Impact of training and exercise intensity on blood antioxidant markers in healthy Standardbred horses

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Abstract

This study investigated the effect of training and exercise intensity on blood antioxidant markers in six healthy Standardbred horses. Markers studied were uric acid (UA), ascorbic acid (AA), α-tocopherol, vitamin A (Vit A), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione − reduced (GSH) and oxidized (GSSG), glutathione redox ratio (GRR), copper (Cu), zinc (Zn) and selenium (Se). The horses performed a standardized exercise test (SET) when they were untrained (T0), after 4 weeks of light training (T4) and after 8 weeks of interval training (T12). Forty-eight hours after SET T4 and SET T12, a SET with run up to fatigue (TTF4 and TTF12) was performed. Maximal oxygen consumption (\(\bar{V}O_{2\text{max}}\)) was determined within 3–5 days after TTF4 and TTF12. At each test (SET \(T(i)\) and TTF\((i)\)), venous blood was sampled at rest (R), peak-exercise (Emax), 15 (E15) and 60 (E60) min after the test. UA, AA and GRR were increased significantly by the exercise, whereas GSH and Vit A were decreased significantly (\(P < 0.05\)). Training-related increases (T0 vs. T4 vs. T12) were observed for UA, SOD, GPx and Se, whereas GSH, α-tocopherol and Zn decreased (\(P < 0.05\)). Exercise intensity (TTF\((i)\) vs. \(T(i)\)) increased UA and AA significantly and decreased GSH significantly (\(P < 0.05\)). A significant correlation between ΔSOD and Δ\(\bar{V}O_{2\text{max}}\) (\(r = 0.86, p < 0.05\)) determined at SET T4 and SET T12 was found. Taken together, these results indicate that training and exercise intensity significantly influence blood antioxidant markers in healthy Standardbred horses.

Keywords: antioxidant markers; treadmill; training; exercise intensity

Introduction

The exercise-induced generation of reactive oxygen species (ROS) is a well-known phenomenon that was described for the first time in humans by Dillard et al.\(^1\). The most important sources of ROS that are generated during exercise are mitochondria (respiratory chain), but activated phagocytes (respiratory burst) and several enzymes (oxidases) might also contribute to an increased ROS release\(^2\). Among the potentially harmful effects of exercise figures ROS-induced tissue damage by oxidation of cellular components, such as membrane lipids, proteins, carbohydrates and deoxyribonucleic acids\(^3\).

On one hand, exercise-induced oxidative stress is believed to contribute to accelerated muscle fatigue and muscle fibre damage, leading to exercise intolerance...
and poor performance in different animal species\(^4\), as well as to a decreased immune defence of the organism\(^5\). On the other hand, epidemiological studies performed in humans clearly demonstrate that regular exercise is beneficial for health and well-being\(^6,7\), which might be at least partially explained by an adaptation and up-regulation of the antioxidant defence system. Indeed, it has been shown in different species that the exercise-induced oxidative burden depends on workload and might be modulated by training\(^8\)–\(^11\).

As most ROS have a short half-life, it remains difficult in practice to quantify increased ROS generation. However, the so-called pro-oxidative burden is counterbalanced by several antioxidant components (such as hydrophilic and lipophilic vitamins, antioxidant scavengers, antioxidant enzymes and their catalysts) aiming at reducing their potentially deleterious effects. Consequently, increased ROS generation might be evidenced by a modification of various antioxidants or by an increase of oxidative markers\(^12\). As ROS-antioxidant interactions as well as the antioxidant interactions themselves are relatively complex, it is difficult to assess whether oxidative processes are increased when a single marker is used. A better approach would be the determination of several antioxidant and oxidant markers, which might also be helpful to improve knowledge of the antioxidant network\(^13,14\).

In the equine species, an increase of exercise-induced ROS generation has been suggested in several experimental studies\(^15\)–\(^19\) as well as in field investigations\(^20\)–\(^24\). Although there is already considerable knowledge about exercise-sensitive antioxidants and oxidant markers and about the impact of different workloads (endurance, race) and external conditions (temperature, ambient humidity), the effect of training on the antioxidant defence system has to our knowledge not yet been investigated in racehorses.

The present study aimed therefore to investigate, in healthy Standardbred horses, the impact of exercise and exercise intensity on different blood antioxidant markers throughout a training period of 12 weeks.

### Materials and methods

#### Horses
Six clinically healthy French Standardbred horses (geldings, age: \(4.7 \pm 0.8\) years, body weight: \(445 \pm 30\) kg; mean \(\pm\) standard deviation (SD)) that had completed one training season were detrained at grass for at least 10 weeks before starting this study. The animals were kept in stalls and were bedded on wood shavings. They were fed grass silage (4 kg) and molassed oats (2 kg) twice daily. Besides the well-defined treadmill training (see Study design), the horses were allowed to move freely in a paddock for 20 min per day. The study was approved by the Animal Ethics Committee of the University of Liège.

