



# OPEN The effect of long term astaxanthin supplementation on the antioxidant status of racing Arabian horses – preliminary study

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Astaxanthin due to its strong antioxidant activity is believed to reduce oxidative stress and therefore is considered as feed additive in pathological conditions and also for the athletes. It is promoted by several equine web portals, however, data supporting that concept in horses is limited. Thus, the aim of this study was to evaluate the effect of astaxanthin supplementation on the parameters of oxidative status in 3 years old, racing Arabian horses during long term observation and the changes related to a single training session of high intensity. Six horses were supplemented with astaxanthin at a dose of 0.52–0.58 mg/kg BW and 7 received no supplementation. Astaxanthin supplementation resulted in the increase in total antioxidant status by 31.5%, accompanied by decreases in the amount of total thiobarbituric acid-reactive substances -TBARS and glutathione reductases - GR values by 34.5% and 45.4%, respectively, after 1 month and this effect persisted until the end of the observation. After individual training session the activities of glutathione peroxidases and GR were lower by 69% and 46%, respectively, and TBARS lower by 38% in supplemented horses. These results directly confirmed the beneficial effects of astaxanthin supplementation on the antioxidant status of race horses. Astaxanthin partially counterbalance the training-related oxidative stress, save the horse natural antioxidant defense, and shift the redox status towards a more reducing environment. At the same time, exercise-induced reactive oxygen species production at certain level was maintained and so that contributed to training progress.

**Keywords** Race horses, Training, Oxidative stress, Antioxidants, Dietary supplementation

## Abbreviations

BAS	Basophil count
CAT	Catalases
CK	Creatine kinase
dROMs	Reactive oxygen metabolites
EOS	Eosinophil count
GPx	Glutathione peroxidases
GR	Glutathione reductases

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HCT	Hematocrit
HGB	Hemoglobin concentration
LA	Lactic acid
LDH	5-lactate dehydrogenase isoenzyme-5
LYM	Lymphocyte count
MDA	Malondialdehyde
MON	Monocyte count
NEU	Neutrophil count
PLT	Platelet count
RBC	Red blood cell count
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
SOD	Dismutases
TAC	Total antioxidant capacity
TAS	Total antioxidant status
TBA	Thiobarbituric acid
TBARS	The amount of total thiobarbituric acid-reactive substances
WBC	White blood cell count

Oxidative stress is defined as an imbalance between increased levels of reactive oxygen species (ROS) generated during aerobic metabolism which cannot be balanced by the activity of antioxidant mechanisms<sup>1–4</sup>. Muscle tissue in both human and equine athletes is particularly vulnerable to ROS-induced damage due to the very rapid metabolism of muscle cells aimed at producing high amount of energy during exercise. Performance horses, regardless of the discipline, undergo this process even in anaerobic efforts e.g. short time racing when the oxygen consumption increases even 30-fold above the basal level<sup>5</sup>. Under normal physiological conditions, cells maintain redox homeostasis by generating and eliminating free radicals including ROS. This can occur through intracellular enzymatic antioxidant mechanisms or through the action of antioxidants. The enzymatic antioxidants include the superoxide dismutases (SOD), catalases (CAT), glutathione peroxidases (GPx), and reductases (GR). In the process of training of performance horses, oxidative stress is somewhat desirable, as moderate ROS production during exercise promotes positive physiological adaptations in active skeletal muscles (e.g. mitochondrial biogenesis, synthesis of antioxidant enzymes and stress proteins)<sup>4</sup>. This is necessary to achieve a high fitness level and the ability to participate in sports competitions. On the other hand, excessive training that does not match the age and condition of the animal has negative consequences and leads to the decrease of athletic performance. Oxidative stress and antioxidant response has been reported both after single bout of strenuous exercise in endurance horses that undergo aerobic effort<sup>5–7</sup> and in race horses that undergo anaerobic effort<sup>8–10</sup>. Training has been shown to affect both, the oxidative stress and antioxidant status, however, routine exercise does not seem to produce the sufficiently high level of oxidative stress to induce considerable damage of muscle cells<sup>8,11,12</sup>. Regardless of the experimental design, the studies indicate that proper nutrition is critical for the welfare of performance horses, including the reduction of oxidative stress.

