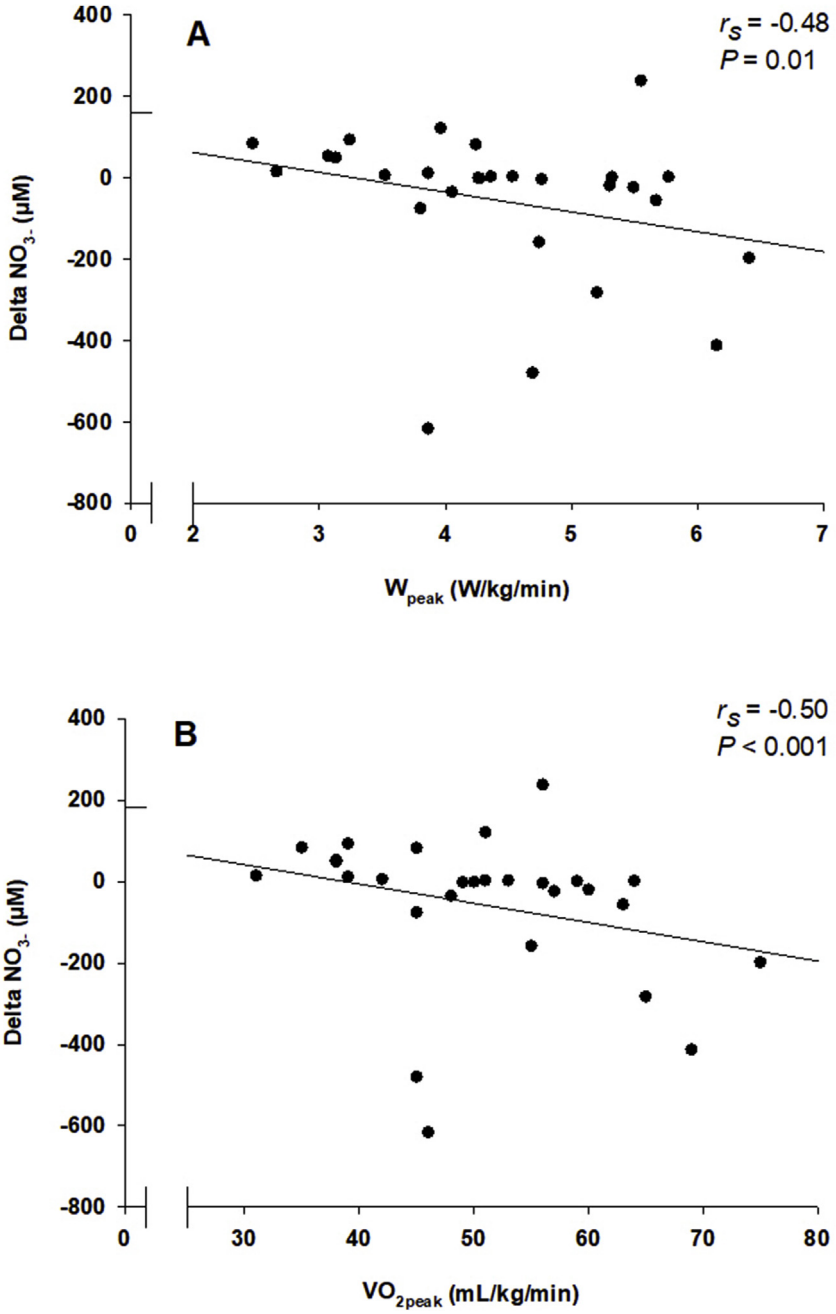

Fig. 1. Spearman's rank correlation between changes (delta) in salivary nitrate (NO_{3^-}) and peak power output (W_{peak}) (A) and peak oxygen uptake (VO_{2peak}) (B) in males (n = 28).

reducing capacity or a significant correlation with aerobic fitness markers. Using plasma samples, previous studies have found a correlation between nitrite concentrations and other parameters related to exercise capacity in healthy individuals [9,10]. However, these studies have some limitations that are worthy of discussion. For instance, in the first study by Rassaf et al. [9] the correlation coefficient between post-exercise plasma nitrite and W_{peak} was only 0.37 which suggests a large inter-individual variability in the cohort. Furthermore, no correlation between plasma nitrite and VO_{2peak} was reported in this study as would be expected as both parameters are closely associated [9]. In a second study by the same group, a stronger correlation was found (r = 0.65) between plasma nitrite and heart rate at lactate anaerobic threshold [10], however, from a physiological perspective this correlation is weak since heart rate at lactate anaerobic threshold cannot be considered a valid marker of performance in humans [23].