#### Study design
The time schedule of exercise tests and variables that were recorded during exercise tests performed on a treadmill\(^1\) is indicated in Table 1. All horses underwent an adaptation period of 4 weeks during which they were familiarized to the treadmill. Exercise

### Table 1  Study design

<table>
<thead>
<tr>
<th>Time schedule of tests</th>
<th>Test duration</th>
<th>Antioxidant markers (R, E_{\text{max}}, E_{\text{15}}, E_{\text{60}})</th>
<th>LA</th>
<th>HR (_9)</th>
<th>HR_{\text{max}}</th>
<th>T(_{8})</th>
<th>(_\text{VO}_2\text{max})</th>
<th>(_\text{VCO}_2\text{max})</th>
<th>(_\text{VT})</th>
<th>(_\text{VE})</th>
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</thead>
<tbody>
<tr>
<td>4 weeks’ adaptation period</td>
<td>SET T0</td>
<td>(x)</td>
<td>(x)</td>
<td>(x)</td>
<td>(x)</td>
<td>(x)</td>
<td>(x)</td>
<td>(x)</td>
<td>(x)</td>
<td>(x)</td>
</tr>
<tr>
<td>4 weeks of light exercise</td>
<td>SET T4</td>
<td>(x)</td>
<td>(x)</td>
<td>(x)</td>
<td>(x)</td>
<td>(x)</td>
<td>(x)</td>
<td>(x)</td>
<td>(x)</td>
<td>(x)</td>
</tr>
<tr>
<td>48 h</td>
<td>TTF(_4)</td>
<td>(x)</td>
<td>(x)</td>
<td>(x)</td>
<td>(x)</td>
<td>(x)</td>
<td>(x)</td>
<td>(x)</td>
<td>(x)</td>
<td>(x)</td>
</tr>
<tr>
<td>3–5 days</td>
<td>(_\text{VO}_2\text{max}) T(_4)</td>
<td>(x)</td>
<td>(x)</td>
<td>(x)</td>
<td>(x)</td>
<td>(x)</td>
<td>(x)</td>
<td>(x)</td>
<td>(x)</td>
<td>(x)</td>
</tr>
<tr>
<td>8 weeks of interval training</td>
<td>SET T12</td>
<td>(x)</td>
<td>(x)</td>
<td>(x)</td>
<td>(x)</td>
<td>(x)</td>
<td>(x)</td>
<td>(x)</td>
<td>(x)</td>
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</tr>
<tr>
<td>48 h</td>
<td>TTF(_{12})</td>
<td>(x)</td>
<td>(x)</td>
<td>(x)</td>
<td>(x)</td>
<td>(x)</td>
<td>(x)</td>
<td>(x)</td>
<td>(x)</td>
<td>(x)</td>
</tr>
</tbody>
</table>

| R, E_{\text{max}}, E_{\text{15}}, E_{\text{60}} | – determined at rest, peak-exercise and 15 and 60 min after the test, respectively; LA | venous blood lactate; HR \(_9\) s\(^{-1}\) | heart rate recorded at a speed of 9 m s\(^{-1}\); HR_{\text{max}} | maximal heart rate; T\(_{8}\) | rectal temperature; V\(_{\text{CO}_2\text{max}}\) | maximal carbon dioxide output; V\(_T\) | tidal volume; V\(_E\) | minute ventilation; SET T\(_i\) | standardized exercise test performed after \(i\) weeks of training; TTF\(_i\) | standardized exercise test with run up to fatigue performed after \(i\) weeks of training; \(\_\text{VO}_2\text{max}\) T\(_i\) | determination of maximal oxygen uptake after \(i\) weeks of training.

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performed twice weekly during this period was of short duration (15–20 min) and low intensity (slope 0% and speed < 4 m s⁻¹).

After the adaptation period, the horses underwent the first standardized exercise test (SET T0). Antioxidant markers (for details see Blood sample collection) were determined in jugular venous blood sampled immediately prior to the SET (rest: R), at peak-exercise (Emax), as well as 15 (E15) and 60 (E60) min after the end of the SET. During the following 4 weeks, the horses underwent a light treadmill exercise (three times weekly, slope 3%, maximal speed 4.5 m s⁻¹, minimum distance during week 1: 3000 m, maximum distance during week 4: 9000 m). As for SET T0, antioxidant markers were sampled at R, Emax, E15 and E60 during SET T4 and 48 h later during TTF4, consisting of an exercise test with run up to fatigue. Three to five days later, maximal oxygen consumption (VO₂max) and maximal heart rate (HRmax) were determined (VO₂max T4).