Astaxanthin, a red pigment commonly extracted from various microorganisms and marine algae such as *Haematococcus pluvialis* or the yeast *Phaffia rhodozyma*, has emerged as a promising new antioxidant that may be beneficial in preventing and/or reducing the risk of negative consequences of oxidative stress-induced cell and tissue damage<sup>13</sup>. Chemically, astaxanthin (3,3'-dihydroxy- $\beta$ -carotene-4,4'-dione, C<sub>40</sub>H<sub>52</sub>O<sub>4</sub>) belongs to the xanthophyll family, the oxygen derivatives of carotenoids. It has 13 conjugated double bonds that are perfectly symmetrical with respect to the 15–15' position, and the backbone is an unsaturated hydrocarbon chain of 40 carbon atoms in length. The most noteworthy feature of astaxanthin's chemical structure is the presence of oxygen atoms in the tetraterpene chain and the conjugated double bonds. This structure is responsible for the molecule's strong antioxidant properties and remarkable polarity<sup>14</sup>. Compared to other carotenoids such as  $\beta$ -carotene and lycopene, astaxanthin has better bioavailability thanks to its amphipathic properties<sup>15</sup>. It can integrate into the lipid bilayer of cell membranes and affect their properties and functions. In fact, astaxanthin is able to quench and scavenge ROS including hydrogen peroxide, superoxide anion, singlet oxygen, etc. in both the inner and outer layers of cell membranes, demonstrating its unique potential compared to other antioxidants. In contrast, ascorbic acid (vitamin C) acts only on the outer layer of cell membranes, whereas  $\beta$ -carotene or  $\alpha$ -tocopherol (vitamin E) act on the inner layer<sup>13</sup>. This explains a high bioactive potential of astaxanthin. Astaxanthin is thought to have a potential antioxidant activity 10-fold higher than  $\beta$ -carotene and 100 times higher than  $\alpha$ -tocopherol<sup>16</sup>. Many reports have already confirmed the beneficial pharmacological properties of astaxanthin not only in terms of its antioxidant activity, but also its anti-inflammatory<sup>17</sup>, immunostimulant, anti-cancer<sup>18</sup>, and anti-diabetic<sup>19</sup> properties. To date most research on astaxanthin supplementation have been conducted to investigate its potential use in humans, including the use of its antioxidant properties to reduce the negative effects of oxidative stress in human athletes<sup>20,21</sup>. Previous research conducted in both in vitro cell-cultures and in vivo animal models provide some evidence to support the use of astaxanthin as a dietary supplement for both athletes and recreationally active people. According to the pharmacological models, metabolism, performance and recovery should improve after 3–5 weeks of intake<sup>20</sup>. However, a recent report found no effect of 4 weeks of astaxanthin supplementation on the markers of muscle damage or soreness in resistance-trained males<sup>22</sup>.

In animal nutrition, astaxanthin-rich algae *H. pluvialis* have been used as feed additives to improve the quality of animal products and the European regulations authorized its consumption up to a certain degree<sup>23,24</sup>. Recently, astaxanthin has achieved the status of a 'super nutrient' and is the subject of an increasing number of scientific studies<sup>14,25</sup>. Its use in animals particularly susceptible to oxidative stress associated with intense skeletal muscle metabolism such as performance horses is a now matter of lively discussion. So far several combinations

of antioxidant vitamins (mainly vitamin E and C), trace elements (zinc, copper, and selenium), SOD and ergothioneine have been used in horses<sup>26–31</sup> and their effects are generally viewed as beneficial.

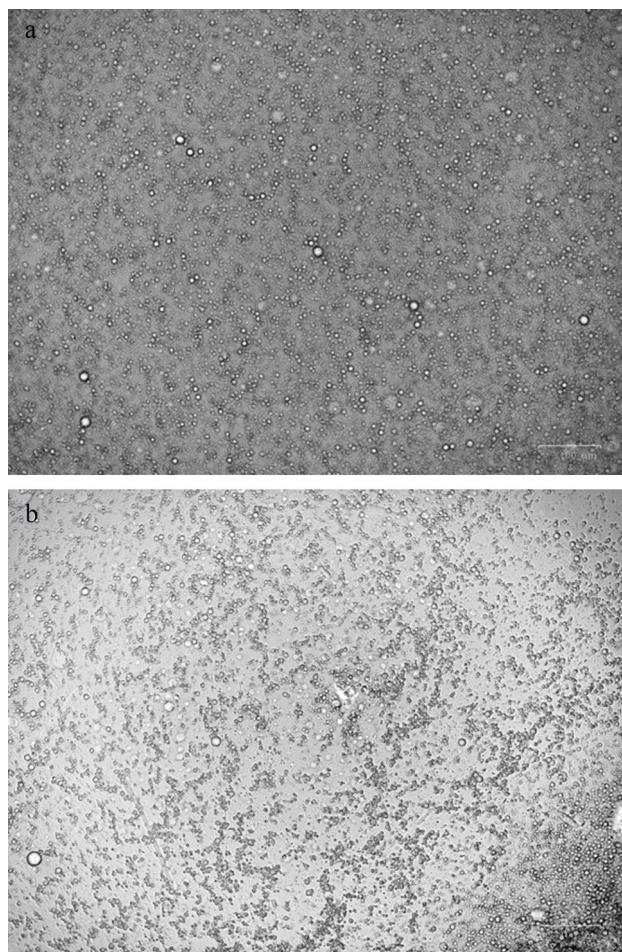
Astaxanthin is recommended as a supplement for sport horses by several equine web portals, but the hypothesis regarding its effect has been studied only indirectly, in combination with L-carnitine, based on the activities of creatine kinase (CK) and lactate dehydrogenase isoenzyme-5 (LDH-5) in blood<sup>32</sup>. Direct effect of astaxanthin on oxidative stress in horses has never been studied. Therefore, this study aimed to assess the effect of micellar form of astaxanthin administered to Arabian race horses at a dose of 250 mg per a horse (0.52–0.58 mg/kg BW) on the parameters of oxidative status during long-term observation and the changes related to a single training session of high intensity. To formulate the first broad characteristics, the panel of parameters of oxidative stress was used: the total antioxidant status (TAS), the amount of total thiobarbituric acid-reactive substances (TBARS) and blood activities of the enzymes involved in antioxidant defense such as GPx, GR, SOD, and CAT.

## Results

The astaxanthin content in the administered supplement was  $0.1520 \pm 0.0052$  µg/mg (coefficient of variation [CV%] of 3.42%) and total carotenoids (as β carotene) content was  $0.9889 \pm 0.0485$  µg/mg (CV% of 4.90%). After a monthly storage of the micelles, no separation of the water and oil phases could be observed. Additionally, imaging with the ZOE™ Fluorescent Cell Imager confirmed the stability of the micelles (Fig. 1) on the day of micelle preparation (Fig. 1a) and after 1 month (Fig. 1b).

