

**Title:** The effects of exercise on alterations in redox homeostasis in elite male and female endurance athletes using a clinical point of care test

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## **Abstract**

Exercise causes alterations in redox homeostasis (ARH). Measuring ARH in elite athletes may aid in the identification of training tolerance, fatigued states and underperformance. To our knowledge no studies have examined ARH in elite male and female distance runners at sea level. The monitoring of ARH in athletes is hindered by a lack of reliable and repeatable in-the-field testing tools and by the rapid turnaround of results. We examined the effects of various exercise intensities on ARH in healthy (non-overreached) elite male and female endurance athletes using clinical point of care (POC) redox tests, referred to as the free-oxygen-radical-test (FORT; pro-oxidant) and the free-oxygen-radical-defence (FORD; anti-oxidant). Elite male and female endurance athletes ( $n=22$ ) completed a discontinuous incremental treadmill protocol at submaximal running speeds and a test to exhaustion. Redox measures were analyzed via blood sampling at rest; warm-up; sub-maximal exercise; exhaustion and recovery. FORD was elevated above rest after sub-maximal, and maximal exercise and recovery ( $p<0.05$ ,  $d = 0.87-1.55$ ), with only maximal exercise and recovery increasing FORT ( $p<0.05$ ,  $d = 0.23-0.32$ ). Overall a decrease in oxidative stress in response to sub-maximal and maximal exercise was evident ( $p<0.05$ ,  $d = 0.46$ ). There were no gender differences for ARH ( $p>0.05$ ). The velocity at lactate threshold (vLT) correlated with the FORD response at rest, maximal exercise and recovery ( $p<0.05$ ). Using the clinical point of care test, an absence of oxidative stress after post-exhaustive exercise is evident in the non-fatigued elite endurance athlete. The blood antioxidant response (FORD) to exercise appears to be related to a key marker of aerobic fitness: vLT.

**Keywords:** oxidative stress, monitoring, biomarkers, antioxidant, fatigue

## Introduction

Exercise is known to be a source of reactive nitrogen and oxygen species (RNOS), leading to post-exercise alterations in redox homeostasis (ARH) (Nikolaidis et al. 2012b). Initially, RNOS were thought to be detrimental to athlete recovery, but it is now clear that RNOS are important for adaptation to endurance training (Finaud et al. 2006, Ristow et al. 2009, Powers et al. 2010a), skeletal muscle hypertrophy and protein signaling (Makanae et al. 2013, Paulsen et al. 2014). Nonetheless, a critical balance (captured in the theory of hormesis; Radak et al., (2008) exists between the sufficient or “optimal” dose of RNOS to drive adaptation, and the overproduction of RNOS that could lead to apoptosis, immunosuppression, excessive fatigue and reduced performance (Levada-Pires et al. 2008, Lewis et al. 2015)

The monitoring of elite athletes is widespread with considerable resources devoted to improving performance (Davison et al. 2009). Longitudinal monitoring of ARH in elite athletes may allow the identification of fatigued states, maladaptation, and under-performance in athletes (Lewis et al. 2015). Indeed, ARH after training phases, are proportional to the increase in training load (Margonis et al. 2007) and training volume (Knez et al. 2013). Furthermore, changes in antioxidant capacity and oxidative stress (OS) have been reported in over-reached and overloaded athletes (Palazzetti et al. 2003, Tanskanen et al. 2010) and in association with environmental and dietary stressors (Watson et al. 2005, Pialoux et al. 2009).

Several point-of-care (POC) tests have been developed to determine OS, with such tests offering convenience, rapid results and diagnosis, however, the validity has been questioned (Lindschinger et al. 2004, Kilk et al. 2014). The free oxygen radical test (FORT) and free oxygen radical defence (FORD) assays provide an accurate and non-invasive method for monitoring ARH, with excellent reliability and repeatability

(Garelnabi et al. 2008, Lewis et al. 2016). FORT captures the concentration of hydroperoxides in a biological sample, being derived from numerous lipid and protein molecules, ubiquitous within human tissues (Dean et al. 1997, Girotti 1998); lipid hydroperoxides derived from phospholipid, cholesterol and fatty acids, and protein hydroperoxides from proteins, peptide, amino acids, DNA and nucleic acids (Mimiyoto et al. 2007). Hydroperoxides are fairly stable, with protein and peptide hydroperoxides said to have a half-life of several hours at room temperature. Furthermore, the peroxidation of proteins and formation of protein hydroperoxides is the most extensive modification by radicals, and exceeds the formation of more commonly used biomarkers of protein oxidation such as protein carbonyls under similar conditions (Gebicki 2016).

FORD is an estimation of plasma antioxidant capacity, with the water-soluble molecules of ascorbic acid, glutathione, and albumin (but not uric acid), accounting for the majority of antioxidant activity (Palmieri and Sblendorio 2007). The ratio of the two tests provides an index of OS. The FORT-FORD Test (FFT) is a POC test that can be undertaken rapidly with athletes in a training environment (Lewis et al. 2016). However, to our knowledge this test has not been used systematically in an exercise context.

Despite the huge body of research in the field of redox biology, few studies describing the redox responses to exercise in female athletes have been published (Lewis et al. 2015) and to our knowledge only one paper has been published in elite female endurance athletes competing at a national and international level (Braakhuis et al. 2013). In sub-elite and recreationally trained athletes, gender differences exist for ARH, with females typically exhibiting lower (OS) compared with males in lipid peroxidation measures (Bloomer and Fisher-Wellman 2008).