During the following 8 weeks, the horses underwent interval training adapted from Lovell. Treadmill training was performed three times per week and consisted twice weekly of three consecutive exercise bouts of 1000 m at 8 m s⁻¹ (slope 3%) and once weekly of a single exercise of 10 000 m at 6 m s⁻¹ (slope 3%). Increasing the distance and the speed progressively increased the workload. At the end of the training period, two consecutive exercise bouts of 1400 m at 9 m s⁻¹ (slope 3%) were performed twice weekly. The third training included the same workload but was finished by a third step of 400 m at maximal speed (ranging from 11 to 13 m s⁻¹ according to the horse). After this training period, the horses performed a further SET (SET T12), followed 48 h later by an exercise test with run up to fatigue (TTF12). Antioxidant markers were sampled according to the same timing schedule as described above. The protocol ended 3–5 days after TTF12 by determination of VO₂max and HRmax (VO₂max T12).

Standardized exercise tests

Standardized exercise tests

SET T0, SET T4 and SET T12 consisted of a warm-up at walk (5 min, 1.7 m s⁻¹, slope 0%) and slow trot (5 min, 4 m s⁻¹, slope 0%) followed by a 4-min high-intensity trot of increasing speed (min 1: 7 m s⁻¹, min 2: 8 m s⁻¹, min 3: 9 m s⁻¹, min 4: 10 m s⁻¹) at a slope of 6%. A cool-down of 5 min walking (1.7 m s⁻¹, slope 0%) finished the SET.

Standardized exercise tests with run up to fatigue

TTF4 and TTF12 were similar to the previous tests but included a last step at constant speed of 11 m s⁻¹ until the horse showed signs of fatigue. The duration of this last step was carefully recorded.

Standardized exercise tests for determination of VO₂max

After a warm-up at walk (5 min, 1.7 m s⁻¹, slope 0%) and slow trot (5 min, 4 m s⁻¹, slope 0%), the slope was set at 6% and the speed was increased stepwise from 4 m s⁻¹ (2 min) to 6 m s⁻¹ (2 min), 8 m s⁻¹ (2 min), 9 m s⁻¹ (1 min), 10 m s⁻¹ (1 min), 11 m s⁻¹ (1 min) and 12 m s⁻¹ (up to fatigue or until VO₂max reached a plateau for at least 1 min). The test was ended by a cool-down walk of 5 min (slope 0%, 1.7 m s⁻¹).

Exercise monitoring

Heart rate monitoring

Heart rate (HR) was determined at each SET using a telemetric electrocardiogram recorder (Life Scope Monitor; Nihon Kohoen Corp., Tokyo, Japan).

Venous blood lactate determination

Venous blood was sampled from an indwelling jugular catheter at each SET at Emax for analysis of plasma lactate (LA) (Accusport®; Boehringer, Mannheim, Germany).

Determination of VO₂max

As described in previous studies, the horses were equipped with a lightweight and tightly fitting facemask constructed from fibreglass. Respiratory flow from each nostril was measured using a pneumotachograph composed of two ultrasonic flow transducers, which were mounted diagonally across a polyvinylchloride tube (flow tube). Each pneumotachograph had a scale range of 60 l s⁻¹, allowing recording of a maximal peak flow of 120 l s⁻¹. Linearity and symmetry of the pneumotachographs had been tested for 1–55 l s⁻¹ and pneumotachographs were calibrated by use of a known flow source and an air velocity transducer. A mass spectrometer was used to sample air of one flow tube and inspired and expired O₂ and CO₂ concentrations were measured continually on a breath-by-breath basis. Oxygen uptake (VO₂), carbon dioxide (VCO₂) output, maximal tidal volume (VTmax) and maximal expired minute volume (VEmax) were analysed and calculated by use of on-line and breath-by-breath computer analysis. The mass spectrometer was calibrated using gas of known composition. VO₂ was displayed continuously on a screen, allowing determination of when horses reached their VO₂max.

Body temperature determination

Rectal temperature was measured after the cool-down walk of each SET.

Blood sample collection

Venous blood was collected through the jugular catheter for analysis of antioxidant markers when the horses were at rest, 30 min before the SET and at peak-exercise as well as 15 and 60 min after the end
of the SET (Emax, E15 and E60, respectively). Blood was aliquoted into Vacutainer tubes containing ethylene-diaminetetraacetic acid (EDTA) for analysis of ascorbic acid (AA), a-tocopherol, vitamin A (Vit A), glutathione peroxidase (GPx), superoxide dismutase (SOD), glutathione (GSH) and haemoglobin (Hb). Trace-element-free NH143 IU tubes were used to collect samples for analysis of selenium (Se), copper (Cu) and zinc (Zn); heparin-containing tubes were used for analysis of cholesterol, uric acid (UA), packed cell volume (PCV) and total protein.