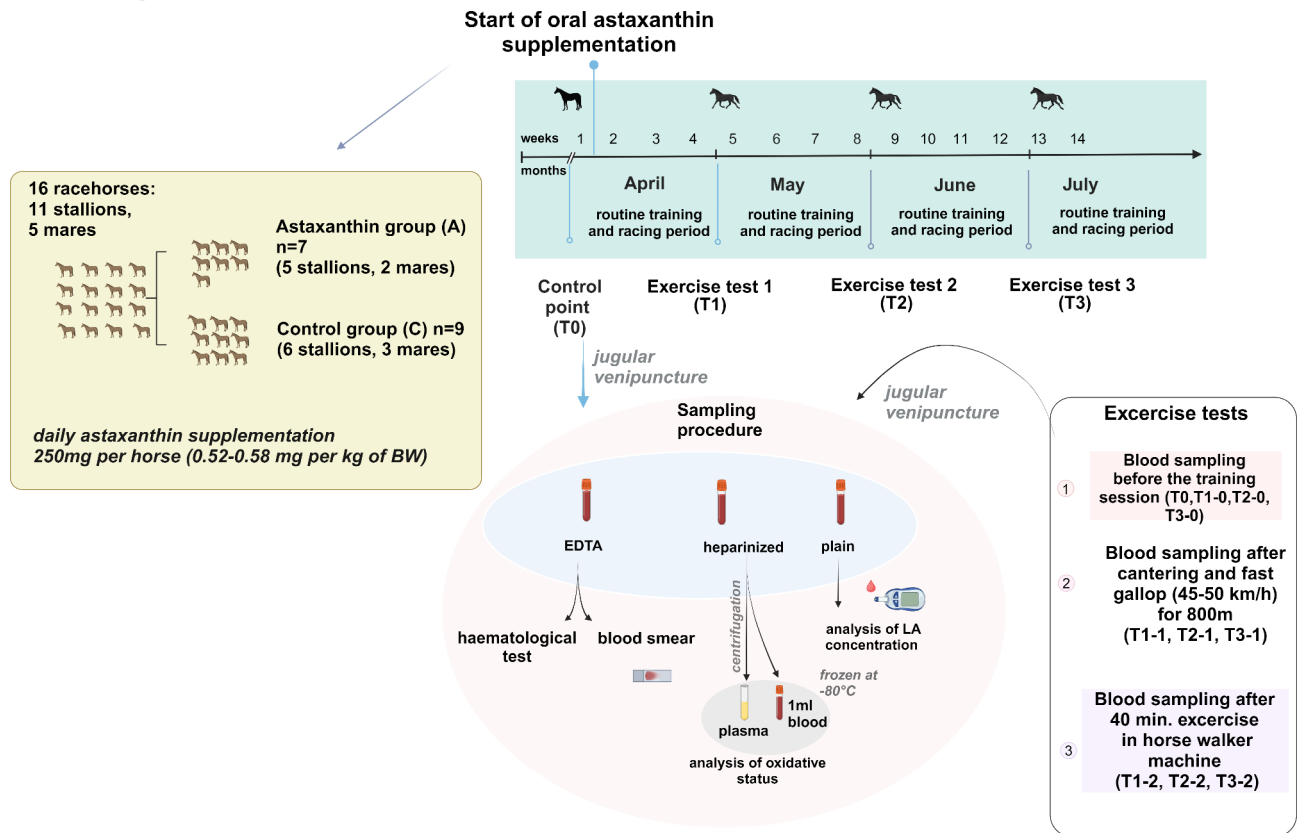
Evaluation of the long-term effect of astaxanthin supplementation (Fig. 2) on the baseline oxidative status, involved examination of horses (including blood tests) at four time-points: in April just before the beginning of intensive training sessions and astaxanthin supplementation (this testing denoted as T0), and then monthly before the intensive training session (pre-session) – in May (after 1 month of training, T1-0), June (after 2 months of training, T2-0), and in July (after 3 months of training, T3-0).

To evaluate the effect of astaxanthin supplementation (Fig. 2) on oxidative status during a single training session, in May the horses were subjected to the field exercise test including blood tests 2 more times: right after the training session involving fast gallop (T1-1), and after 40-min. restitution in the horse walker (T1-2).



**Fig. 1.** Visualization of micelles, (a) day 1, (b) day 30.

# Study scheme



**Fig. 2.** Study design. Created with BioRender.com.

Of the initial number of 16 Arabian horses (11 stallions and 5 mares), 2 stallions and 1 mare were excluded due to the accidental contusions that interrupted their training, so finally the astaxanthin (A) group consisted of 6 horses (4 stallions and 2 mares) and the control (C) group consisted of 7 horses (5 stallions and 2 mares). There was no difference in sex distribution between groups ( $p = 0.999$ ). In June and July, the horses were already involved in racing, so the analyses were performed on smaller number of animals: 11 horses (5 from A group and 6 from C group) in June (T2-0), and 9 horses (4 from A group and 5 from C group) in July (T3-0). The numbers of starts as well as the time of the first start and the numbers of won races were similar in both groups ( $p = 0.850$ ).

The increases in lactic acid (lactate, LA) concentration in blood after training sessions were significant ( $p = 0.001$ ) and similar in both groups ( $p = 0.210$ ) which confirmed that the effort was similar and relatively high for both groups.

