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Published in:
Nitric Oxide: Biology and Chemistry

DOI (link to publication from Publisher):
10.1016/j.niox.2019.01.011

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Publication date:
2019

Document Version
Accepted author manuscript, peer reviewed version

Link to publication from Aalborg University

Citation for published version (APA):
Rokkedal-Lausch, T., Franch, J., Poulsen, M. K., Thomsen, L. P., Weitzberg, E., Kamavuako, E. N., Karbing, D. S., & Larsen, R. G. (2019). Chronic high-dose beetroot juice supplementation improves time trial performance of well-trained cyclists in normoxia and hypoxia. *Nitric Oxide: Biology and Chemistry*, *85*, 44-52. Advance online publication. https://doi.org/10.1016/j.niox.2019.01.011

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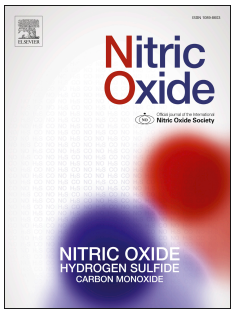

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PII: S1089-8603(18)30337-9

DOI: https://doi.org/10.1016/j.niox.2019.01.011

Reference: YNIOX 1857

To appear in: *Nitric Oxide*

Received Date: 7 November 2018

Revised Date: 14 January 2019

Accepted Date: 18 January 2019

Please cite this article as: T. Rokkedal-Lausch, J. Franch, M.K. Poulsen, L.P. Thomsen, E. Weitzberg, E.N. Kamavuako, D.S. Karbing, R.G. Larsen, Chronic high-dose beetroot juice supplementation improves time trial performance of well-trained cyclists in normoxia and hypoxia, *Nitric Oxide* (2019), doi: https://doi.org/10.1016/j.niox.2019.01.011.

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Title

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Declarations of interest: EW is a co-applicant on patents related to the therapeutic use of nitrate and nitrite. Other authors, none.

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Abstract

Dietary nitrate (NO_3^-) supplementation via beetroot juice (BR) is known to improve endurance performance in untrained and moderately trained individuals. However, conflicting results exist in well-trained individuals. Evidence suggests that the effects of NO_3^- are augmented during conditions of reduced oxygen availability (e.g., hypoxia), thereby increasing the probability of performance improvements for well-trained athletes in hypoxia vs. normoxia. This randomized, double-blinded, counterbalanced-crossover study examined the effects of 7 days of BR supplementation with 12.4 mmol NO_3^- per day on 10-km cycling time trial (TT) performance in 12 well-trained cyclists in normoxia (N) and normobaric hypoxia (H). Linear mixed models for repeated measures revealed increases in plasma NO_3^- and NO_2^- after supplementation with BR (both $p<0.001$). Further, TT performance increased with BR supplementation (~1.6%, $p<0.05$), with no difference between normoxia and hypoxia ($p=0.92$). For respiratory variables there were significant effects of supplementation on VO_2 ($p<0.05$) and VE ($p<0.05$) such that average VO_2 and VE during the TT increased with BR, with no difference between normoxia and hypoxia ($p\geq0.86$). We found no effect of supplementation on heart rate, oxygen saturation or muscle oxygenation during the TT. Our results provide new evidence that chronic high-dose NO_3^- supplementation improves cycling performance of well-trained cyclists in both normoxia and hypoxia.

Keywords:

Nitrate,

Nitrite,

Endurance exercise,

Cycling performance,

Hypoxia,

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1.1 Introduction

There is general consensus regarding the physiological factors that limit endurance performance [1,2]. These factors include maximal oxygen consumption (VO_{2max}), the fractional utilization of VO_{2max}, and exercise efficiency. Even minor improvements in these factors can enhance performance of endurance athletes. One strategy proposed to improve performance is inorganic nitrate (NO_3^-) supplementation, most often in the form of concentrated beetroot juice (BR) [3]. When ingested, nitrate is reduced to nitrite and nitric oxide (NO). This pathway differs from the classical pathway for NO generation which involves specific enzymes, NO-synthases (NOS) that use L-arginine and molecular oxygen to generate NO. Nitric oxide has been demonstrated to alter several physiological processes such as blood flow, mitochondrial function and contractile properties [3-8]. Recently, several studies have provided evidence that dietary intake of NO_3^- can improve exercise efficiency (reduction in VO_2 at same work rate) [9-12] and endurance performance [9,10,13-17]. Notably, the majority of studies reporting beneficial effects of NO_3^- has been conducted in untrained and moderately trained individuals (VO_{2max} < 60 ml/min/kg) [10,15,16,18], whereas studies in highly trained individuals (VO_{2max} > 60 ml/min/kg) have shown minor [16,19-21] or no improvements [22-27], indicating that NO_3^- may be less effective in this population [28,29]. In addition to this, recent studies in hypoxia have also provided evidence that NO_3^- improves exercise efficiency [17,21,30,31], muscle oxygenation [31] and elevates oxygen saturation (SpO_2) [21,30,31]. The lower O_2 availability in hypoxia impairs the L-Arginine-NOS pathway, and potentiates the nitrate-nitrite-NO pathway, suggesting that BR may be more effective in hypoxia

than in normoxia [3,32-34]. Supporting the notion that BR is more effective in hypoxia, Kelly et al. [30] showed that, in healthy individuals, BR improved time to exhaustion during severe intensity exercise in hypoxia but not in normoxia. In addition, BR has been shown to attenuate the decrease in muscle oxygenation and muscle metabolic perturbation in hypoxia in untrained and moderately trained subjects [31,35]. Hence, highly trained athletes may also experience greater performance improvements with BR in hypoxia compared with normoxia. Recently, few studies have examined this idea with conflicting results. In well-trained athletes NO_3^- supplementation had no effect on 10-km or 15-km cycling performance, 10-km running performance or roller-skiing treadmill performance in hypoxia [36-39]. Contrary to this, two studies have reported positive effects of BR in hypoxia on 16.1-km cycling performance and 1500m running performance in trained athletes [17,21]. The discrepancy could be due to different supplementation strategies for NO_3^-. Specifically, the effects of NO_3^- supplementation seems to be potentiated with BR as source of NO_3^- [40,41], with chronic loading over several days [42,43], and by using a dose of >8mmol per day [13,20,44]. Optimizing the supplementation strategy of NO_3^- may be even more important in trained athletes, as this population already exhibit adaptations elicited by endurance training and diet, including higher NO_3^- plasma levels [45,46], NO release [47], NOS activity[48] and a higher percentage of type I fibers [8,49], that altogether may attenuate the response to NO_3^- supplementation.