**Blood sample processing**

The blood samples were processed immediately after collection as follows. One part of whole blood (EDTA) was kept on ice for analysis of GPx, SOD and Hb within 8 h of collection; further samples of whole blood (100 [micro]l + 10 [micro]l of 1-methyl-2-vinylpyridinium-trifluoromethanesulphonate for analysis of oxidized glutathione (GSSG); 50 [micro]l for analysis of reduced glutathione (GSH)) were immediately frozen on dry ice and stored at −80°C until analysis. After centrifugation of EDTA blood (15 min, 900×g), one part of the plasma was stabilized with 10% metaphosphoric acid (MPA; plasma/MPA 1:1) and was kept on dry ice to be analysed for ascorbic acid within 6 h of collection. The second part was frozen immediately on dry ice and kept at −80°C until analysis. After centrifugation of EDTA blood (15 min, 900×g), one part of the plasma was stabilized with 10% metaphosphoric acid (MPA; plasma/MPA 1:1) and was kept on dry ice to be analysed for ascorbic acid within 6 h of collection.

**Blood marker analysis**

**Ascorbic acid**

Plasma concentrations of AA were determined spectrophotometrically according to the method described by Omaye et al., using the reduction of 2,6-dichlorophenol-indophenol (Merck no. 1.03028). The intra-assay coefficient of variation (CV) was 4% and inter-assay CV was 6%.

**a-Tocopherol**

Plasma concentrations of a-tocopherol were determined by high-performance liquid chromatography (HPLC) on reversed-phase columns (C18, 120 mm, 100×45 mm) with an isotropic elution (methanol/water 98:2) and ultraviolet (UV) detection at 280 nm. The intra- and inter-assay CVs were 2.6% and 6.2%, respectively.

**Vitamin A**

Plasma Vit A concentration was determined by HPLC and UV detection using the kit provided by Chromsystems® (Munich, Germany). The intra- and inter-assay CVs were 3.6% and 6.8%, respectively.

**Selenium, copper, zinc**

Plasma concentrations of trace elements were determined by inductively coupled plasma mass spectrometry (Agilent 7500a instrument; Agilent Technologies, Palo Alto, CA) according to the method described by Chappuis et al. The intra- and inter-assay CVs were 2.7–4.3% and 3.8–6.5%, respectively.

**Superoxide dismutase and glutathione peroxidase**

Whole blood was analysed spectrophotometrically using a RANSOD and RANSEL kit for SOD and GPx, respectively (Randox Laboratories, Antrim, UK). The intra- and inter-assay CVs were lower than 5% and 7%, respectively.

**Glutathione**

Whole blood glutathione (GSH and GSSG) was determined spectrophotometrically using the Bioxytech® GSH/GSSG 412 assay kit (Oxis, Portland, OR). The intra- and inter-assay CVs were 1% and 3%, respectively, for GSH and 6.5% and 7.6% respectively, for GSSG.

**Uric acid**

Plasma concentration of UA was analysed spectrophotometrically using an automatic analyser (Modular, Roche Hitachi, Roche Diagnostic, Vilvorde, Belgium). The intra- and inter-assay CVs were 2.1% and 4.2%, respectively.

**Standardization of blood markers’ concentration**

a-Tocopherol was standardized by calculating the a-tocopherol/cholesterol ratio and GPx and SOD by expressing their activities per gram of Hb. The concentration of hydrophilic plasma markers (AA, UA) and trace elements was standardized by total protein concentration, whereas lipophilic plasma markers (a-tocopherol and Vit A) were standardized by cholesterol. As GSH and GSSG were determined in whole blood, their concentrations were adjusted for changes of PCV. Adjustment was performed using the following equation:

\[
X_{\text{adj}} = X_{\text{obs}} + (X_{\text{obs}} \times \frac{(M_{R} - M_{obs})}{M_{R}}),
\]

where \(X_{\text{adj}}\) is adjusted marker concentration, \(X_{\text{obs}}\) is measured marker concentration at \(t_{i}\), \(M_{R}\) is concentration of the standardizing element determined at rest and \(M_{obs}\) is concentration of the standardizing element determined at \(t_{i}\).