Recent studies have found that salivary nitrite does not necessarily reflect plasma nitrite levels [24,25]. Additionally, a greater abundance of oral nitrate-reducing bacteria and nitrite synthesis has not been associated with an increase of plasma nitrite availability in healthy individuals [14]. To explain this, it has been suggested that excess nitrite formation in the mouth could be excreted via the urinary system to prevent a change in NO homeostasis [14]. Furthermore, Porcelli et al. [26] found that subjects with higher values of VO_{2peak}, and with presumably greater oral nitrate-reducing capacity, presented lower changes in plasma nitrite after taking 5.5 mmol of sodium nitrate for six days as compared to individuals with low VO_{2peak} levels. On the other hand, greater salivary nitrite concentrations have been recently linked to higher intensity and load of training athletes [27]. In the current experiment, we only found a positive and significant correlation between salivary nitrite and W_{peak} in females after exercise however this

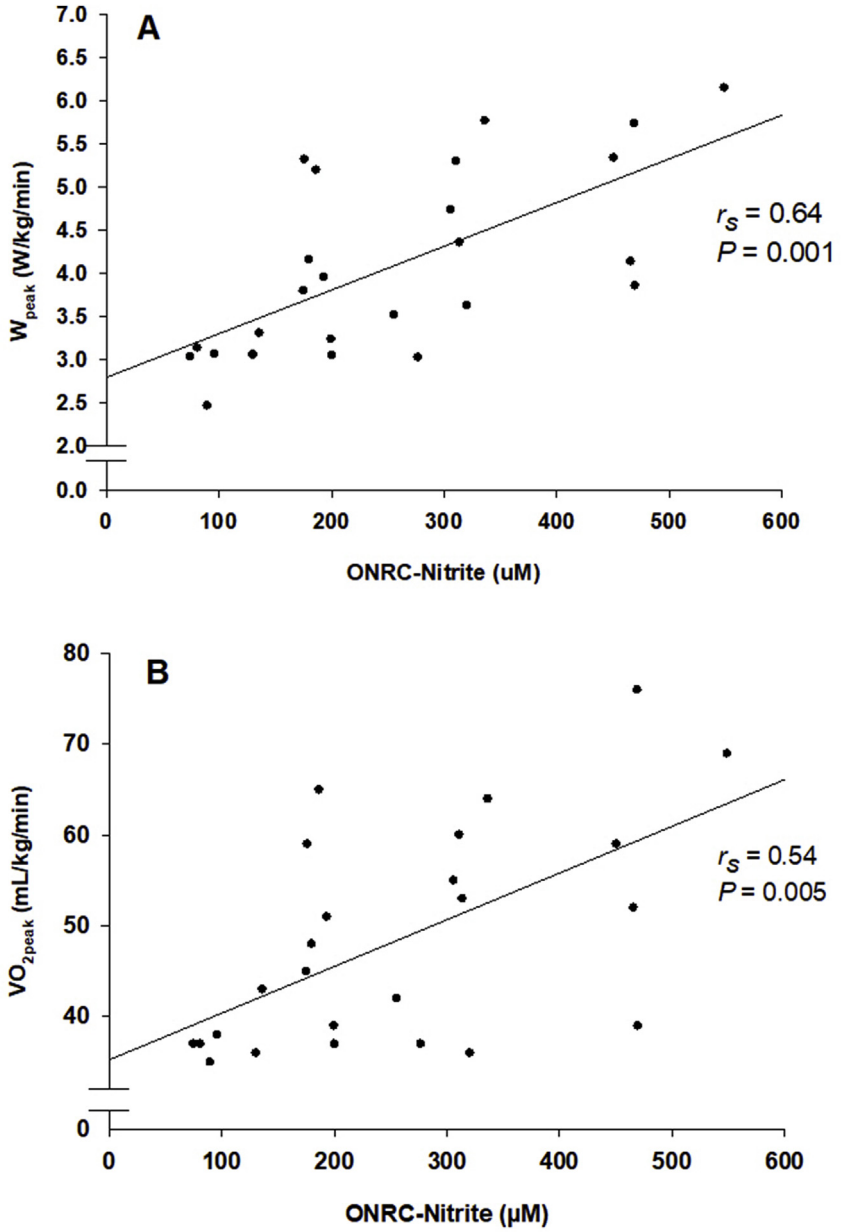

Fig. 2. Spearman's rank correlation between the oral nitrate-reducing capacity (ONRC) in absolute nitrite values and peak power output (W_{peak}) (A) and peak oxygen uptake (VO_{2peak}) (B) in males and females (n = 25).

correlation was not strong (r_s = 0.44). Additionally, we did not see the same effect between these two variables before exercise or in relation to the VO_{2peak}. Thus, this correlation in females should be treated with caution and further studies are needed to analyse it in more depth.