The purpose of the present study was to examine the effects of several days supplementation with a high-dose BR on cycling time trial performance in well-trained cyclists, with continuous measurements of SpO_2, muscle oxygenation and

105 oxygen uptake in normoxia and normobaric hypoxia. We hypothesized that BR
106 would improve TT cycling performance in hypoxia but not in normoxia.

107 **2.1 Material and Methods**

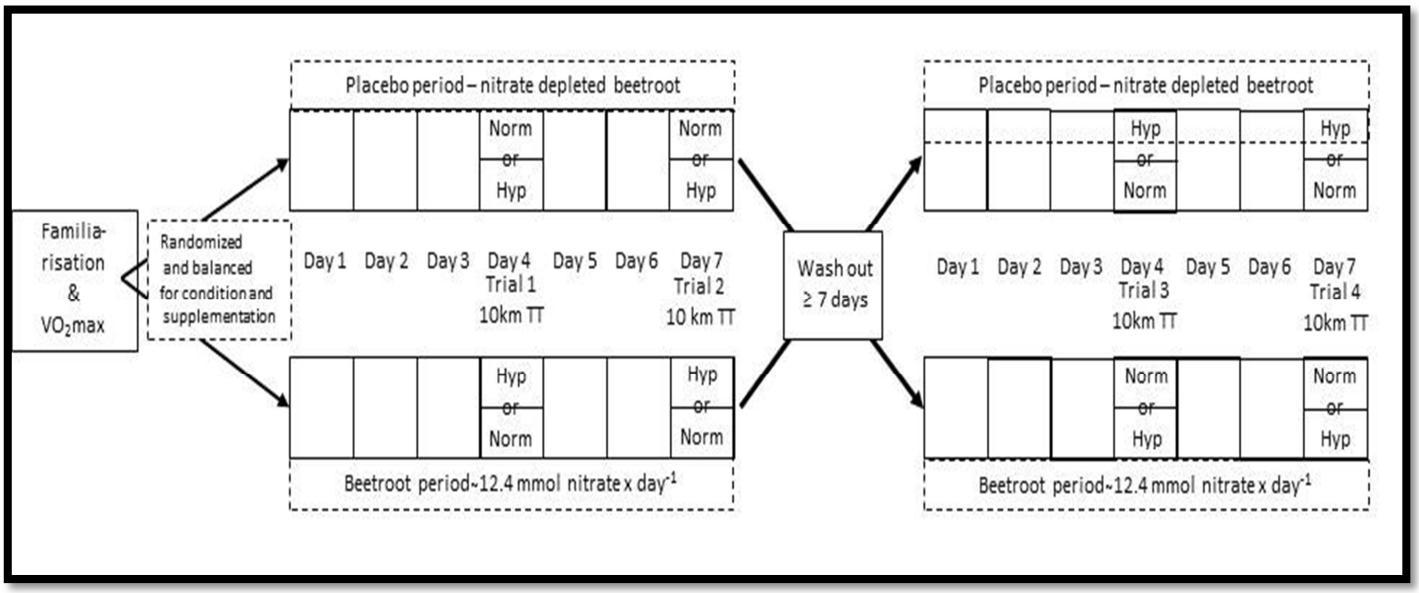
108 *2.1.1 Participants*

109 Twelve healthy male cyclists at the age of 29.1 ± 7.7 yrs (range 22 to 44 yrs) were
110 enrolled in the study. Participants had a VO_{2max} of 5.09 ± 0.47 $L \cdot min^{-1}$
111 corresponding to 66.4 ± 5.3 $ml \cdot min^{-1} \cdot kg^{-1}$ and a wattmax of 430 ± 35 watt
112 corresponding to 5.6 ± 0.3 $watt \cdot kg^{-1}$ (mean ± SD). Participants were best classified
113 as well-trained in performance level 4 as defined by Jeukendrup et al. [50] and De
114 Pauw et al. [51], respectively. The protocol and test procedures used in the current
115 study were conducted in accordance with the Declaration of Helsinki and
116 approved by the Ethics Committee of Northern Jutland (N-20150049). All
117 participants signed informed consent prior to enrollment.

118 *2.1.2 Study design*

119 Participants reported to the laboratory on five separate occasions. Experimental
120 trials followed a randomized counterbalanced-crossover design and were double-
121 blinded for supplementation and single-blinded for inspiratory conditions. The
122 first visit consisted of a maximal exercise performance test to ensure participants
123 were familiar with testing procedures and to ensure participants met the inclusion
124 criteria (i.e., $VO_{2max} > 60$ $ml \cdot kg \cdot min^{-1}$ or wattmax $\geq$ 5 w/kg). Visits 2-5 involved
125 four experimental trials (Fig 1). Each trial consisted of a 10-km time trial
126 performed in conditions of normoxia or hypoxia, with supplementation of BR or
127 nitrate-depleted BR as placebo (PLA). Specifically, supplementations were
128 ingested in periods of seven days, separated by a wash out period of at least seven

129 days. During each supplementation period, 10-km time trials were performed on
130 day four and day seven, in different conditions. The order of condition was
131 maintained for each individual for the first and second supplementation period
132 such that visits 1 and 3 (and visit 2 and 4) were performed in the same condition.
133 The design was counterbalanced for condition and supplementation such that half
134 of the participants started with normoxia and half of the participants started with
135 BR. All exercise trials were performed on the Cyclus2 ergometer (RBM Cyclus 2,
136 Germany) using the participants' own bike.