**Statistical analysis**

Blood antioxidant concentrations measured at R, Emax, E15, E60 during the different tests (T0, T4,
Training, exercise and blood antioxidant markers in horses

TTF4, T12, TTF12 were normally distributed and were analysed by a mixed linear model for repeated measures (SAS® software; SAS Institute Inc., Cary, NC), allowing analysis of the effect of sampling time, test and time–test interaction. Physiological variables were analysed by analysis of variance (ANOVA) for repeated measures and by post hoc paired Student’s tests (Statview® software; SAS Institute, Inc.). Correlation analyses (Pearson’s) were performed either between antioxidant markers and LA or between inter–test variation of antioxidant markers (change of (Δ) marker concentration) and inter–test variation of running time (Δrunning time) or VO2max (ΔVO2max). Differences were considered significant when P < 0.05. Data are shown as least-square means with associated standard error of the mean (SEM) for antioxidant markers and as mean ± SEM for physiological variables.

Results

Physiological variables

Data related to HR recorded at a speed of 9 m s⁻¹, plasma LA, rectal temperature and running time are shown in Table 2. Heart rate recorded at 9 m s⁻¹ did not differ significantly between exercise tests. Plasma LA was increased significantly in tests with run up to fatigue, the value determined at TTF12 being the highest. Rectal temperature was also significantly increased after tests including a run up to fatigue when compared with SETs of 4 min duration. Running time up to fatigue (RTF) was about 4 min 50 s after 4 weeks of light training (TTF4) and about 5 min 30 s after the last test (TTF12).

Regarding ventilation data and metabolic measurements, all variables recorded before (VO2 T4) and after (VO2 T12f) the interval training (VTmax 20.9 ± 2.2 vs. 22.6 ± 2.41, P < 0.05), Vemax (1638 ± 27 vs. 1745 ± 111 min⁻¹, P < 0.05), VO2max (117 ± 6 vs. 139 ± 7 ml kg⁻¹ min⁻¹, P < 0.05) and VO2max (144 ± 12 vs. 177 ± 11 ml kg⁻¹ min⁻¹, P < 0.05) were increased significantly. Maximal heart rate (HRmax, 225 ± 10 vs. 227 ± 6) remained unchanged by the interval training (P > 0.05).

Blood antioxidants

Least-square mean concentrations (± SEM) of blood markers assessed at rest, peak-exercise, 15 and 60 min after exercise, as well as at the different exercise tests (T0, T4, TTF4, T12 and TTF12), are shown in Table 3. Due to the statistical analysis by a mixed linear model, only one SEM value was attributed to least-square mean values calculated at R, Emax, E15 and E60, as well as to the least-square mean values calculated at T0, T4, TTF4, T12 and TTF12.

Exercise induced a significant increase in AA and UA at E15 and E60, whereas Vit A was significantly decreased at Emax. Activity of SOD and GPx as well as plasma trace element concentrations (Se, Cu, Zn) remained unchanged, whereas concentration of GSH was decreased significantly at Emax and E15. The glutathione redox ratio (GRR) was slightly but significantly increased at Emax, whilst modifications of GSSG remained non-significant.

Among the markers whose concentration or activity was significantly increased by training (T0 vs. T4 and/or T12) were UA, Se, Cu, SOD and GPx. A significant decrease of α-tocopherol, GSSG and GRR% was observed at T4; at T12, a further decrease of α-tocopherol as well as an increase of Vit A were noted.

After the light exercise training with run up to fatigue (T4 vs. TTF4), a significant increase of AA and UA as well as a significant decrease of GPx, GSH and Zn were observed. After 8 weeks of interval training and run up to fatigue (T12 vs. TTF12), AA and AU were significantly increased, whereas Vit A and GSH were significantly decreased.

Correlation analyses between physiological variables, running time and antioxidant markers

Plasma UA concentration determined at E15 was positively correlated with plasma LA determined at Emax (r = 0.85, P < 0.0001; n = 24).

Among the correlations investigating the variation of antioxidant marker concentration and the increase of running time to fatigue (ΔRTF), the following results are worthwhile to mention: ΔGPx at rest – ΔRTF (SET TTF4 vs. SET TTF12): r = 0.93, P < 0.05, n = 6; ΔUA

Table 2 Heart rate recorded at a speed of 9 m s⁻¹ (HR 9), plasma lactate (LA) determined at peak-exercise, rectal temperature (T*) recorded at post-exercise and running time assessed during different exercise tests of six healthy Standardbred horses. Data are shown as mean ± standard error of the mean