An exercise challenge has been used consistently as a valid means of assessing redox responses and OS in the following groups or settings: young and old untrained and trained participants (Cobley et al. 2014), healthy and fatigued athletes (Tanskanen et al. 2010), and in response to environmental challenges (i.e. simulated altitude; Debevec et al. 2014). We chose to include a maximal exercise challenge and to push the athletes to exhaustion because (i) elite athletes are well adapted to their exercise modality and (ii) we wanted to assess the redox response in elite healthy athletes (i.e. not in fatigued, overreached or overtrained athletes) to generate normative OS data (i.e. FORT and FORD) for elite healthy endurance athletes in response to exercise. Previous studies have used maximal exercise tests as a means of distinguishing between healthy and fatigued athletes in terms of physiological and biomarker responses, including OS (Tanskanen et al. 2010, Meeusen et al. 2010). Finally, the majority of studies in elite athletes examining ARH employed cycling and rowing as the exercise modality (Lewis et al. 2015). We are unaware of any studies that have specifically looked at acute effects of exercise on ARH in elite male and female runners.

The aims of the current study were to, for the first time, (i) assess for ARH in response to sub-maximal and maximal exercise using the FFT, in elite non-fatigued endurance athletes, further validating the FFT in elite sport, and (ii) assess using the FFT whether differences in ARH exist between elite males and elite females.

## **Materials and methods**

### **Subjects**

Elite endurance athletes were recruited to the study; see table 1 for athlete characteristics (female n=7; male n=15). The group was made up of elite runners and triathletes including Olympic finalists, European and Commonwealth medalists from distances of 400m to marathon, and a European Ironman triathlon champion. All athletes provided written informed consent and completed a health questionnaire. Testing was carried out between December and September and the athletes described themselves as free from injury, illness, and under performance; most of the participants were tested in the competition preparation phase (May-August). The ethics committee of St Mary's University approved the study.

### **Experimental protocol**

On the day of the test, the athletes arrived in the laboratory between 0700 and 0900 hours. They were well hydrated and had been instructed to undertake only light exercise during the previous 24 hours (classified as an “easy” aerobic session) and to abstain from high-intensity and resistance exercise during the previous 72 hours. Following completion of the informed consent and medical questionnaire, the participants were allowed to consume a standard breakfast (see ‘Diet’ section). Approximately 1.5-2 hours after ingestion of the standard breakfast, the athletes entered the exercise phase.

### **Diet**

To control for the effect of the various breakfast choices on redox balance (RB), athletes were instructed to arrive fasted, having consumed a maximum of 500ml of water only on waking. A standard high carbohydrate and protein breakfast was

provided on arrival, in the form of a formulated high-energy sports nutrition bar (Powerbar Energise, Nestle Powerbar U.K.) and 500ml milk shake (For Goodness Shakes, U.K.), thus ensuring no recognized sources of antioxidants (e.g. testing e.g. fruits, vegetables, high fibre cereal grains, seeds and nuts) were consumed immediately before the testing. Moreover, no tea, coffee or fruit juices were allowed. Water was allowed *ad libitum*. The athletes were instructed not to take any vitamin or mineral, or sports nutrition products (e.g. vitamin C tablets, iron) during the 24 hours before the testing or on the morning of the test. In addition, they were required to maintain their normal diet, and avoid unusual consumption of caffeinated drinks and foods and the consumption of alcohol in the 24 hours prior to testing.

### **Sub-maximal and maximal exercise protocol**

After a 10 min warm-up on a motorised treadmill (Woodway ELG, Woodway USA, Forester Court, WI, 53209), the athletes completed a discontinuous incremental test involving 3 min work efforts, each  $1\text{km}\cdot\text{hr}^{-1}$  faster than the previous stage, separated by a 30 second rest period to allow for the measurement of blood lactate, and rate of perceived exertion (RPE; Borg, 1970). Each athlete completed between 5 and 9 submaximal stages, starting at an intensity below lactate threshold (typical male speed:  $14\text{ km}\cdot\text{hr}^{-1}$ ; female speed:  $11\text{ km}\cdot\text{hr}^{-1}$ ; Blood lactate was checked after the warm up, and the starting speed of the incremental test was reduced if lactate was above  $2\text{mMol}\cdot\text{L}^{-1}$ ). The warm up was conducted at the same speed as the first 3 min stage of the incremental test. The incremental test was terminated once blood lactate exceeded  $4\text{mMol}\cdot\text{L}^{-1}$ . Prior to the submaximal test, the athletes were fitted with a mask for breath-by-breath expired air analysis (Jaeger Oxycon Pro, Hoechberg, Germany) and a heart rate (HR) monitor. HR was measured continuously and

recorded throughout the exercise protocol (Polar Team System<sup>®</sup>, Polar U.K.). Following completion of the submaximal exercise test, athletes were given a 5 min rest period, whereby additional redox measures were immediately taken, before undergoing the maximal progressive exercise test to exhaustion at a constant speed, 2km•hr<sup>-1</sup> slower than the final speed of the sub-maximal test. The test began at a 1% gradient and increased by 1% every minute until volitional exhaustion. Heart rate peak (HR<sub>PEAK</sub>) was the highest HR value derived by Polar ProTrainer 5<sup>®</sup> software set at a 5 second sampling rate. Maximal aerobic capacity ( $\dot{V}O_{2\max}$ ) was defined as the highest 30 second average during the maximal exercise test.

Height and body mass were recorded and skinfold thickness (mm) measured by the same researcher, 7 sites were measured for the calculation of body fat (%) using the equation of Jackson and Pollock (1978). The researcher was accredited through the International Society for the Advancement of Kinanthropometry (ISAK).