The hematological parameters before entering the intensive training (T0) and before training sessions (T1-0, T2-0, and T3-0) fell within reference intervals for horses<sup>33</sup> and did not differ significantly between the A and C groups (Table 1). In both groups, erythrogram parameters i.e. red blood cell count (RBC), hemoglobin concentration (HGB), hematocrit (HCT) as well as white blood cell count (WBC) increased significantly after the training session (at T1-1;  $p \leq 0.001$  and  $p = 0.013$ , respectively) and reverted to the baseline value after 40-min. restitution in horse walker (at T1-2; Table 2).

## Long-term effect of astaxanthin supplementation on the oxidative status

None of resting oxidative status measurements in April (before supplementation) differed significantly between groups (Table 3). The long-term effect of astaxanthin supplementation on the oxidative status measurements at rest involved significant increase in TAS, and significant decrease in GR and TBARS (Fig. 3).

TAS measurements in the C group remained unchanged compared to the resting (T0) value until July, when it increased, however, this change was not significant ( $p = 0.056$ ). In the A group, TAS increased after one month of supplementation and was significantly higher than in the C group on May ( $p < 0.001$ ) (Fig. 3a). GR activity remained unchanged in the C group for the whole study, with slight (insignificant) decrease in June. In the A group, GR activity markedly decreased ( $p < 0.001$ ) and remained lower than in the C group till the end of observation (Fig. 3b). GPx activity remained unchanged in the C group for the whole study, however, large variations among individuals were noted. In the A group, GPx activity decreased between resting (T0) and pre-session in May (T1-0), however the differences were not significant and the variations among individuals were considerable (Fig. 3c). SOD activity remained unchanged in the C group until July, when it increased significantly



Hematological measurement	Group	Time points at which hematological measurements were taken				Comparison between				Interaction between time and group	
						Time points (T0, T1-0, T2-0, T3-0)		Groups (A, C)			
		T0	T1-0	T2-0	T3-0	F-statistic (df <sub>1</sub> ,df <sub>2</sub> )	p-value	F-statistic (df <sub>1</sub> ,df <sub>2</sub> )	p-value	F-statistic (df <sub>1</sub> ,df <sub>2</sub> )	p-value
RBC [T/L]	A	10.0 (9.0, 10.9)	9.9 (9.0, 10.9)	10.0 (9.0, 10.9)	9.9 (8.9, 10.8)	0.72 (3,37)	0.545	0.04 (1,37)	0.839	0.82 (3,37)	0.490
	C	10.3 (9.5, 11.2)	10.3 (9.4, 11.1)	9.6 (8.6, 10.6)	10.1 (9.1, 11.0)						
	RSE	0.55	0.58	0.30	0.35						
HGB [g/dl]	A	14.7 (13.5, 15.9)	14.8 (13.7, 16.0)	14.8 (13.6, 16.0)	14.6 (13.4, 15.8)	0.70 (3,37)	0.560	1.22 (1,37)	0.729	0.67 (3,37)	0.577
	C	15.2 (14.1, 16.3)	15.4 (14.3, 16.4)	14.4 (13.1, 15.6)	14.9 (13.8, 16.1)						
	RSE	0.79	0.86	0.41	0.58						
HCT [%]	A	42 (39, 45)	41 (38, 44)	41 (38, 44)	40 (37, 44)	1.10 (3,37)	0.361	0.11 (1,37)	0.742	0.54 (3,37)	0.661
	C	43 (40, 46)	42 (39, 45)	40 (36, 43)	42 (38, 45)						
	RSE	2.6	3.0	1.3	1.6						
PLT [G/l]	A	187 (161, 213)	147 (121, 173)	82 (55, 110)	174 (146, 202)	24.86 (3,37)	<0.001*	1.33 (1,37)	0.256	1.49 (3,37)	0.233
	C	175 (151, 199)	113 (89, 137)	95 (64, 125)	149 (122, 177)						
	RSE	20.3	30.5	17.4	19.7						
WBC [G/l]	A	10.0 (9.1, 10.9)	8.9 (8.1, 9.8)	9.3 (8.3, 10.2)	9.4 (8.5, 10.4)	1.19 (3,37)	0.327	0.11 (1,37)	0.740	0.65 (3,37)	0.586
	C	9.5 (8.7, 10.3)	9.4 (8.6, 10.2)	9.0 (7.9, 10.0)	9.3 (8.3, 10.2)						
	RSE	0.85	1.06	0.68	0.42						
NEU [G/l]	A	4.9 (4.1, 5.7)	4.6 (3.8, 5.4)	4.8 (4.0, 5.6)	4.6 (3.8, 5.4)	0.08 (3,37)	0.972	2.36 (1,37)	0.133	0.72 (3,37)	0.549
	C	4.0 (3.3, 4.7)	4.1 (3.4, 4.8)	3.9 (3.0, 4.7)	4.3 (3.5, 5.1)						
	RSE	0.60	0.63	0.59	0.40						
LYM [G/l]	A	4.3 (3.7, 4.8)	3.6 (3.1, 4.2)	3.8 (3.2, 4.4)	4.0 (3.4, 4.6)	3.70 (3,37)	0.020*	3.26 (1,37)	0.079	1.07 (3,37)	0.373
	C	4.8 (4.3, 5.3)	4.5 (4.0, 5.0)	4.3 (3.7, 4.9)	4.3 (3.7, 4.8)						
	RSE	0.35	0.51	0.19	0.24						
MON [G/l]	A	0.60 (0.51, 0.69)	0.55 (0.46, 0.64)	0.50 (0.40, 0.60)	0.51 (0.41, 0.61)	2.07 (3,37)	0.121	0.04 (1,37)	0.839	1.28 (3,37)	0.297
	C	0.53 (0.44, 0.61)	0.56 (0.48, 0.65)	0.53 (0.43, 0.63)	0.49 (0.40, 0.59)						
	RSE	0.061	0.047	0.080	0.049						
EOS [G/l]	A	0.18 (0.11, 0.24)	0.12 (0.06, 0.19)	0.14 (0.07, 0.21)	0.26 (0.19, 0.33)	4.03 (3,37)	0.014*	4.53 (1,37)	0.040*	1.07 (3,37)	0.375
	C	0.12 (0.06, 0.18)	0.09 (0.03, 0.15)	0.09 (0.01, 0.16)	0.13 (0.06, 0.20)						
	RSE	0.035	0.021	0.043	0.111						
BAS [G/l]	A	0.05 (0.03, 0.07)	0.08 (0.06, 0.10)	0.07 (0.05, 0.09)	0.07 (0.05, 0.09)	5.53 (3,37)	0.003*	0.01 (1,37)	0.991	1.10 (3,37)	0.360
	C	0.06 (0.05, 0.08)	0.08 (0.06, 0.10)	0.06 (0.04, 0.08)	0.08 (0.06, 0.10)						
	RSE	0.011	0.016	0.008	0.013						