137 **Figure 1: Experimental design**

138 *2.1.3 Maximal exercise performance*

139 Participants completed a 10-minute warm up at 100 watts and hereafter an
140 incremental exercise test to exhaustion to determine gas exchange threshold
141 (GET[30]), VO_{2max} and wattmax (Fig 1).The incremental exercise test
142 commenced at 100 watts and increased by 30 watts each minute until voluntary
143 exhaustion. Following a 10-minute rest, participants completed a familiarization
144 trial for the 10-km TT. While a VO_{2max} validation bout is recommended [52], this

145 was not performed in this present study as these well-trained cyclists routinely
146 achieve maximal effort during exercise. Respiratory breath-by-breath data were
147 measured throughout the test using a metabolic cart (Jaeger, Vyntus CPX,
148 Carefusion). The metabolic cart was calibrated before each test according to the
149 manufacturer's recommendations. Maximal oxygen uptake (VO_{2max}) was
150 determined as the highest 30-second average, Wattmax as peak power output from
151 the last minute of the test ((watt) + time in last stage (s)/60 × 30 (W)) and heart
152 rate (HR) as the peak value attained during the test. GET was determined from a
153 number of measurements, including 1) the first disproportionate increase in VCO_2
154 from visual inspection of plotting VCO_2 and VO_2 and 2) an increase in expired
155 ventilation (V_E/VO_2) with no increase in V_E/VCO_2 [30]. HR was recorded
156 continuously using a heart rate sensor (Polar Electro, Oy, Finland).
157
158 *2.1.4 Experimental trials*
159 Participants ingested BR or PLA for seven consecutive days (Fig 1). Specifically,
160 participants consumed 140ml of concentrated BR (~12.4 mmol nitrate) or 140ml
161 of nitrate-depleted BR (PLA; ~0 mmol nitrate) (Beet It Sport, James White Drinks
162 Ltd., Ipswich, UK) per day; one dose (70 ml) in the morning and one dose (70 ml)
163 in the evening. On the days of the experimental trials (i.e., days four and seven),
164 participants were instructed to consume the total dose (i.e., 140 ml) 2-h prior to
165 arriving at the laboratory (approx. 2.75-h. before commencing the time trial).
166 During the 24-h preceding the first experimental trial, each participant recorded
167 their diet and was told to replicate this diet for the remaining three trials.
168 Participants were also instructed to avoid the intake of specific nitrate-rich foods.

169 The use of antibacterial mouthwash products was not permitted and caffeine
170 intake was prohibited for 12-h preceding each test. For each individual, all
171 experimental trials were performed at the same time of day.
172 Upon arrival at the laboratory, participants rested for 5-minutes before a resting
173 blood sample was drawn into two 4 ml lithium heparin vacutainers
174 (Becton Dickinson, Plymouth, UK). Blood samples were immediately centrifuged
175 for 10 min at 4°C, 3000g after which plasma was extracted and stored at -80 ^{0}C
176 for later determination of plasma nitrate and nitrite according to the method
177 described by Hezel et al. [53]. A near infrared spectroscopy (NIRS) probe
178 (Oxymon MK III, Artinis Medical Systems, Netherlands) was placed on the belly
179 of the Vastus Lateralis of the right leg in order to measure changes in muscle
180 oxygenation. Probe position was marked with a permanent pen to ensure identical
181 probe placement for subsequent trials, and the NIRS probe was placed with
182 double-sided adhesive tape. Further, elastic bandages were used to ensure a fixed
183 placement of the probe. An earlobe pulse oximeter (Nonin XPod 8000Q2, Nonin
184 Medical, Inc, Plymouth, MN) was used to measure SpO_2 throughout the tests.
185 Participants then rested 5-minutes on the bike while breathing the gas mixture
186 corresponding to the condition for that specific trial. Throughout each trial,
187 participants breathed trough a facemask (Hans Rudolph, V-982185) connected to
188 a low resistance y-valve (Hans Rudolph, two way Y-shape non-rebreathing valve,
189 2730L), with the inspiration valve connected to a closed reservoir. The inspired
190 gas was modified via the closed reservoir using a custom built setup consisting of
191 a mechanical ventilator (SV-300, Maquet, Solna, Sweden) modified such that
192 mixing of gas (pressurized room air and nitrogen) was controlled by manipulating

193 the inspired oxygen setting on the ventilator. The participants breathed through
194 the same circuit for all experimental trials. The fraction of inspired oxygen was
195 adjusted to 15 ± 0.1% in hypoxia (~2500m of altitude) and 20.9 ± 0.1% in
196 normoxia (sea level). Warm-up consisted of three six-minute exercise bouts at the
197 power output corresponding to 70% of GET measured in normoxia. A six-minute
198 rest separated each bout. After the third bout, participants rested for 10 minutes
199 without the facemask. Prior to the TT, participants sat on the bike for five minutes
200 while breathing the gas mixture corresponding to the conditions for that specific
201 trial. Then participants completed a 10-km TT with the instruction of finishing
202 with the highest average power output and as fast as possible. Participants were
203 blinded to all information except cadence and remaining distance of the TT, and
204 were verbally encouraged at each km completed. VO_2 and HR were measured
205 continuously during the TT. For all physiological variables, average values from
206 the 10km-TT were calculated and used for further analyses. Further, peak values
207 for VO_2, RER (both highest 30-s average) and HR (highest 1-s value) during the
208 TT were calculated and used for further analyses. The ratio of average power to
209 average oxygen uptake (PO/VO_2) during the time trial was used as an index of
210 exercise efficiency [15]. NIRS variables of oxygenated (HbO_2), deoxygenated
211 (HHb) and total (THb) hemoglobin were recorded continuously at 2 Hz and
212 expressed as relative changes (Δ) from the baseline value measured during the
213 final 90-seconds pre-exercise rest period.