Another interesting finding of this study was the lack of changes in salivary nitrite levels as well as the oral nitrate-reducing capacity after exercise. It has been suggested that the oral nitrate/nitrite pathway is enhanced under acidic conditions [2], but in spite of the low salivary pH and higher lactate concentrations observed after exercise, this did not induce any change in salivary nitrate and nitrite. It is possible that these variations in the oral pH balance were too small to stimulate the nitrate/nitrite reduction response compared to the changes that have been observed in the stomach under much more acidic conditions [28].

Comparing our results to previous data in plasma [9,10] and saliva [27], the oral nitrate-reducing capacity seems to be the strongest parameter related to the main markers of aerobic fitness levels. However this does not elicit greater salivary and plasma concentrations of nitrate and nitrite in baseline conditions. Overall, current research in this field suggests that NO homeostasis is tightly regulated and it may differ according to fitness levels [29]. Repeated exercise and shear stress stimulation improves endothelial function by several mechanisms such as upregulation of endothelial NOS (eNOS) activity and enhanced antioxidant balance [29]. These exercise-induced adaptations may help trained individuals to maintain NO homeostasis more efficiently under different physiological situations, and to be less dependent on exogenous sources of NO donors such as inorganic nitrate or L-Arginine [6]. Our current results suggest that enhanced oral nitrate-reducing capacity may be another factor to add to the list of exercise training-induced adaptations that play a key role in maintaining NO homeostasis. While the enzymatic L-Arginine/NOS pathway may be the main source of NO during exercise at low-moderate intensities (aerobic conditions), the oral nitrate/nitrite pathway may be the principal system ensuring sufficient NO formation at high exercise intensities when oxygen supply is limited [2]. This oral pathway can be analogous of anaerobic glycolysis in bioenergetics in order to maintain NO homeostasis, and it may be more efficient in trained individuals due to repeated stimulation.