**Table 1.** Analysis of hematological measurements at rest using the mixed linear model. Hematological measurements are presented as the estimated marginal means with 95% confidence intervals (CI 95%) in parentheses and residual standard error (RSE). Hematological measurements were performed in April just before the beginning of intensive training sessions and astaxanthin supplementation (T0), and then monthly before the intensive training session (pre-session) – in May (after 1 month of training, T1-0), June (after 2 months of training, T2-0), and in July (after 3 months of training, T3-0). A – astaxanthin group, C – control group, RBC – red blood cell count, HGB – hemoglobin concentration, HCT – hematocrit, PLT – platelet count, WBC – white blood cell count, NEU – neutrophil count, LYM – lymphocyte count, MON – monocyte count, EOS – eosinophil count, BAS – basophil count, RSE – residual standard error, df<sub>1</sub> – degrees of freedom associated with the among-group sum of squares, df<sub>2</sub> – degrees of freedom associated with the within-group (error) sum of squares. \*Significant at  $\alpha = 0.05$ .

( $p < 0.001$ ), in contrast to the A group where it remained unchanged during whole time of observation, however, large variations among individuals were also noted (Fig. 3e). TBARS (Fig. 3d) remained unchanged in the C group with large variations among individuals. In the A group, TBARS decreased significantly one month after the beginning of astaxanthin supplementation ( $p < 0.001$ ) and remained lower than in the C group till the end of the observation. Neither time nor astaxanthin supplementation affected the CAT activity, however, the values largely varied among individuals.

### The effect of astaxanthin supplementation on oxidative status during a single training session

Significant changes occurred in all oxidative status measurements except CAT (Table 4). The training session was analyzed after one month of astaxanthin supplementation and therefore the measurements before training session already differed significantly between groups. TAS before the training was significantly higher in the A