214 *2.1.5 Statistical analysis*

215 Differences in performance and physiological parameters were analyzed using
216 linear mixed models for repeated measures. This method of data analysis was

used as it has the advantage of preventing listwise deletion due to missing data (md). For clarification, md for each variable has been noted in table 1. As the dependent variable, the variable of interest was entered (watt, VO_2, VE, VCO_2, SpO_2, etc.) into the model. To investigate the effects of supplementation (BR vs. PLA), condition (hypoxia vs. normoxia) and supplementation-by-condition, these were entered as fixed effects. Subject id was included in the model as a random effect to control for the within-subject nature of the 4 trials. Further, paired t-tests were used to compare differences between the VO_{2peak} obtained during the normoxic time trials and the VO_{2max} from the ramp incremental test. Within group effect sizes were calculated as the difference in means (BR vs. PLA) divided by the pooled SD of the change score, using the following definitions: trivial effect d < 0.2, small effect > 0.2, moderate effect > 0.5, large effect > 0.8 [54]. Associations between changes in TT performance and changes in NO_3^-, NO_2^-, VO_2, and SpO_2 from PLA to BR were assessed using Pearson correlation coefficient.

All data are presented as means ± SE, unless stated otherwise, with statistical significance being accepted when $P \leq 0.05$. All statistical tests were performed using SPSS 25 (IBM Corp., Armonk, USA) or STATA (Texas, USA) version SE 12.1.

3.1 Results

3.1.1 Plasma nitrate and nitrite

There were significant main effects of supplementation on NO_3^- and NO_2^- (both $p<0.001$) such that BR elevated NO_3^- and NO_2^- (Fig 2). There were no effects of

condition (NO_3^- p=0.858; NO_2^- p=0.542) or supplementation-by-condition interaction (NO_3^- p<0.907; NO_2^- p=0.687). Further, there were no differences in levels of NO_3^- (p=0.234) or NO_2^- (p=0.231) between 4 and 7 days of supplementation (Fig 3).

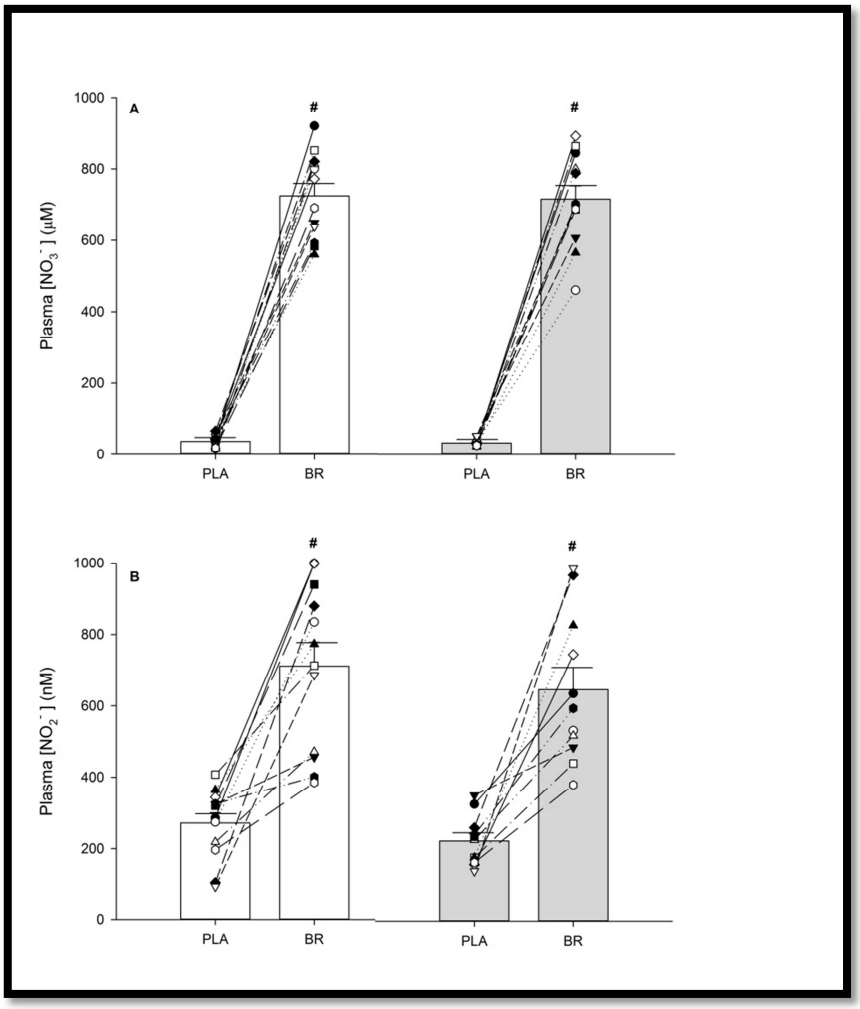


Figure 2: Individual and mean plasma levels of NO_3^- (A) and NO_2^- (B) (mean±SE) prior to time trial tests in normoxia (open bars) and hypoxia (filled bars), after supplementation with beetroot juice (BR) or placebo (PLA). (#, p < 0.001, PLA vs. BR, N=11 in hypoxic conditions).