### **Blood sampling**

Capillary blood samples were obtained at rest from the earlobe at the following time points: baseline (pre-exercise), immediately post sub-maximal exercise, immediately post-maximal exercise, and after recovery from the maximal exercise test (static recovery, 20 minutes post maximal test, supine).

Athletes were allowed access to sips of water post-exercise and into the recovery period should they complain of a dry mouth and thirst. Pre- and post-exercise plasma volume (PV) changes were estimated via the determination of hematocrit (Hct) and hemoglobin (Hb) concentration, using the formula of Dill and Costill (1974).



Whole blood capillary samples, 50  $\mu$ L for FORD, and 20 $\mu$ L for FORT were sampled from the ear lobe in heparinized capillary tubes. These were immediately mixed with reagent and centrifuged at 5,000 rpm for 1 minute, and analysed according to the manufacturers instructions using a Callegari analyser (Callegari SpA, Catellani Group, Parma, Italy) controlled at 37°C with absorbance set at a wavelength of 505nm for the calculation FORT and FORD. We have previously published in detail the methodology for the FORT and FORD assay (Lewis et al. 2016). Intra- and inter-assay coefficients of variation (CV) for FORT and FORD were <5% and 7% respectively.

Hematocrit was determined by capillary collection using 60  $\mu$ L sodium heparinised tubes, then centrifuged at 3000 rpm for 3 min. The packed cell volume was measured using a micro-haematocrit reader (Hawksley, UK). A 10  $\mu$ L blood sample was collected in a Hemocue™ 201+ microcuvette and analysed in a Hemocue™ 201+ (AB Leo Diagnostics, Helsinborg, Sweden) dual wavelength photometer for haemoglobin readings.

Capillary blood samples were also obtained following every phase of exercise and immediately analysed for blood lactate using a Biosen C-Line analyser (EFK Diagnostic, Barleben, Germany). These were used to identify the running speed corresponding to the lactate threshold (LT; defined as the first rise in blood lactate exceeding 0.4 mM), and the running speed corresponding to 3 mM (vLTP). The Lactate-OR web based application (<https://orreco.shinyapps.io/lactate/>) was used to define these points (Newell et al. 2014).

## **Statistical analysis**

All statistics were carried out using Minitab Inc. version 16. (USA). The distributions of all variables were assessed for normality with box-plots, and calculated using the Anderson-Darling test. Following normality tests, a general linear model (GLM) was used to test for an effect of exercise (5 levels: rest, warm-up, sub-max, maximal exercise and recovery) on FFT measures, and for an effect for gender (2 levels). In addition, a GLM was used to test for differences between short distance events (400m, 800m, 1500m) and long distance athletes (5k, 10k, marathon, triathlon) for FORT and FORD responses to exercise (rest, warm-up, sub-max, maximal exercise and recovery). If a significant interaction was evident, then pairwise comparisons were performed using the Tukey *post hoc* test. All FFT measures at rest and exercise data were analysed both with and without adjusting for PV to assess the need to control for changes in PV in relation to exercise and redox measures. Cohen's *d* effect sizes (*d*) were then used to calculate the magnitude of the standardised difference in means where significant, and reported as 0.2 (small), 0.5 (moderate), 0.8 (large), and 1.3 (very large). Significant relationships between variables were explored, for gender, and then combined male and female FORT, FORD, age and exercise intensity variables using Pearson's correlation coefficients. Data are presented as mean  $\pm$  SD with significance accepted at  $p < 0.05$ .

## Results

Subject characteristics and physiological variables are presented in table 1, which shows significant differences between male and female athletes for speeds at lactate threshold ( $p = 0.05$ ,  $d = 1.03$ ), and lactate turn point ( $p = 0.03$ ,  $d = 1.22$ ) and velocity at  $\dot{V}O_{2\max}$  ( $v\dot{V}O_{2\max}$ ) ( $p = 0.02$ ,  $d = 1.36$ ) (table 1).

### Insert table 1 here

#### FORD and FORT

There were no effects for gender on plasma FORD ( $p = 0.48$ ) and FORT ( $p = 0.42$ ); thus male and female data were combined. The combined results adjusted for PV at rest, and after warm up, sub-maximal exercise, maximal exercise and recovery are presented in figure 1. The importance of correcting for PV changes with exercise was evident, because un-adjusted PV FORD and FORT concentrations resulted in additional interactions not otherwise present. Significant relationships between the redox and physiological variables are reported in table 2.

### Insert table 2 here

#### OS index

The OS index refers to the ratio of FORT to FORD and provides a basic indication of the pro-antioxidant balance in plasma. An interaction was evident for the OS index with time ( $p < 0.001$ ), with no effect observed for gender ( $p = 0.35$ ); see figure 1.

There were no significant differences among short distance events (400m, 800m, 1500m) or among long distance athletes (5k, 10k, marathon, triathlon) for FORT and FORD across any of the time points measured ( $p>0.05$ ).

## Discussion

We report evidence of significant ARH in elite male and female endurance athletes in response to sub-maximal and maximal exercise, with no differences between genders. Using a clinical redox POC test to assess ARH, significant increases in both FORD and FORT were evident, with the ratio of the two measures indicating an overall reduction in OS in response to exercise. The increase in FORD was greater than the increase in FORT. Furthermore, we identified a significant relationship between plasma FORD both at rest, and at intensities reflective of the athletes aerobic conditioning. Thus we can conclude that aerobic fitness influences plasma FORD in elite athletes. To the best of our knowledge, this is the first study to experimentally evaluate the FORD and FORT tests in relation to exercise in male and female athletes.