<table>
<thead>
<tr>
<th>Variable</th>
<th>SET T0</th>
<th>SET T4</th>
<th>TTF4</th>
<th>SET T12</th>
<th>TTF12</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR 9 (beats min⁻¹)</td>
<td>208 ± 3ᵃ</td>
<td>216 ± 2ᵃ</td>
<td>216 ± 2ᵃ</td>
<td>218 ± 5ᵃ</td>
<td>217 ± 3ᵃ</td>
</tr>
<tr>
<td>LA (mmol⁻¹)</td>
<td>9.8 ± 0.6ᵃ</td>
<td>12.2 ± 0.7ᵃ</td>
<td>17.8 ± 0.7ᵇ</td>
<td></td>
<td>–</td>
</tr>
<tr>
<td>T* (°C)</td>
<td>39.3 ± 0.1ᵃ</td>
<td>39.1 ± 0.2ᵃ</td>
<td>39.5 ± 0.1ᵇ</td>
<td>39.2 ± 0.1ᵃ</td>
<td>40.0 ± 0.1ᶜ</td>
</tr>
<tr>
<td>Running time (min)</td>
<td>4.00 ± 0.0⁰</td>
<td>4.00 ± 0.0⁰</td>
<td>4.82 ± 0.1³</td>
<td>4.00 ± 0.0⁰</td>
<td>5.49 ± 0.1²</td>
</tr>
</tbody>
</table>

SET – standardized exercise tests; SET T0 = SET before exercising period; SET T4 = SET after 4 weeks of light exercise; TTF4 = exercise test to fatigue performed 48 h after T4; SET T12 = SET after 8 weeks of interval training; TTF12 = exercise test to fatigue performed 48 h after T12. Within-line data with different superscripts are statistically different (P < 0.05); – indicates missing value.
Table 3  Blood antioxidant marker concentrations determined in venous jugular blood of six healthy Standardbred horses at rest (R), peak-exercise (Emax), and 15 (E15) and 60 (E60) min after the end of the different standardized exercise tests (SETs). Data are shown as least-square mean ± associated standard error of the mean (SEM).

<table>
<thead>
<tr>
<th>Effect tested</th>
<th>AA (μmol l⁻¹)</th>
<th>UA (μmol l⁻¹)</th>
<th>α-Tocopherol (mg g⁻¹ cholesterol)</th>
<th>α-Tocopherol (mg g⁻¹ cholesterol)</th>
<th>Vit A (IU l⁻¹)</th>
<th>SOD (IU g⁻¹ Hb)</th>
<th>GPx (IU g⁻¹ Hb)</th>
<th>Se (μg l⁻¹)</th>
<th>Cu (mg l⁻¹)</th>
<th>Zn (mg l⁻¹)</th>
<th>GSH (μmol l⁻¹)</th>
<th>GSSG (μmol l⁻¹)</th>
<th>GRR (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sampling time</td>
<td>Associated SEM</td>
<td>± 2.01 ± 11.2</td>
<td>± 0.31 ± 0.15 ± 25 ± 77 ± 17 ± 12 ± 0.04 ± 0.02 ± 32 ± 5.8 ± 0.6</td>
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</tr>
<tr>
<td>R</td>
<td>16.6</td>
<td>9.9</td>
<td>4.04</td>
<td>2.31</td>
<td>1031</td>
<td>1366</td>
<td>201</td>
<td>137</td>
<td>1.06</td>
<td>0.46</td>
<td>958</td>
<td>23.2</td>
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<tr>
<td>Emax</td>
<td>18.9</td>
<td>24.3</td>
<td>4.15</td>
<td>2.29</td>
<td>974*</td>
<td>1261</td>
<td>175</td>
<td>135</td>
<td>1.12</td>
<td>0.48</td>
<td>577*</td>
<td>23.7</td>
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<td>E15</td>
<td>20.6*</td>
<td>95.1*</td>
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<td>2.21</td>
<td>1024</td>
<td>1298</td>
<td>180</td>
<td>133</td>
<td>1.09</td>
<td>0.45</td>
<td>833*</td>
<td>30.5</td>
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<td>E60</td>
<td>20.0*</td>
<td>114.3*</td>
<td>3.97</td>
<td>2.24</td>
<td>1043</td>
<td>1459</td>
<td>190</td>
<td>132</td>
<td>1.07</td>
<td>0.42</td>
<td>1021</td>
<td>15.7</td>
<td>1.4</td>
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<tr>
<td>SET</td>
<td>Associated SEM</td>
<td>± 2.1 ± 11.9 ± 0.25 ± 0.14 ± 27 ± 83 ± 18 ± 10 ± 0.04 ± 0.02 ± 34 ± 6.2 ± 0.66</td>
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<tr>
<td>T0</td>
<td>16.6</td>
<td>27.14</td>
<td>4.3</td>
<td>2.43</td>
<td>996</td>
<td>854</td>
<td>158</td>
<td>109</td>
<td>0.98</td>
<td>0.47</td>
<td>887</td>
<td>33.1</td>
<td>3.6</td>
</tr>
<tr>
<td>T4</td>
<td>13.6</td>
<td>27.8</td>
<td>3.81*</td>
<td>2.15*</td>
<td>1021</td>
<td>1299*</td>
<td>202a</td>
<td>152a</td>
<td>1.15a</td>
<td>0.47</td>
<td>794*</td>
<td>7.2a</td>
<td>1.4a</td>
</tr>
<tr>
<td>TTF4</td>
<td>19.0b</td>
<td>63.1b</td>
<td>3.91</td>
<td>2.21</td>
<td>1028</td>
<td>1287</td>
<td>165b</td>
<td>139</td>
<td>1.14</td>
<td>0.41b</td>
<td>709b</td>
<td>11</td>
<td>1.9</td>
</tr>
<tr>
<td>T12</td>
<td>19.1</td>
<td>54.22a</td>
<td>3.73a</td>
<td>2.11a</td>
<td>1055</td>
<td>1710a</td>
<td>199a</td>
<td>139a</td>
<td>0.94</td>
<td>0.49</td>
<td>951</td>
<td>39</td>
<td>3.9</td>
</tr>
<tr>
<td>TTF12</td>
<td>27.1c</td>
<td>132.2c</td>
<td>3.2</td>
<td>1.81</td>
<td>988c</td>
<td>1618a</td>
<td>209</td>
<td>140</td>
<td>0.96</td>
<td>0.46</td>
<td>895c</td>
<td>26</td>
<td>2.8</td>
</tr>
<tr>
<td>Time x SET interaction</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
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<td>*</td>
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</tr>
</tbody>
</table>