Hematological measurement	Group	Time points at which hematological measurements were taken			Mauchly's test for sphericity ( <i>p</i> -value)	Comparison between				Interaction between time and group	
		T1-0	T1-1	T1-2		Time points (T1-0, T1-1, T1-2)		Groups (A, C)		F-statistic (df <sub>1</sub> ,df <sub>2</sub> )	<i>p</i> -value
						F-statistic (df <sub>1</sub> ,df <sub>2</sub> )	<i>p</i> -value	F-statistic (df <sub>1</sub> ,df <sub>2</sub> )	<i>p</i> -value		
RBC [T/l]	A	10.1 (9.0, 11.1)	12.1 (10.8, 13.5)	10.3 (9.4, 11.1)	0.169	16.22 (2,20)	< 0.001*	0.31 (1,10)	0.589	0.55 (2,20)	0.585
	C	10.3 (9.4, 11.1)	11.7 (10.6, 12.9)	9.7 (9.0, 10.4)							
	RSE	1.04	1.37	0.86							
HGB [g/dl]	A	15.1 (13.7, 16.4)	18.5 (16.4, 20.6)	15.5 (14.3, 16.7)	0.148	17.30 (2,20)	< 0.001*	0.57 (1,10)	0.469	0.64 (2,20)	0.540
	C	15.4 (14.2, 16.5)	17.8 (16.0, 19.6)	14.5 (13.5, 15.5)							
	RSE	1.35	2.13	1.16							
HCT [%]	A	41 (37, 46)	54 (47, 62)	43 (40, 46)	0.032*	18.0 (1.30,13.0) <sup>a</sup>	0.001*	1.05 (1,10)	0.330	0.80 (1.30,13.0) <sup>a</sup>	0.421
	C	42 (39, 46)	50 (44, 57)	40 (37, 42)							
	RSE	4.2	7.5	3.0							
PLT [G/l]	A	147 (106, 187)	140 (110, 170)	154 (118, 190)	0.800	2.46 (2,20)	0.111	0.46 (1,10)	0.515	1.73 (2,20)	0.202
	C	113 (79, 147)	141 (115, 167)	152 (121, 182)							
	RSE	40.5	30.4	36.2							
WBC [G/l]	A	9.0 (7.7, 10.4)	11.2 (9.5, 12.9)	9.9 (8.8, 11.0)	0.019*	7.61 (1.26,12.6) <sup>a</sup>	0.013*	0.08 (1,10)	0.780	0.52 (1.26,12.6) <sup>a</sup>	0.528
	C	9.4 (8.3, 10.5)	10.7 (9.2, 12.1)	9.6 (8.7, 10.5)							
	RSE	1.31	1.72	1.07							
NEU [G/l]	A	4.8 (4.0, 5.6)	5.1 (4.1, 6.1)	5.6 (4.5, 6.6)	0.008*	2.96 (1.20,12.0) <sup>a</sup>	0.107	7.86 (1,10)	0.100	1.36 (1.20,12.0) <sup>a</sup>	0.276
	C	4.1 (3.4, 4.8)	4.2 (3.4, 5.1)	4.3 (3.4, 5.2)							
	RSE	0.83	0.99	1.06							
LYM [G/l]	A	3.5 (2.6, 4.3)	5.2 (4.2, 6.2)	3.7 (3.0, 4.3)	0.007*	14.8 (1.20,12.0) <sup>a</sup>	0.002*	5.12 (1,10)	0.047*	0.58 (1.20,12.0) <sup>a</sup>	0.492
	C	4.5 (3.8, 5.2)	5.7 (4.8, 6.6)	4.6 (4.1, 5.1)							
	RSE	0.86	1.04	0.61							
MON [G/l]	A	0.57 (0.46, 0.68)	0.71 (0.60, 0.83)	0.48 (0.38, 0.58)	0.312	8.86 (2,20)	0.002*	0.39 (1,10)	0.547	3.76 (2,20)	0.041*
	C	0.56 (0.47, 0.66)	0.58 (0.48, 0.67)	0.52 (0.44, 0.61)							
	RSE	0.111	0.117	0.101							
EOS [G/l]	A	0.13 (0.08, 0.17)	0.12 (0.08, 0.15)	0.12 (0.09, 0.15)	0.936	0.12 (2,20)	0.890	1.92 (1,10)	0.196	1.31 (2,20)	0.293
	C	0.09 (0.05, 0.13)	0.10 (0.07, 0.12)	0.09 (0.06, 0.12)							
	RSE	0.044	0.034	0.035							
BAS [G/l]	A	0.09 (0.06, 0.11)	0.08 (0.05, 0.1)	0.06 (0.04, 0.08)	0.176	5.29 (2,20)	0.014*	0.03 (1,10)	0.867	2.11 (2,20)	0.147
	C	0.08 (0.06, 0.10)	0.08 (0.06, 0.1)	0.07 (0.05, 0.09)							
	RSE	0.027	0.026	0.023							
LA [mmol/l]	A	0.05 (0.05, 0.05)	17.3 (8.12, 26.5)	1.79 (0.42, 3.16)	< 0.001*	23.2 (1.02,10.2) <sup>a</sup>	0.001*	1.79 (1,10)	0.210	2.57 (1.02,10.2) <sup>a</sup>	0.140
	C	0.05 (0.05, 0.05)	9.12 (1.35, 16.9)	1.87 (0.72, 3.03)							
	RSE	0.000	9.234	1.370							

**Table 2.** Analysis of hematological measurements using repeated-measure analysis of variance. Hematological measurements are presented as the estimated marginal means with 95% confidence intervals (CI 95%) in parentheses and residual standard error (RSE). Hematological measurements were performed right after the training session involving fast gallop (T1-1), and after 40-min. restitution in the horse walker (T1-2). A – astaxanthin group, C – control group, RBC – red blood cell count, HGB – hemoglobin concentration, HCT – hematocrit, PLT – platelet count, WBC – white blood cell count, NEU – neutrophil count, LYM – lymphocyte count, MON – monocyte count, EOS – eosinophil count, BAS – basophil count, LA – lactate concentration, RSE – residual standard error, df<sub>1</sub> – degrees of freedom associated with the among-group sum of squares, df<sub>2</sub> – degrees of freedom associated with the within-group (error) sum of squares. <sup>a</sup> Greenhouse–Geisser correction for sphericity applied. \*Significant at  $\alpha = 0.05$ .

group ( $p = 0.024$ ), increased significantly immediately after the training in both groups ( $p < 0.001$ ), reaching similar values in both groups and then decreased after 40-min. restitution, remaining significantly higher in the A group ( $p < 0.001$ , Fig. 4a). GR activity was significantly lower in the A group ( $p < 0.001$ ) before the training (pre-session, T1-0), then slightly increased in both groups remaining significantly lower in the A group ( $p = 0.002$ ) and after restitution decreased to similar values in both groups (Fig. 4b). GPx activity was at similar level in both groups before the training and after exercise it increased significantly only in the C group ( $p < 0.001$ ) being also significantly higher than in the A group ( $p < 0.001$ ) and decreased after restitution to similar level in both groups (Fig. 4c). SOD activity remained unaffected by training except for restitution value, which decreased significantly in the A group ( $p = 0.007$ ), being also significantly lower than in the C group ( $p < 0.001$ ) (Fig. 4e). TBARS remained unaffected by the training effort, however was significantly lower in the A group in all time