The observed increases in the male and female athlete FORD after sub-maximal and maximal exercise are in agreement with those reported by Braakhuis and colleagues (2013), who studied a large cohort of elite male and female rowers. The authors observed a moderate (~12%) increase in plasma total antioxidant capacity (TAC) in response to a 30-min rowing ergometer time trial. The plasma biochemical components contributing to the plasma TAC assay were not reported by the authors; however, uric acid and vitamin C are commonly noted as explaining most of the variability in the assay response (Sies 2007). In addition, in their study, acute rowing performance and average training hours were negatively correlated with plasma TAC at rest, with the most highly trained rowers having the lowest resting TAC but the greatest rise in plasma TAC in response to the time trial. Our findings for our measure of plasma antioxidant capacity (FORD) at rest contrast with those of Braakhuis and colleagues (2013). However, our observations of the FORD responses to exercise are in accordance, because significant moderate relationships between plasma FORD and

measures of training status (LT, LTP,  $\dot{V}O_{2\max}$ ) were evident. This suggests that a higher level of aerobic conditioning is related to the plasma FORD response to exercise. This might be explained on the basis that the more highly trained the athlete (i.e. those attaining a higher velocity at LT, LTP and  $\dot{V}O_{2\max}$ ) the greater their resting GSH concentrations, and capacity for the mobilisation of endogenous antioxidant enzymes (i.e. GSH) into the blood in response to maximal exercise, to combat increases in RNOS. A strong relationship ( $r=0.65$ ) between endurance performance and glutathione has been reported in well-trained triathletes (Margaritis et al. 1997). Braakhuis and colleagues (2013) concluded that training status as opposed to dietary factors has the largest impact on antioxidant reserves and the associated mobilisation of antioxidant compounds in response to exercise; our findings lend support to such a contention.

The rise in plasma FORD after exercise may be accounted for by the rise in plasma ascorbic acid (vitamin C), released from the adrenal glands into the circulation which occurs with exercise. Plasma vitamin C is known to increase in response to stress hormones (Padayatty et al. 2007), which increase with exercise duration and intensity, and the plasma vitamin C concentration is reported to contribute to the antioxidant activity of the FORD assay (Palmieri and Sblendorio 2007). Another important factor contributing to the rise in plasma FORD is the tri-peptide glutathione (GSH; (Palmieri and Sblendorio 2007). Several studies in elite athletes have reported acute and chronic changes in blood GSH in response to exercise (Lewis et al. 2015), with exercise training known to elicit the up-regulation of intra-cellular GSH (Elokda and Nielsen 2007), and intra-cellular GSH being a significant source of blood GSH (Giustarini et al. 2008). Thus plasma changes in the athletes FORD values may largely reflect changes in vitamin C and GSH; this was not tested experimentally in

the present study. However, a significant relationship between resting FORD and red blood cell GSH in well-trained athletes has been reported (Lewis et al. 2016), with 15-20% of RBC GSH exported into plasma on a daily basis (Giustarini et al. 2008).

Other large and small molecular weight molecules reported to make a significant contribution to the antioxidant capacity of blood include, ceruloplasmin, albumin, bilirubin, and melatonin (Atanasiu et al. 1998, Benitez et al. 2002, Benot et al 1999). Evidence for albumin and caeruloplasmin explaining the increase in FORD (plasma antioxidant capacity) with exhaustive exercise is lacking. In fact, a large proportion of the reduced fraction of albumin (mercaptalbumin) is oxidized with intense exercise (Lamprecht et al. 2008). However, bilirubin, an antioxidant, increases following intense exercise and in association with the rise in plasma antioxidant capacity (Benitez et al. 2002). In addition, there is evidence that melatonin might have contributed to the observed acute rise in blood antioxidant capacity (i.e. FORD) with exercise. Melatonin is a potent antioxidant, and is known to increase acutely with heavy exercise performed in natural daylight hours (Atkinson et al. 2003). Furthermore, the diurnal variation in blood antioxidant capacity reflects the changes in melatonin, with maximal nocturnal values reported for both (Benot et al. 1999). Finally, using bright light to blunt the nocturnal rise in melatonin prevents the accompanying rise in serum antioxidant capacity (Benot et al. 1999). The contribution of various serum proteins, bilirubin and melatonin to the antioxidant capacity of the FORD assay warrants investigation.

The participants of the current study were healthy and were training and competing on a national and international stage. Athletes diagnosed as over-trained, and suffering from a decrement in performance and excessive fatigue may display a blunted rise in plasma antioxidant capacity in response to a maximal exercise test

(Tanskanen et al. 2010). Palazzetti and colleagues (2003) reported an absence in the post-exercise rise in plasma TAC in over-reached athletes, an observation that was not evident prior to the period of overload training. The athletes recruited in the current study did not report fatigue or performance concerns on interview, and we observed a significant rise in FORD following exercise, thus supporting the notion that the athletes were not fatigued excessively at the time of the study. Therefore, this study provides reference data for which the responses in fatigued or under-recovered athletes may be compared against, however, more research is needed to characterize the FORD and FORT responses in fatigued athletes.

Increases in FORT occurred only with maximal exercise, remaining elevated following 20 minutes recovery. There were no differences between rest and warm up and submaximal exercise. Therefore, it appears that unless the athlete's physiological systems become acutely stressed when exercising to exhaustion, there may be sufficient reserve with the endogenous antioxidant enzymatic systems to combat any exercise induced increase in RNOS, in the healthy elite athlete.