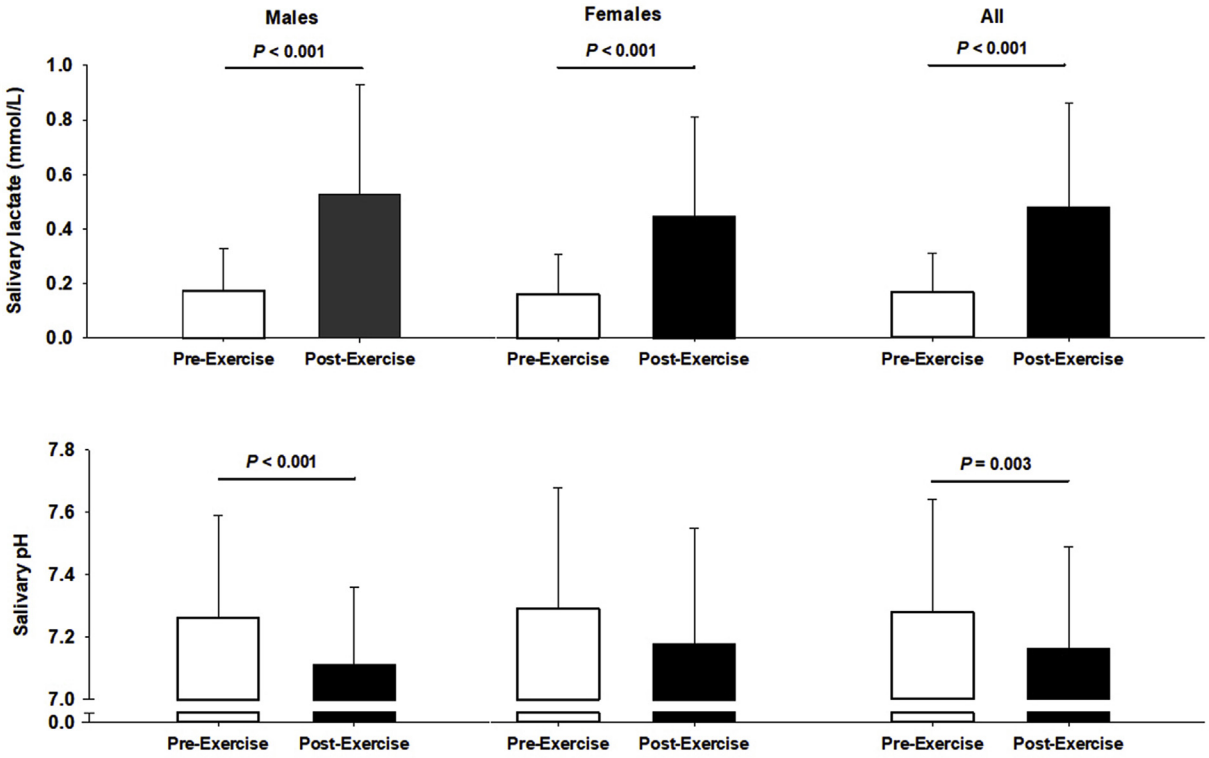

Fig. 3. Salivary lactate concentrations and pH in males ($n = 28$), females ($n = 22$) and in all the participants ($n = 50$) before and 20 min after exercise (mean ± SD).

To the best of our knowledge, this is the first evidence linking markers of aerobic fitness level with the oral nitrate-reducing capacity in healthy humans. Although we did not analyse the oral microbiome in the current study, we suggest that higher oral nitrate-reducing capacity could be associated with three different factors: 1) enhanced activity of oral bacteria, 2) increased diversity and abundance of species that confer a higher nitrate-reducing capacity, and 3) a combination of both factors. A recent study by Burleigh et al. (2018) [14] has shown that higher oral nitrite production was linked to a greater abundance of nitrate-reducing species in healthy humans. Perhaps, exercise training can modulate the oral microbiome in a similar way increasing the abundance of oral nitrate-reducing bacteria, which could explain our correlation between higher nitrate-reducing capacity and greater fitness levels. However, it is not possible to discard changes on the activity of oral bacteria either. From this viewpoint, a recent study by Kapil et al. [21] found that higher oral nitrate-reducing capacity in females was not associated with a different composition of the oral microbiome. Thus, further research is needed to elucidate these key questions in regards to nitrate and nitrite metabolism.

How exercise modulates the oral microbiome and/or the nitrate-reducing capacity requires further research. Changes in the acid/base balance may be a key mechanism explaining this interesting phenomenon. An increase in muscle lactate concentration commonly occurs during exercise, even at moderate intensity, which is reflected by higher blood levels of this metabolite [30]. Circulatory lactate is rapidly distributed among different tissues and fluids such as saliva [31] and we found a significant increase of salivary lactate in both groups after exercise. In males, this was accompanied by a significant decrease of salivary pH, but despite a similar pattern being observed in females, differences were not statistically significant. A reason that may explain, at least partially, these differences between genders is exercise-induced stress levels [32]. Limitations in female leg muscle strength as opposed to cardiovascular capacity could limit their performance on the cycle ergometer test and explain some of the differences in the biological and physiological markers observed in this study [32]. Although we did not assess the salivary kinetics of lactate and pH during the recovery period, it may be expected that salivary lactate dropped later in the recovery period due to the activation of buffering mechanisms which may help to increase the oral pH levels back to baseline values [33]. Small changes in the acid/base balance during exercise and the recovery period may have a significant impact on microbiota composition especially when exercise is practiced repeatedly over time [16].

Diet is another key factor that has been associated with changes in the diversity of the gut microbiome [34] however research relating to diet and the oral microbiome is presently limited. We estimated the dietary nitrate intake in this study using a 3 day-dietary record and the average consumption was below 43.1 mg/day. This amount was below the values reported in the general population (~108 mg/day) [35] and athletes (~106 mg/day) [36]. However, we only estimated nitrate intake from vegetables. Other sources such as water were not taken into account, which may add further nitrate. Additionally, participants were encouraged to avoid vegetables rich in inorganic nitrate at least a day prior the exercise test, which may also explain the low consumption of nitrate of our participants. Furthermore, no participant of this study was taking supplements containing NO donors such as nitrate or L-Arginine prior to testing.

This study has some limitations that are important to highlight. While participants completed a medical questionnaire to assess their physical and oral health status before the start of the study, this did not exclude participants carrying some degree of gum disease. It has been estimated that prevalence of gum disease in adults living in the UK is over 45% [37]. This is relevant since periodontal disease can seriously disrupt the equilibrium of the oral microbiome [38] and can also increase the levels of salivary nitrate and nitrite due to an immune-mediated inflammatory response [39]. Thus, future studies should examine the oral health of participants in more depth. In addition, we did not analyse the salivary flow rate and salivary buffering capacity. It may be beneficial, in future studies, to assess whether a higher salivary flow rate after exercise is a key mechanism for increasing the availability of circulatory nitrite. Furthermore, we did not assess the