Oxidative status measurements	Group	Time points at which oxidative status measurements were taken				Comparison between				Interaction between time and group	
						Time points (T0, T1-0, T2-0, T3-0)		Groups (A, C)			
		T0	T1-0	T2-0	T3-0	F-statistic (df <sub>1</sub> ,df <sub>2</sub> )	p-value	F-statistic (df <sub>1</sub> ,df <sub>2</sub> )	p-value	F-statistic (df <sub>1</sub> ,df <sub>2</sub> )	p-value
TAS [μmol/l]	A	78.9 (67.3, 90.5)	103.8 (92.2, 115)	93.6 (82.0, 105)	116 (103, 130)	12.36 (3,39)	< 0.001*	11.01 (1,39)	0.002*	1.63 (3,39)	0.197
	C	71.0 (60.3, 81.8)	74.9 (64.2, 85.7)	77.3 (65.8, 88.8)	98.1 (85.6, 111)						
	RSE	12.13	6.01	15.27	8.24						
TBARS [nmol/l]	A	8.27 (7.30, 9.25)	5.42 (4.45, 6.39)	5.33 (4.36, 6.31)	5.31 (4.12, 6.50)	6.72 (3,39)	< 0.001*	12.86 (1,39)	< 0.001*	5.13 (3,39)	0.004*
	C	7.49 (6.59, 8.39)	7.64 (6.74, 8.54)	6.31 (5.34, 7.29)	8.00 (6.93, 9.07)						
	RSE	1.015	1.107	1.368	1.139						
GR [U/l]	A	341 (306, 376)	186 (152, 221)	207 (173, 242)	217 (175, 260)	11.50 (3,39)	< 0.001*	29.04 (1,39)	< 0.001*	10.84 (3,39)	< 0.001*
	C	315 (283, 347)	328 (296, 360)	272 (238, 307)	337 (299, 374)						
	RSE	32.62	37.53	44.17	38.91						
GPx [U/l]	A	326 (282, 370)	258 (214, 302)	271 (228, 315)	258 (205, 310)	0.77 (3,39)	0.520	2.47 (1,39)	0.124	2.67 (3,39)	0.061
	C	296 (255, 336)	317 (277, 358)	302 (259, 346)	323 (276, 370)						
	RSE	44.93	39.56	46.68	36.75						
SOD [U/l]	A	2.71 (2.42, 3.00)	2.48 (2.20, 2.77)	2.56 (2.28, 2.85)	2.98 (2.65, 3.30)	12.32 (3,39)	< 0.001*	2.24 (1,39)	0.143	2.41 (3,39)	0.081
	C	2.69 (2.42, 2.95)	2.85 (2.58, 3.11)	2.70 (2.42, 2.98)	3.45 (3.15, 3.74)						
	RSE	0.238	0.171	0.216	0.194						
CAT [U/l]	A	11.3 (8.2, 14.3)	12.2 (9.2, 15.3)	11.6 (8.5, 14.6)	10.1 (6.80, 13.3)	2.10 (3,34)	0.118	7.10 (1,34)	0.012*	0.44 (3,34)	0.725
	C	15.7 (12.4, 19.0)	17.2 (13.9, 20.6)	17.6 (14.3, 21.0)	15.9 (12.6, 19.2)						
	RSE	1.29	0.98	1.68	2.28						

**Table 3.** Analysis of oxidative status measurements at rest using the mixed linear model. Oxidative status measurements are presented as the estimated marginal means with 95% confidence intervals (CI 95%) in parentheses and residual standard error (RSE). Oxidative status measurements were performed in April just before the beginning of intensive training sessions and astaxanthin supplementation (T0), and then monthly before the intensive training session (pre-session) – in May (after 1 month of training, T1-0), June (after 2 months of training, T2-0), and in July (after 3 months of training, T3-0). A – astaxanthin group, C – control group, TAS – total antioxidant status, TBARS – thiobarbituric acid-reactive substances, GR – glutathione reductases, GPx – glutathione peroxidases, SOD – superoxide dismutases, CAT – catalases, RSE – residual standard error, df<sub>1</sub> – degrees of freedom associated with the among-group sum of squares, df<sub>2</sub> – degrees of freedom associated with the within-group (error) sum of squares. \*Significant at α = 0.05.