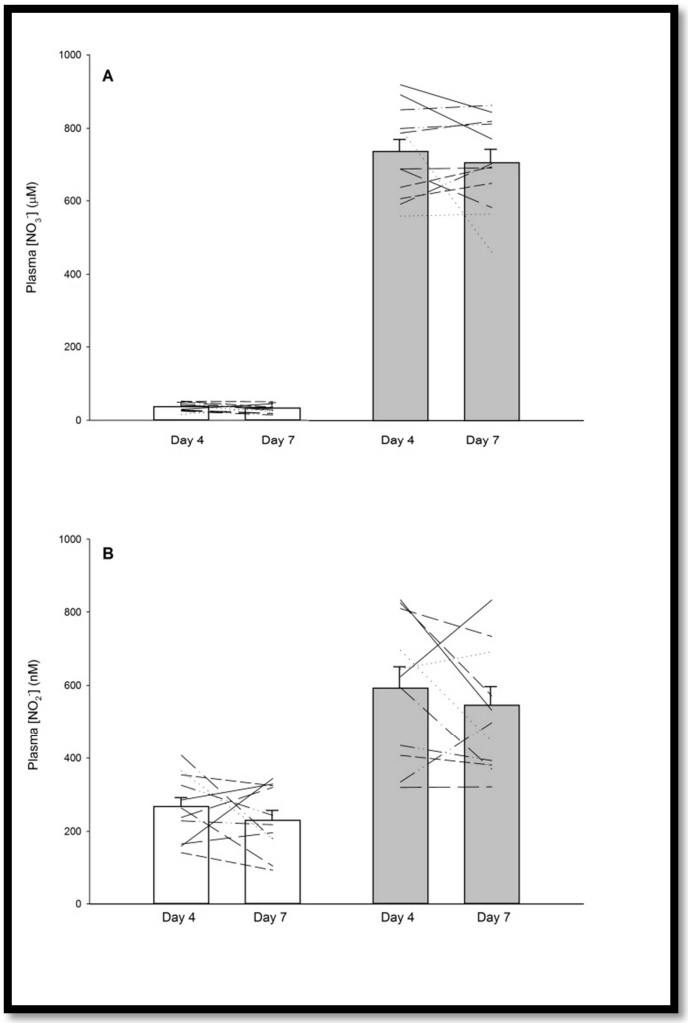
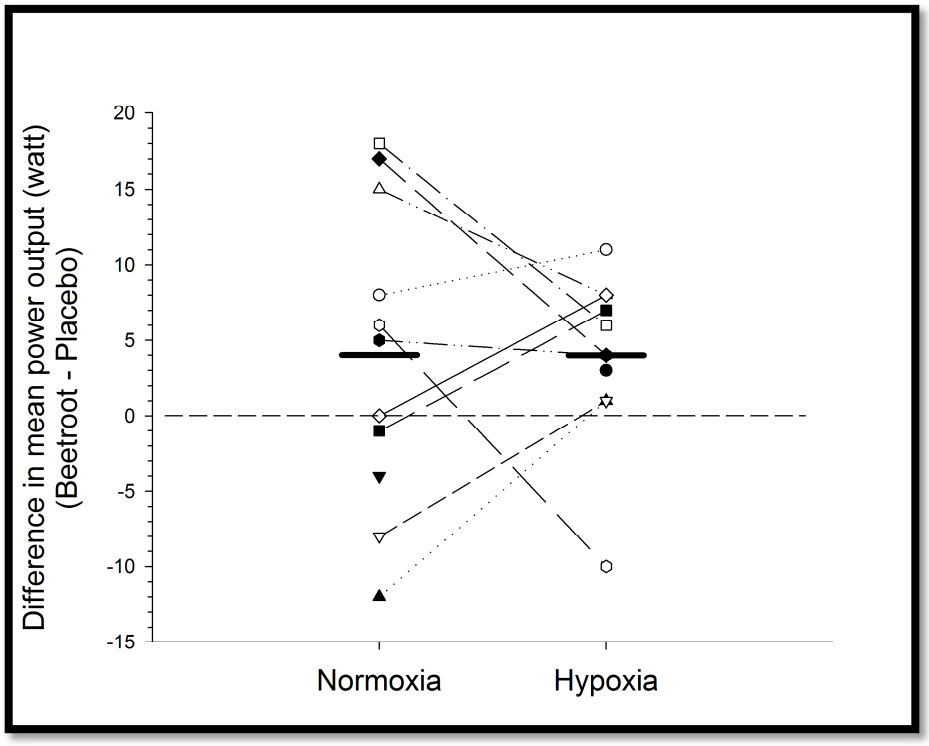


Figure 3: Individual and mean plasma levels of NO_3^- (A) and NO_2^- (B) (mean±SE) prior to time trial tests at day 4 and day 7 after supplementation with beetroot juice (filled bars) or placebo (open bars). (#, p < 0.001, PLA vs. BR, N=11 in hypoxic conditions).

3.1.2 Time trial performance

All participants completed all four TT`s. However, two tests were discarded due to measurement error (n=1 in N-BR and n=1 in H-PLA). Time trial performance data are presented in Table 1. There was a main effect of condition ($p<0.001$) on time trial performance such that hypoxia lowered power output by ~15% and ~6 %, respectively. Further, there was a main effect of supplementation on time trial power output ($p=0.019$) and completion time ($p=0.024$) showing an overall 1.6%

263 increase in power output and 0.6% reduction in completion time with BR (Fig 4),
264 with no condition-by-supplementation interaction (both p=0.923). Notably, 10 out
265 of 11 participants increased power output in H-BR compared to H-PLA, whereas
266 6 out of 11 increased power output in N-BR compared to N-PLA (Fig 4). Effect
267 size calculations for within group differences between BR and PLA show
268 moderate (0.703) and small (0.398) effects for hypoxia and normoxia,
269 respectively.



270

271 **Figure 4. Individual and mean differences in power output (watt) during 10 km TT**
272 **performance between placebo and beetroot supplementations in normoxic and hypoxic**
273 **conditions. Bold horizontal lines indicate mean values for each condition. Single dotted line**
274 **indicates no difference between beetroot and placebo supplementation**