Vit A = vitamin A; SOD = superoxide dismutase; GPx = glutathione peroxidase; Se = selenium; Cu = copper; Zn = zinc; GSH = reduced glutathione; GSSG = oxidized glutathione; GRR = glutathione redox ratio (GSSG/[GSH + GSSG]); T0 = SET before exercising period; T4 = SET after 4 weeks of light exercise; TTF4 = SET to fatigue realized 48 h after T4; T12 = SET after 8 weeks of interval training; TTF12 = SET to fatigue realized 48 h after T12. For time effect analysis: *, significantly different from value at R. For test effect analysis: a, significantly different from T0, a, significantly different from T0 and T4; b, significantly different from T4; c, significantly different from T12. Time x SET interactions are shown in the last line. All P < 0.05.
Training, exercise and blood antioxidant markers in horses

Effect of training on physiological variables and antioxidant markers

The impact of training was evaluated by determining antioxidant markers during and after a SET when the horses were completely untrained (T0), slightly trained (T4) and trained (T12). Similar to previous studies\textsuperscript{26-34,35}, VO$_{2\text{max}}$ was significantly increased after 8 weeks of interval training. For completeness of this study, it would have been interesting to record VO$_{2\text{max}}$, or at least a ’pseudo–VO$_{2\text{max}}$’ at T0. However, as the horses were untrained at T0, the determination of a real VO$_{2\text{max}}$ value would have been questionable because the animals were unable to keep a constant speed.

All antioxidant markers underwent training-related modifications (Table 3); however, it is worthwhile to remember that the least-square mean indicated for each marker at the different SETs takes into account the variations that occurred between R, Emax, E15 and E60 and does not correspond to the value at R. Accordingly, a variation of a SET-associated value in the absence of time-related changes indicates a pure training effect ($\alpha$-tocopherol, Vit A, SOD, GPx, Se, Zn and Cu), whereas changes of both time- and test-related values imply an interaction between both effects (UA, AA, GSH, GSSG, GRR).

The increase during the tests in UA can be attributed to training, as already described by Essen-Gustavsson et al.\textsuperscript{36}. Training-related increases of AA have also been described in humans\textsuperscript{37}, as well as an increase of GPx and SOD activity in man\textsuperscript{38-41} and laboratory animals\textsuperscript{42,43}. It is interesting to point out that SOD activity was increased by 100% within 12 weeks, suggesting that this enzyme is a sensitive marker of repeated exercise, but does not respond within 60 min to a single exercise bout (values at R, Emax, E15 and E60 being similar during SET). A similar benefit of training on SOD activity in skeletal muscle has been reported after 12 weeks of physical training in human patients suffering from chronic heart failure. In this study, not only SOD and GPx activities were increased, but also the expression of their respective genes\textsuperscript{44}.