The plasma FORD increase in response to exercise was greater than the FORT response, reflecting a mobilization of anti-oxidants. It is well documented that endurance training increases antioxidant enzyme activity in blood, red blood cells and skeletal muscle, with elite athletes having well adapted antioxidant enzymatic systems leading to a reduced ARH and OS increasing across the season (Lewis et al. 2015). Indeed, some of the largest reported changes in redox homeostasis occurred in the general preparation phases of periodised training programs, such as when athletes are returning to training after a transition period of minimal training and detraining (Kyparos et al. 2009; Kyparos et al. 2011).



In studies examining redox responses to exercise consideration needs to be given to the timing of the post-exercise sample. Unfortunately there is no single time point post-exercise which best describes the response across a spectrum of OS assays (Michailidis et al. 2007). The ideal sampling point will depend on the assay used (Michailidis et al. 2007), the mode of exercise (Nikolaidis et al. 2012a), and the training status of the participants (Bloomer and Fisher-Wellman 2008). Given that we chose to assess elite athletes, we selected a sampling point immediately post-maximal exercise and 20 minutes into recovery, as used by other studies in elite athletes (Palazzetti et al. 2003, Tanskanen et al. 2010). However, in view of no significant differences between exhaustion and recovery for FORD and FORT in our study, future studies should look to extend the recovery period beyond 20 minutes to identify the time point at which complete homeostatic recovery occurs.

There are a number of limitations with our study that need to be addressed. We only used two biomarkers and their ratio to assess ARH for the presence of OS. In redox biology there are numerous assays available to assess ARH (Powers et al. 2010b). However, we choose the FFT on the basis of the test being a POC and thus potential for in-the-field-testing with athletes, with good reliability (Garelnabi et al. 2008). Such a POC test may prove advantageous in elite sport on the basis of recent findings identifying proportional increases in biomarkers of oxidative status (malondialdehyde) and antioxidant “defense” (catalase; CAT) in tandem with endurance performance in response to progressive increases in training volume (Knez et al. 2013). Indeed, the authors reported no further increases in performance when the single resting measure of oxidative status (MDA) exceeded that of the single measure of antioxidant defence (CAT); indicating increased OS (ratio of MDA/CAT), which corresponded to a plateau in performance. Future research should carry out

longitudinal monitoring and testing with the FFT in elite endurance athletes in order to understand the relationship between FORD and FORT, fatigue and performance.

We chose to control for PV changes in our study and carried out the analysis with and without adjustments. It has been reported that failure to control for PV changes may in part account for some of the discrepancies reported in the literature in studies of exercise and OS (Farney et al. 2012). Indeed, adjusting for PV influenced the results for both the FORD and FORT, and thus a failure to control for PV in exercise studies may influence the outcome and increase the chances of significant findings (type 1 error) in relation to pre- vs. post- exercise observations for ARH.

Finally, we chose to pool the data from endurance athletes, and from those who run short vs. long distances. In fact, we observed no significant differences among short distance events (400m, 800m, 1500m) or among long distance athletes (5k, 10k, marathon, triathlon) for FORT and FORD across any of the time points measured. Although Koury et al. (2004) observed no significant differences in antioxidant enzymes between elite short distance and long distance runners, they noted that the triathletes had significantly greater concentrations of superoxide dismutase in red blood cells than did both the other running groups. It is a limitation that we did not have a large enough number of triathletes to examine this further.

## Conclusions

ARH occurs in healthy elite male and female world-class endurance athletes well adapted to endurance training. The increase in FORD (antioxidant status) were greater and occurred earlier (with sub-maximal and maximal exercise) than the increase in FORT (oxidant status), such that the overall response to a single exhaustive bout of exercise could be characterized as inducing a decrease in OS in healthy elite

endurance athletes. The contribution of various serum proteins, bilirubin and melatonin to the antioxidant capacity of the FORD assay in athletes warrants investigation.

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There are no conflicts of interest

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## References

- Atanasiu, R.L., Stea, D., Mateescu, M.A., Vergely, C., Dalloz, F., Briot, F. et al. 1998. Direct evidence of caeruloplasmin antioxidant properties. *Molecular and cellular biochemistry*, **189**: 127-135.
- Atkinson, G., Drust, B., Reilly, T., & Waterhouse, J. 2003. The relevance of melatonin to sports medicine and science. *Sports Medicine*, **33**: 809-831.
- Benítez, S., Sánchez-Quesada, J.L., Lucero, L., Arcelus, R., Ribas, V., Jorba, O. et al. 2002. Changes in low-density lipoprotein electronegativity and oxidizability after aerobic exercise are related to the increase in associated non-esterified fatty acids. *Atherosclerosis*, **160**: 223-232.
- Benot, S., Gobema, R., Reiter, R. J., Garcia-Mauriño, S., Osuna, C., & Guerrero, J. M. 1999. Physiological levels of melatonin contribute to the antioxidant capacity of human serum. *Journal of pineal research*, **27**: 59-64.
- Bloomer, R.J., and Fisher-Wellman, K.H. 2008. Blood oxidative stress biomarkers: influence of sex, exercise training status, and dietary intake. *Gend Med* **5**: 218–228. doi:10.1016/j.genm.2008.07.002.
- Braakhuis, A.J., Hopkins, W.G., and Lowe, T.E. 2013. Effect of Dietary Antioxidants, Training and Performance Correlates on Antioxidant Status in Competitive Rowers. *Int J Sports Physiol Perform*. **8**: 565-575.