275

276

277

278

	Md	N-PLA	N-BR	H-PLA	H-BR	Linear mixed model effects		
						Supplement	Condition	Interaction
		Time Trial						
Performance variable								
Power output, Watt	2	311.3 ± 13.2	315.8 ± 13.2	264.4 ± 13.2	269.3 ± 13.2	p=0.019	p<0.001	p=0.923
Completion time, sec	2	890.1 ± 16	884.5 ± 16	945.6 ± 16	939.5 ± 16	p=0.024	p=0.001	p=0.923
Average values								
PO/VO_2, $W/L^{-1}\cdot min^{-1}$	10	71.1 ± 1.8	70.8 ± 1.8	68.0 ± 1.8	68.0 ± 1.8	p=0.777	p=0.001	p=0.757
VO_2, $ml\cdot min^{-1}$	10	4364 ± 140	4443 ± 139	3855 ± 142	3948 ± 142	p=0.030	p<0.001	p=0.862
$\%VO_{2max}$	10	85.9 ± 1.6	87.4 ± 1.6	75.8 ± 1.7	77.7 ± 1.7	p=0.038	p<0.001	P=0.798
VCO_2, $ml\cdot min^{-1}$	10	4300 ± 151	4498 ± 150	4012 ± 153	4067 ± 153	p=0.005	p<0.001	P=0.120
VE, $L\cdot min^{-1}$	10	129.9 ± 7.0	135.8 ± 7.0	136.4 ± 7.1	142.4 ± 7.1	p=0.019	p=0.010	P=0.998
RER	10	0.99 ± 0.01	1.01 ± 0.01	1.04 ± 0.01	1.03 ± 0.01	p=0.462	p=0.003	P=0.082
$HR\cdot min^{-1}$,	3	168.5 ± 3.1	171.2 ± 3.1	169.4 ± 3.1	169.5 ± 3.1	p=0.118	p=0.486	P=0.072
SpO_2, %	9	97.1 ± 0.9	97.1 ± 0.9	84.5 ± 0.9	84.3 ± 0.9	p=0.787	p=0.000	P=0.779
Peak values								
VO_{2peak}, $ml\cdot min^{-1}$	10	4925 ± 151	4895 ± 150	4225 ± 152	4304 ± 152	p=0.443	p<0.001	p=0.111
HR_{peak}, $\cdot min^{-1}$	3	183.9 ± 2.9	185.5 ± 2.9	181.1 ± 2.9	181.5 ± 2.9	p=0.153	p<0.001	p=0.308
RER_{peak}	10	1.07 ± 0.02	1.1 ± 0.02	1.14 ± 0.02	1.14 ± 0.02	p=0.334	p=0.003	p=0.246
NIRS								
ΔHbO_2, AU	3	-28.5 ± 2.6	-27.6 ± 2.6	-30.7 ± 2.6	-29.4 ± 2.6	p=0.543	p=0.061	p=0.849
ΔHHb, AU	3	24.5 ± 2.6	23.9 ± 2.6	26.3 ± 2.6	26.6 ± 2.6	p=0.885	p=0.042	p=0.633
ΔTHb, AU	3	-4.3 ± 2.0	-3.4 ± 2.0	-3.9 ± 2.0	-2.7 ± 1.9	p=0.527	p=0.766	p=0.934
$\Delta HHb/VO_2$, $AU\cdot L\cdot min^{-1}$	12	5.68 ± 0.73	5.75 ± 0.71	7.01 ± 0.78	6.78 ± 0.74	p=0.851	p=0.017	p=0.728

Table 1- Average and peak performance, ventilatory and cardiopulmonary data during the TT. md denotes the number of missing data points from each variable (complete number of data points = 48).

3.1.3 TT physiological data

Physiological data obtained during the TT are presented in Table 1. There were significant effects of condition on SpO_2 (p<0.001), VE (p=0.010), RER

285 ($p=0.003$), VCO_2 ($p=0.001$), VO_2 ($p<0.001$), PO/VO_2($p=0.001$) and $\%VO_{2max}$
286 ($p<0.001$) such that hypoxia decreased SpO_2, VCO_2, VO_2, PO/VO_2, VO_{2peak},
287 HR_{peak} and $\%VO_{2max}$ while VE,RER and RER_{peak} increased. There were
288 significant effects of supplementation on VO_2 ($p=0.030$) (Fig 5), VE ($p=0.019$),
289 VCO_2 ($p=0.005$) and $\%VO_{2max}$ ($p=0.038$) such that VO2, VE, VCO_2 and $\%VO_{2max}$
290 increased with BR. The VO_{2peak} attained during the time trials in normoxia were
291 significantly lower than the VO_{2max} measured from the incremental test (N-PLA
292 ~3.3%, $p=0.03$; N-BR ~3.7%, $p=0.02$).
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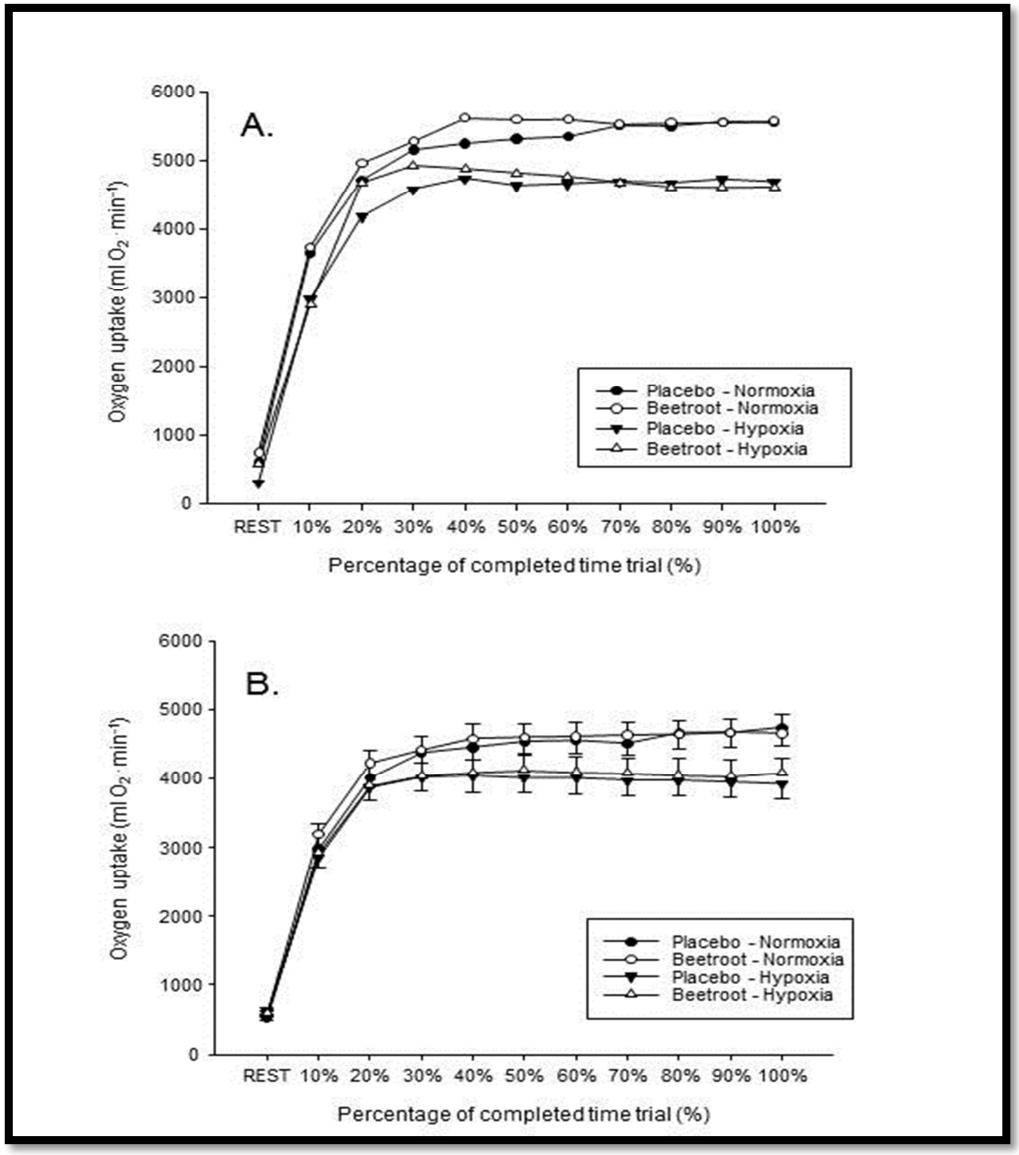