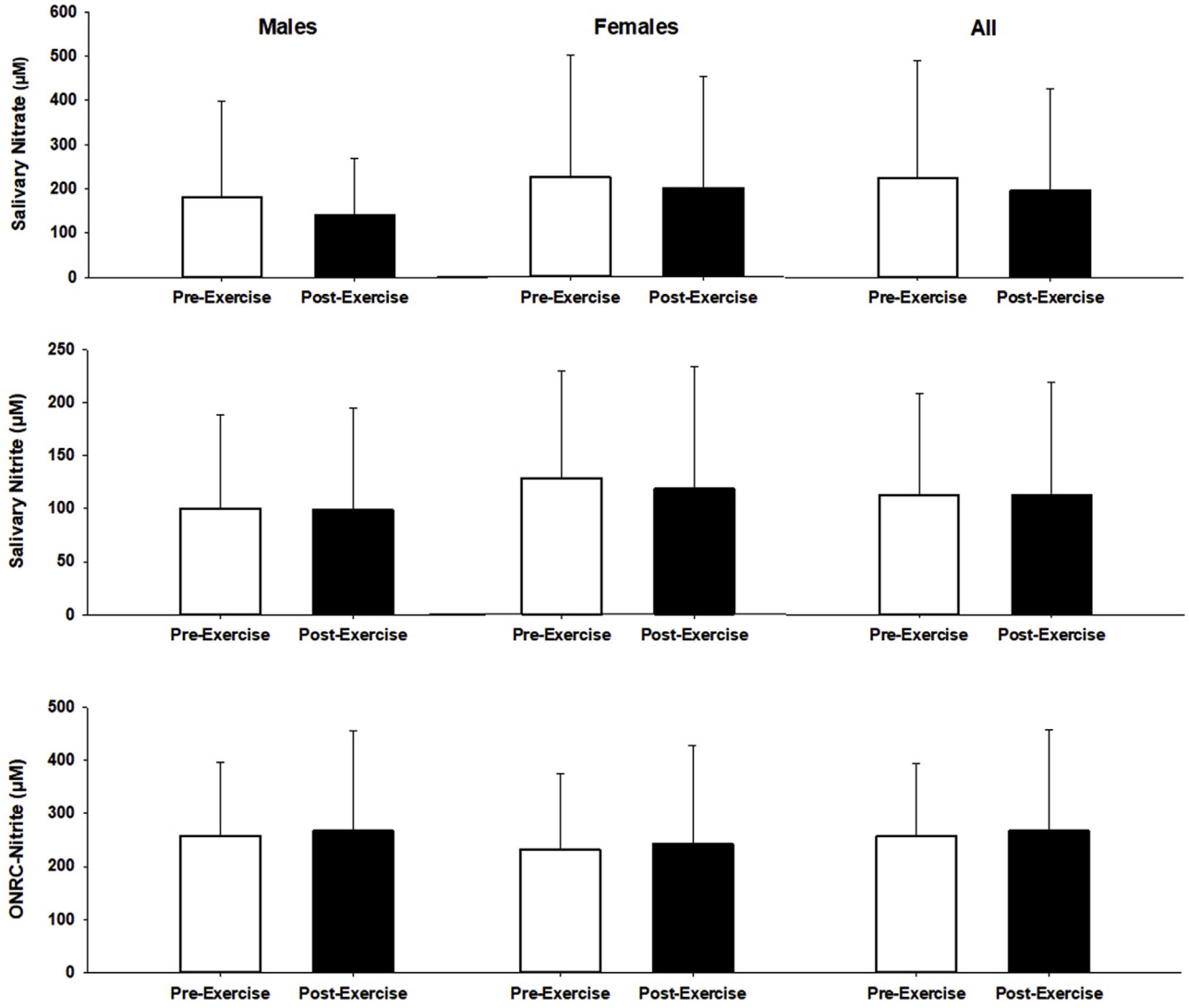

Fig. 4. Salivary nitrate and nitrite concentrations in males (n = 28) and females (n = 22) as well as oral nitrate-reducing rate (ONRC) in absolute nitrite values (males = 15; females = 10) before and after exercise (mean ± SD).

diversity of the oral microbiome due to funding constraints and because this question was not part of our original investigation. It would have also been interesting to assess circulatory levels of nitrate and nitrite to compare it with previous studies. Measurement of cardiovascular parameters such as blood pressure and flow-mediated arterial dilation may also have been of value. The questions arising from this study are shaping our current research.

6. Conclusion

This is the first study showing that the oral nitrate-reducing capacity is related to the main markers of aerobic fitness such as W_{peak} and VO_{2max} in healthy humans. Further research is needed to investigate in more depth the effects of exercise on the activity and diversity of the oral microbiome.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.niox.2019.03.001.

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