- Cobley, J.N., Sakellariou, G.K., Owens, D.J., Murray, S., Waldron, S., Gregson, W. et al. 2014. Lifelong training preserves some redox-regulated adaptive responses after an acute exercise stimulus in aged human skeletal muscle. *Free Radical Biology and Medicine* **70**: 23–32. doi:10.1016/j.freeradbiomed.2014.02.004.
- Davison, R.C.R., van Someren, K.A., and Jones, A.M. 2009. Physiological monitoring of the Olympic athlete. *Journal of Sports Sciences* **27**: 1433–1442. doi:10.1080/02640410903045337.
- Dean, R., FU, S., Stocker, R., and Davies, M. 1997. Biochemistry and pathology of radical-mediated protein oxidation. *Biochem. J.* **324**: 1–18.
- Debevec, T., Pialoux, V., Mekjavic, I.B., Eiken, O., Mury, P., and Millet, G.P. 2014. Moderate Exercise Blunts Oxidative Stress induced by Normobaric Hypoxic Confinement. *Medicine & Science in Sports & Exercise*: **46**: 33.41. doi:10.1249/MSS.0b013e31829f87ef.
- Dill, D.B., and Costill, D.L. 1974. Calculation of percentage changes in volumes of blood, plasma, and red cells in dehydration. *Journal of Applied Physiology* **37**: 247–248.
- Elokda, A.S., and Nielsen, D.H. 2007. Effects of exercise training on the glutathione antioxidant system. *European Journal of Cardiovascular Prevention & Rehabilitation* **14**: 630–637. doi:10.1097/HJR.0b013e32828622d7.

- Farney, T.M., McCarthy, C.G., Canale, R.E., Schilling, B.K., Whitehead, P.N., and Bloomer, R.J. 2012. Absence of Blood Oxidative Stress in Trained Men after Strenuous Exercise. *Medicine & Science in Sports & Exercise* **44**: 1855–1863. doi:10.1249/MSS.0b013e3182592575.
- Garelnabi, M.O., Brown, W.V., and Le, N.-A. 2008. Evaluation of a novel colorimetric assay for free oxygen radicals as marker of oxidative stress. *Clinical Biochemistry* **41**: 1250–1254. doi:10.1016/j.clinbiochem.2008.07.009.
- Gebicki, J. M. 2016. Oxidative stress, free radicals and protein peroxides. *Archives of Biochemistry and Biophysics*, **595**: 33-39.
- Girotti, A.W. 1998. Lipid hydroperoxide generation, turnover, and effector action in biological systems. *J. Lipid Res.* **39**: 1529–1542.
- Giustarini, D., Milzani, A., Dalle-Donne, I., and Rossi, R. 2008. Red blood cells as a physiological source of glutathione for extracellular fluids. *Blood Cells, Molecules, and Diseases* **40**: 174–179. doi:10.1016/j.bcmed.2007.09.001.
- Jackson, A.S., and Pollock, M.L. 1978. Generalized equations for predicting body density of men. *Br. J. Nutr.* **40**: 497–504.
- Kilk, K., Meitern, R., Härmson, O., Soomets, U., and Hõrak, P. 2014. Assessment of oxidative stress in serum by d-ROMs test. *Free Radic Res* **48**: 883–889.

doi:10.3109/10715762.2014.919390.

- Koury, J. C., de Oliveira, A. V., Portella, E. S., de Oliveira, C. F., Lopes, G. C., & Donangelo, C. M. 2004. Zinc and copper biochemical indices of antioxidant status in elite athletes of different modalities. *International journal of sport nutrition and exercise metabolism*, **14**: 358-372.
- Knez, W.L., Jenkins, D.G., and Coombes, J.S. 2013. The Effect of an Increased Training Volume on Oxidative Stress. *Int J Sports Med*. **35**: 8-13. doi:10.1055/s-0033-1333746.
- Kyparos, A., Riganas, C., Nikolaidis, M.G., Sampanis, M., Koskolou, M.D., Grivas, G.V. et al. 2011. The effect of exercise-induced hypoxemia on blood redox status in well-trained rowers. *Eur J Appl Physiol* **112**: 2073–2083. doi:10.1007/s00421-011-2175-x.
- Kyparos, A., Vrabas, I.S., Nikolaidis, M.G., Riganas, C.S., and Kouretas, D. 2009. Increased oxidative stress blood markers in well-trained rowers following two thousand-meter rowing ergometer race. *J Strength Cond Res* **23**: 1418–1426. doi:10.1519/JSC.0b013e3181a3cb97.
- Lamprecht, M., Greilberger, J.F., Schwaberg, G., Hofmann, P. and Oetl, K., 2008. Single bouts of exercise affect albumin redox state and carbonyl groups on plasma protein of trained men in a workload-dependent manner. *Journal of Applied Physiology*, **104**:1611-1617.