The observed increase of Se at T4 and T12 remains difficult to explain because the horses were fed in an identical manner for at least 4 weeks prior to the protocol, as well as throughout the whole study. Therefore, diet-related changes leading to an increased Se uptake were not expected. Regarding the decrease of $\alpha$-tocopherol at T4 and T12, the turnover of this lipophilic antioxidant necessary for membrane protection etc. might be increased by repeated exercise\textsuperscript{50} and training\textsuperscript{57}. It would be interesting to test whether $\alpha$-tocopherol supplementation allows a training-related decrease to be prevented.
**Effect of exercise intensity during training**

The impact of exercise intensity and training was evaluated by performing TTF4 and TTF12, both SETs including a fifth step at a speed of 11 m s⁻¹, which was maintained as long as the horse did not show signs of fatigue. Although somewhat subjective, running time at T12 was significantly higher than at T4, which was in agreement with other indices of training, such as increase of VO₂max and LA (Table 2). Increased VO₂max indicates an improvement of the aerobic metabolism, whereas increased LA values determined at run up to fatigue are suggestive of an increased anaerobic metabolism. Among the antioxidant markers, UA underwent significant exercise intensity-related changes as well as a training effect, the concentration at TTF4 and TTF12 being higher than at T4 and T12 and the concentration at T12 being higher than at T4. A similar, but less pronounced effect was observed for AA.

One of the best described correlations is that between VO₂max and LA (Table 2). Increased VO₂max indicates an improvement of the aerobic metabolism, whereas increased LA values determined at run up to fatigue are suggestive of an increased anaerobic metabolism. Among the antioxidant markers, UA underwent significant exercise intensity-related changes as well as a training effect, the concentration at TTF4 and TTF12 being higher than at T4 and T12 and the concentration at T12 being higher than at T4. A similar, but less pronounced effect was observed for AA.

The fact that TTF4 and TTF12 were systematically performed 48h after respectively, SET T4 and SET T12 introduced a bias complicating the interpretation of the results. Indeed, is it not known whether the blood antioxidant status of a healthy horse at 48 h post-SET is identical to that pre-SET. Deaton et al. observed significant modifications of plasma AA in antioxidant-complemented healthy horses at 24 h post-exercise. Another study performed in endurance horses showed that this type of exercise induced significant reductions of blood total GSH, GRR and AA for at least 16 h. It has furthermore been demonstrated that GPx activity is decreased after exercise in equine erythrocytes. On one hand, a carry-over effect of the first SET is expected and might have influenced the results of TTF(i) and this effect might have been even more important at TTF4 because the horses were only slightly trained. On the other hand, the present study design does allow us to identify whether a carry-over effect exists for some markers, which would not have been the case in a randomly assigned order of SET T(i) and TTF(i). An alternative might have been an increase of the time interval between SET T(i) and TTF(i), but this measure would have weakened the standardization of the thoroughly respected training programme.

A significant carry-over effect of the SET preceding the run up to fatigue test was observed for GPx and Zn, the test-associated least-square mean values of TTF4 being significantly lower than those of SET T4. Regarding GSH, the decrease observed at TTF4 and TTF12 was due to a pronounced decrease at Emax and E15, the values at R being similar to T4 and T12 (data not shown).

**Correlation analyses**

One of the best described correlations is that between plasma UA and plasma LA in racehorses. The same observation was made in the present study; the statistical approach was further improved by considering increases in plasma UA and increase of running time between SET TTF4 and SET TTF12.

The most interesting correlation found is certainly that between the increase of SOD activity determined at rest at T4 and T12 and the increase of corresponding VO₂max values (Fig. 1). Indeed, to the best of our knowledge, this is the first time that a positive correlation between an antioxidant marker and a valuable indicator of aerobic performance has been described in the equine species. A similar correlation has been shown between SOD activity in muscle biopsies and VO₂max in human athletes. Tessier et al. demonstrated a positive correlation between VO₂max and GPx in humans. The increase of the enzymatic antioxidant activity by repeated exercise is due to enhanced transcription of antioxidant genes by activation of redox-sensitive pathways, contributing thereby to an increased tolerance of exercise-induced oxidative burden, as occurring when VO₂max increases. Owing to red cell turnover and the absence of a cell nucleus in erythrocytes, it is possible that a training-related increase of SOD activity in erythrocytes is delayed in comparison to that occurring in skeletal muscle. Nevertheless, our results show that an increase of red blood cell SOD activity also parallels an increase of VO₂max. Therefore, following blood SOD activity appears as an interesting indicator of performance during training of healthy horses.

**Conclusion**

In summary, it has been shown in this study that a single exercise test did significantly affect the antioxidant markers AA, UA, Vit A, GSH and GRR% in healthy Standardbred horses, whereas α-tocopherol, SOD, GPx, Se, Cu, Zn and GSSG remained unchanged. The antioxidant markers UA, SOD, GPx, Se and α-tocopherol underwent training-related modifications and a prolonged effect (lasting at least 48 h) of a single exercise test was shown for GPx and Zn. The positive and significant correlation between increase in VO₂max and SOD activity suggests that increased activity of this antioxidant enzyme might be a potential indicator of an improved training level.

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