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297 **Figure 5- Oxygen uptake profiles from an exemplar subject (A) and mean data (B) from all**
298 **conditions.**

299

300 *3.1.4 Near infrared spectroscopy measures of muscle oxygenation*

301 Data reflecting changes in muscle oxygenation during the TT are presented in

302 Table 1. There was a main effect of condition on ΔHHb ($p=0.042$) and

303 $\Delta HHb/VO_2$ ($p=0.017$) such that the increase in ΔHHb and $\Delta HHb/VO_2$ during the

304 TT was greater in hypoxia (Table 1). We also found a near-significant main effect

of condition on ΔHbO_2 ($p=0.061$) indicating a greater reduction of ΔHbO2 during TT in hypoxia.

3.1.5 Correlations

There were no significant correlations between changes in performance and changes in plasma NO_3^- or NO_2^- after BR supplementation in normoxia or hypoxia. Further, there were no significant correlations between changes in performance (BR vs. PLA) and changes in VO_2 or SpO_2 nor between changes in performance (BR vs. PLA) and VO_{2max}.

4.1 Discussion

This is the first study to examine the effects of chronic supplementation with high-dose NO_3^-, in the form of BR, on time trial performance in well-trained athletes in both hypoxia and normoxia.

We show a significant main effect of BR on 10-km TT performance, indicating that well-trained cyclists improve power output and completion time with BR in both normoxia and hypoxia. Supplementation with BR also increased VO_2 during the TT in hypoxia and normoxia, showing that the participants were able to utilize a higher fraction of VO_{2max} with BR.

4.1.1 Effects of BR supplementation on TT performance

We found a main effect of BR supplementation on TT performance with no condition-by-supplementation interaction, indicating that BR increased TT performance with no difference between hypoxia and normoxia. However, from a practical perspective, it is worth highlighting that 10 out of 11 participants had higher power output in H-BR vs. H-PLA, while only 6 out of 11 had higher power output in N-BR vs. N-PLA (Figure 3). In support of a small effect of BR, a recent

329 meta-analysis, including studies performed in hypoxia and normoxia, reported a
330 non-significant 0.8% improvement in time trial endurance performance following
331 BR supplementation [55]. The improvement in 10-km TT completion time and
332 power output of 0.6% and 1.6%, respectively, in the present study, is of practical
333 relevance for elite and well-trained athletes. Specifically, only 0.9% separated
334 first and fourth position during the 13.8-km TT of stage 1 at the 2015 Tour De
335 France cycling race [56], and only 0.3% separated the first and third position
336 during the 9.7-km TT of stage 1 at the 2018 Giro d´Italia cycling race [57].
337 Further, 0.6% is the smallest worthwhile change in completion time for road TT
338 cyclists proposed by Paton and Hopkins [58].
339 Few other studies have examined the effects of NO_3^- on TT performance in well-
340 trained athletes in both normoxia and hypoxia within the same study. None of
341 these studies have reported significant improvements in TT performance after BR
342 supplementation [36,38,39]. Nonetheless, the study by Bourdillion et al. [39]
343 reported statistically non-significant improvements in 15-km TT performance of
344 16s (~1%) and 151s (~7%) in normoxia and hypoxia, respectively.
345 In general, studies on TT performance performed in well-trained athletes in
346 hypoxia or in normoxia have reported mixed results. In hypoxia, two studies
347 found statistically significant improvements of 2.2-3.2% (~2.2%) [17,21], while
348 one study reported no effect [37]. In normoxia, numerous studies show no effect
349 [22-27,59-61], while a few studies report a significant effect [15,16,20]. The
350 discrepancy in the literature may partly be due to the use of different NO_3^-
351 supplementation strategies that vary in terms of source, dose, and duration (e.g.,
352 chronic vs. acute). Many of the previous TT studies have not used an optimized

supplementation strategy. Specifically, some studies have used sodium nitrate as the source of NO_3^- [23,39], while there is evidence suggesting that supplementation with NO_3^- in concentrated BR is more effective [40,62]. Several studies have used an acute dose of BR [17,25,26,36-38,59-61], however, a chronic loading protocol consisting of BR supplementation over several days, as used in the present study, has been suggested to be more effective in raising plasma levels of NO_3^- and NO_2^-, and improving performance [11,43]. Finally, several studies have used a low-to-moderate dose of NO_3^- [36,37,59-61], while a higher dose (8-16 mmol), as used in the present study, may be more effective in raising plasma levels and improving performance [13,20,44]. The high dose of NO_3^- used in the present study was tolerated without any adverse events or complaints, demonstrating the efficacy of this supplementation strategy for 7 days. However, there is currently no evidence demonstrating additional benefits with doses higher than 8 mmol. In support of the notion that supplementation strategy is important, studies utilizing an optimized supplementation strategy with chronic supplementation of high dose NO_3^- in the source of BR have reported a significant 2.1% [16] and a non-significant 1.7% [24] improvement in TT power output in trained cyclists.