- Levada-Pires, A.C., Cury-Boaventura, M.F., Gorjao, R., Hirabara, S.M., Puggina, E.F., Peres, C.M. et al. 2008. Neutrophil death induced by a triathlon competition in elite athletes. *Medicine & Science in Sports & Exercise* **40**: 1447–1454.  
doi:10.1249/MSS.0b013e31816dc89e.
- Lewis, N.A., Howatson, G., Morton, K., Hill, J., and Pedlar, C.R. 2015. Alterations in redox homeostasis in the elite endurance athlete. *Sports Med* **45**: 379–409.  
doi:10.1007/s40279-014-0276-5.
- Lewis, N.A., Newell, J., Burden, R., Howatson, G., and Pedlar, C.R. 2016. Critical Difference and Biological Variation in Biomarkers of Oxidative Stress and Nutritional Status in Athletes. *PLoS ONE* **11**: e0149927.  
doi:10.1371/journal.pone.0149927.t002.
- Lindschinger, M., Nadlinger, K., Adelwöhrer, N., Holweg, K., Wögerbauer, M., Birkmayer, J. et al. 2004. Oxidative stress: potential of distinct peroxide determination systems. *Clin. Chem. Lab. Med.* **42**: 907–914.  
doi:10.1515/CCLM.2004.147.
- Makanae, Y., Kawada, S., Sasaki, K., Nakazato, K., and Ishii, N. 2013. Vitamin C administration attenuates overload-induced skeletal muscle hypertrophy in rats. *Acta Physiol* **208**: 57–65. doi:10.1111/apha.12042.



- Margaritis, I., Tessier, F., Richard, M.-J., and Marconnet, P. 1997. No evidence of oxidative stress after a triathlon race in highly trained competitors. *Int J Sports Med* **18**: 186–190.
- Margonis, K., Fatouros, I.G., Jamurtas, A.Z., Nikolaidis, M.G., Douroudos, I., Chatzinikolaou, A. et al. 2007. Oxidative stress biomarkers responses to physical overtraining: Implications for diagnosis. *Free Radical Biology and Medicine* **43**: 901–910. doi:10.1016/j.freeradbiomed.2007.05.022.
- Meeusen, R., Nederhof, E., Buyse, L., Roelands, B., de Schutter, G., and Piacentini, M.F. 2010. Diagnosing overtraining in athletes using the two-bout exercise protocol. *British Journal of Sports Medicine* **44**: 642–648. doi:10.1136/bjsm.2008.049981.
- Michailidis, Y., Jamurtas, A.Z., Nikolaidis, M.G., Fatouros, I.G., Koutedakis, Y., Papassotiriou, I. et al. 2007. Sampling Time is Crucial for Measurement of Aerobic Exercise-Induced Oxidative Stress. *Medicine & Science in Sports & Exercise* **39**: 1107–1113. doi:10.1249/01.mss.0b013e318053e7ba.
- Miyamoto, S., Ronsein, G.E., Prado, F.M., Uemi, M., Corrêa, T.C., Toma, I.N. et al. 2007. Biological hydroperoxides and singlet molecular oxygen generation. *IUBMB life*, **59**: 322-331.
- Newell, J., Korir, P., Moore, B., and Pedlar, C. 2014. App for the calculation of blood lactate markers. *Journal of Sports Sciences*: 1–2.

doi:10.1080/02640414.2014.953982.

Nikolaidis, M.G., Kyparos, A., Dipla, K., Zafeiridis, A., Sambanis, M., Grivas, G.V. et al. 2012a. Exercise as a model to study redox homeostasis in blood: the effect of protocol and sampling point. *Biomarkers* **17**: 28–35.  
doi:10.3109/1354750X.2011.635805.

Nikolaidis, M.G., Kyparos, A., Dipla, K., Zafeiridis, A., Sambanis, M., Grivas, G.V. et al. 2012b. Exercise as a model to study redox homeostasis in blood: the effect of protocol and sampling point. *Biomarkers* **17**: 28–35.  
doi:10.3109/1354750X.2011.635805.

Padayatty, S.J., Doppman, J.L., Chang, R., Wang, Y., Gill, J., Papanicolaou, D.A. et al. 2007. Human adrenal glands secrete vitamin C in response to adrenocorticotrophic hormone. *Am. J. Clin. Nutr.* **86**: 145–149.

Palazzetti, S., Richard, M.-J., Favier, A., and Margaritis, I. 2003. Overloaded training increases exercise-induced oxidative stress and damage. *Can J Appl Physiol* **28**: 588–604.

Palmieri, B., and Sblendorio, V. 2007. Oxidative stress tests: overview on reliability and use. Part I *Eur Rev Med Pharmacol Sci* **11**: 309–342.

Paulsen, G., Hamarsland, H., Cumming, K.T., Johansen, R.E., Hulmi, J.J., Børsheim, E. et al. T. 2014. Vitamin C and E supplementation alters protein signalling after

a strength training session, but not muscle growth during 10 weeks of training.

The Journal of Physiology **592**: 5391–5408. doi:10.1113/jphysiol.2014.279950.

Pialoux, V., Brugniaux, J.V., Rock, E., Mazur, A., Schmitt, L., Richalet, J.-P. et al.

2009. Antioxidant status of elite athletes remains impaired 2 weeks after a simulated altitude training camp. Eur J Nutr **49**: 285–292. doi:10.1007/s00394-009-0085-z.

Powers, S.K., Duarte, J., Kavazis, A.N., and Talbert, E.E. 2010a. Reactive oxygen

species are signalling molecules for skeletal muscle adaptation. Experimental Physiology **95**: 1–9. doi:10.1113/expphysiol.2009.050526.

Powers, S.K., Smuder, A.J., Kavazis, A.N., and Hudson, M.B. 2010b. Experimental

guidelines for studies designed to investigate the impact of antioxidant supplementation on exercise performance. International journal of sport nutrition and exercise metabolism **20**: 2.

Radak, Z., Chung, H. Y., Koltai, E., Taylor, A. W., & Goto, S. 2008. Exercise,

oxidative stress and hormesis. Ageing research reviews, **7**: 34-42.

Ristow, M., Zarse, K., Oberbach, A., Klöting, N., Birringer, M., Kiehntopf, M., et al.

2009. Antioxidants prevent health-promoting effects of physical exercise in humans. Proc. Natl. Acad. Sci. U.S.A. **106**: 8665–8670. doi:10.1073/pnas.0903485106.

- Sies, H. 2007. Total antioxidant capacity: appraisal of a concept. *The Journal of Nutrition* **137**: 1493–1495.
- Tanskanen, M., Atalay, M., and Uusitalo, A. 2010. Altered oxidative stress in overtrained athletes. *Journal of Sports Sciences* **28**: 309–317.  
doi:10.1080/02640410903473844.
- Watson, T.A., Callister, R., Taylor, R.D., Sibbritt, D.W., MacDonald-Wicks, L.K., and Garg, M.L. 2005. Antioxidant Restriction and Oxidative Stress in Short-Duration Exhaustive Exercise. *Medicine & Science in Sports & Exercise* **37**: 63–71. doi:10.1249/01.MSS.0000150016.46508.A1.

## Tables

**Table 1.** Athlete characteristics

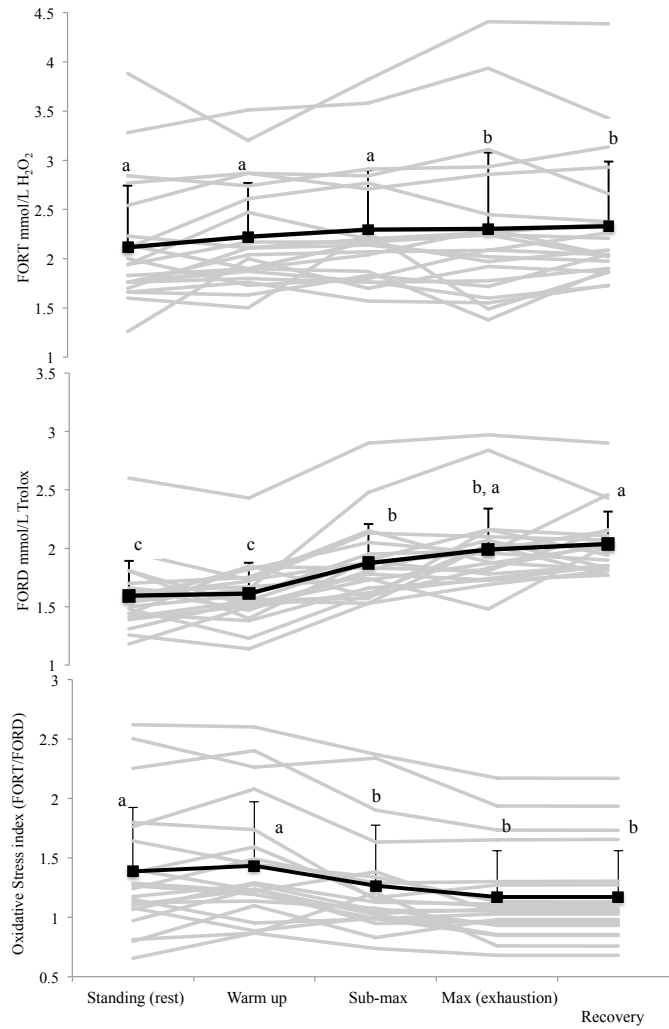
Variable	Female athletes (n=7)	Male athletes (n=15)
Age (y)	27.9±5.3	30.7±9.1
Weight (kg)	59.8±5.9	68.8±6.1
Height (cm)	170.9±6.4	178.2±5.2
Body fat (%)	10.6±3.2	7.7±3.4
Sum $\Sigma$ 7 skinfolds (mm)	59.2±19.7	43.6±19.0
Lactate threshold (km·h <sup>-1</sup> )	13.0±1.2	14.9±2.3*
Lactate turnpoint (km·h <sup>-1</sup> )	15.1±1.2	17.2±2.1*
$\dot{V}O_{2\max}$ (km·min <sup>-1</sup> )	17.2±1.4	19.4±1.8*
$\dot{V}O_{2\max}$ (ml·kg <sup>-1</sup> ·min <sup>-1</sup> )	61.4±7.3	68.7±5.8
$\dot{V}O_{2\max}$ range (ml·kg <sup>-1</sup> ·min <sup>-1</sup> )	53-71	67-80

\* p < 0.05

**Table 2.** Correlation matrix for FORD and physiological variables (LT, LTP and  $\dot{V}O_{2\max}$ )

	FORD rest	FORD max	FORD recovery	$\dot{V}O_{2\max}$	LTP	LT
FORD at rest			.526*	-0.093	.443	.480*
FORD maximal	.742**		.658**	.162	.404	.444*
FORD recovery	.526*	.658**		.457*	.517*	.489*
$\dot{V}O_{2\max}$	-0.093	.162	.457*		.219	.253
LTP	.443	.404	.517*	.219		.975**
LT	.480*	.444*	.489*	.253	.975**	
	.028	.044	.024	.269	.000	

\* p<0.05 \*\* p<0.01



**Figure 1.** FORT, FORD, and OS Index at rest, post warm-up, sub-maximal and maximal exercise and into 20 minutes of static recovery (PV adjusted data presented only). Letters (a, b, c) that differ denote significant differences between time points for each respective biomarker ( $p < 0.05$ ).  $\text{FORT}^{a, b} d = 0.23\text{-}0.32$ ;  $\text{FORD}^{c, b, a} d = 0.87\text{-}1.55$ ;  $\text{OS Index}^{a, b} d = 0.46$ .