4.1.2 Plasma levels of NO_3^- and NO_2^-

In the present study, plasma levels of NO_3^- and NO_2^- after placebo (i.e., nitrate-depleted BR) supplementation, were similar to results from other studies using nitrate-depleted BR [17,21,22,37,38,63].

Four and seven days of BR supplementation increased NO_3^- and NO_2^- to levels reported in studies using a similar supplementation strategy [13,22], with no

differences between 4 and 7 days. Notably, NO_3^- and NO_2^- levels, in the present study, were higher than those reported in studies using acute supplementation [17,21,37,38,63]) or lower dosage of NO_3^- [17,37,59,60]. Taken together, markedly elevated levels of NO_3^- and NO_2^-, in the present study, indicate that BR supplementation was effective in providing an abundant source of NO via the nitrate-nitrite-NO pathway. Plasma levels of nitrite displayed a higher variability compared to plasma nitrate (Fig 2 and Fig 3). This is a common finding and is most likely due to the shorter half-life of nitrite (less than 1h)[64] compared to nitrate (5-8h)[65]. This may be explained by a much higher reactivity of nitrite being subjected to both enzymatic reduction to NO and oxidation to nitrate [33]. Moreover, due to the markedly lower concentration of nitrite in plasma, measuring techniques display more variable results compared to nitrate.

4.1.3 Physiological effects of beetroot juice supplementation

We found a main effect of supplementation on VO_2, VE, VCO_2 and $\%VO_{2max}$ such that BR supplementation resulted in higher VO_2, VE, VCO_2 and $\%VO_{2max}$ during the TT in both hypoxia and normoxia. As studies generally show unchanged [10,12,13,30] or reduced [66,67] VO_{2max} following BR supplementation, these results indicate that the participants were able to utilize a higher proportion of their maximal aerobic capacity during the TT with BR. Further, in the present study, a proxy of exercise efficiency (PO/VO_2) during the TT was unaffected by BR supplementation, suggesting that changes in exercise efficiency did not contribute to improved TT performance. In agreement with this, several studies, in well-trained athletes (>60 $ml \cdot min^{-1} \cdot kg^{-1}$), have shown unchanged exercise efficiency during submaximal exercise following BR

supplementation [24,37,38,63], while only a single study has reported improved efficiency (lower VO_2 during submaximal exercise) in well-trained athletes [21]. In club-level cyclists (56.0 $ml \cdot min^{-1} \cdot kg^{-1}$) [15], BR supplementation improved power output with unchanged VO_2 (greater PO/VO_2), indicating improved exercise efficiency. The discrepancy between these results could be due to the training level of the subjects, as our study included well-trained athletes (66.4 $ml \cdot min^{-1} \cdot kg^{-1}$). Thus, the increase in $\%VO_{2max}$ with BR was likely the main factor contributing to increased TT performance. In accordance with these results, Bourdillion et al. [39] reported greater VO_2 and VE with nitrate supplementation in trained cyclists during a 15-km TT in normoxia and hypoxia, which was accompanied by a non-significant increase (1-7%) in performance (discussed above). Contributing to the increased VO_2 with BR, the increase in VE (~6L/min) is estimated to account for 10-15 $ml/O_2/min$ (~10-20%) of the increase in VO_2, due to greater oxygen demands of the respiratory muscles [68-70].

The active skeletal muscles are the primary site for O_2 usage during the TT, and oxygenation in the vastus lateralis was monitored continuously using NIRS. During the TT, ΔHHb increased in hypoxia compared with normoxia, indicating increased O_2 extraction. However, in agreement with Kelly et al. [30] and Bourdillion et al. [39], ΔHHb was unaffected by BR supplementation, indicating that fractional O_2 extraction in vastus lateralis was not different between BR and PLA. Hence, according to the Fick principle, the increased oxygen uptake in the present study may be a result of increased total O_2 extraction due to increased blood flow. This interpretation is consistent with results demonstrating that NO_3^-

supplementation enhances vascular control and muscle blood flow redistribution during exercise [8,49,72].

5.1 Conclusion

In summary, our results provide novel evidence that chronic high-dose BR supplementation improves 10 km time trial performance of well-trained cyclists in both normoxia and hypoxia. Further, BR supplementation resulted in higher VO_2 and VE during the TT, suggesting that utilization of a greater proportion of the aerobic capacity contributed to the improved performance. While our results do not identify the underlying mechanisms, enhanced vascular control and muscle blood flow redistribution may contribute to higher VO_2 and improved time trial performance with BR supplementation.

6.1 Acknowledgements

We would like to thank all the participants for the contribution to the present study. Further, we thank Merete Fredsgaard, Brita Holst Serup, Hanne Krone Nielsen and Ditte Beck Christensen for the support in blood sample collections. We thank Carina Nihlen for support in analyzing blood samples. All authors approved the final version of the paper, and have no conflicts of interest.

7.1 Conflict of interest statement

The authors declare: no support from any organization for the submitted work; no financial relationships with any organizations that might have an interest in the submitted work in the previous 3 years; no other relationships or activities that could appear to have influenced the submitted work. EW is a co-applicant on patents related to the therapeutic use of nitrate and nitrite.

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679

ACCEPTED MANUSCRIPT

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- High-dose NO_3^- supplementation improved time trial performance of cyclists
- Oxygen uptake during the time trial was elevated with NO_3^- supplementation
- The effects of NO_3^- supplementation were not different between hypoxia and normoxia

ACCEPTED MANUSCRIPT