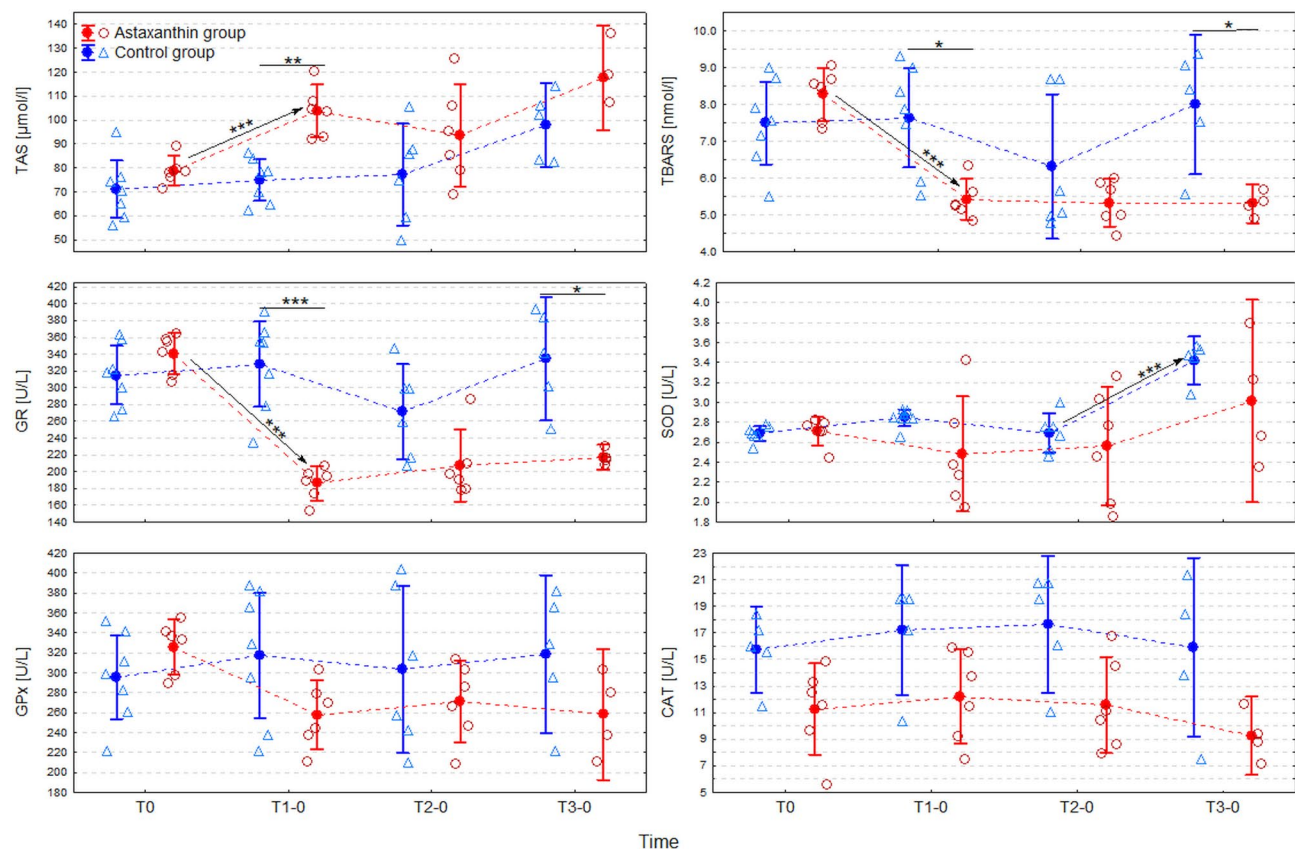
points due to its significant decrease between resting (T0) and pre-session testing in May (T1-0) ( $p = 0.004$ ) (Fig. 4d). CAT remained unaffected by training and at similar level in both groups (Fig. 4f).

Discussion

The beneficial effect of astaxanthin in the horses has been suggested by in vitro studies<sup>34</sup> and in vivo observations<sup>32</sup>, but these data did not show the direct antioxidant effect of supplementation. Moreover, the recommended dosage of commercially available supplements is wide. Sato et al.<sup>32</sup> administered to the horses supplement that contained 37.5 mg of astaxanthin, however, the manufacturer of AstaReal<sup>®</sup> mentioned the studies with 75 mg and 30–100 mg doses<sup>35</sup> and other company recommended much higher dose: 937.5 mg twice daily<sup>36</sup>. Astaxanthin has been proven to be safe and beneficial at very high doses: 50 mg/kg and even 200 mg/kg in rats<sup>37,38</sup>. Beagle dogs and cats were safely dosed with up to 40 mg and 10 mg, respectively, which corresponds to 3–4 mg/kg<sup>39</sup>. However, in the study lasting for 6 weeks the dose of 0.3 mg/kg was used<sup>40</sup>. In the present study the dose of 250 mg per horse was chosen, which on one hand, seems to be high enough to clearly evaluate the effect of astaxanthin supplementation and on the other hand completely safe in the 3 months supplementation period according to currently available data.

The presented results have shown the effect of astaxanthin supplementation on general antioxidant status and the antioxidant defense measured as TAS, the activities of antioxidant enzymes and lipid peroxidation reflected by TBARS as well as the effect of the high-intensity training session on these measurements. The study design allowed also to investigate the exercise-related changes in the level of antioxidant status related to the beginning of race training in Arabian horses without supplementation. Previously, antioxidant status in Arabian horses has been described only in endurance effort (aerobic in nature), so this study is the first report on antioxidant status in racing Arabians (anaerobic effort).

Many methods have been proposed for measuring exercise-induced stress, including the markers of lipid peroxidation, reactive oxygen metabolites, antioxidant potential/status, the antioxidant enzyme activity, and the muscle enzyme activity being an indirect measurement of oxidative damage<sup>5,9</sup>. The methods are limited by the study protocol, and the most common measurements are the activities of antioxidant enzymes and muscle enzymes in blood. However, the presence of reactive oxygen metabolites, expressed as reactive oxygen metabolites (dROMs) in blood, has also been shown as a result of exertion<sup>5,10</sup>.



**Fig. 3.** The long-term effect of astaxanthin supplementation on the parameters of oxidative status. Statistical significance is indicated above the lines as follows: \*  $0.01 \leq p < 0.5$ , \*\*  $0.001 \leq p < 0.01$ , \*\*\*  $p < 0.001$ .

The indirect beneficial effect of astaxanthin supplementation in race horses has been reported as a reduction of muscle enzymes creatine phosphokinase (CK) and lactate dehydrogenase (LDH-5) leakage into circulation<sup>32</sup>. Continuous astaxanthin supplementation for 8 weeks led to the decreases in CK and LDH activities measured 4 h after an intense training session. In this study, we looked deeper in the mechanisms by measuring antioxidant parameters which give the insight into the level of oxidative stress that occurs as a direct result of the training as well as single bout of exercise. The activities of muscle enzymes were not measured, as marked CK increases occur at least 2 h after the training session<sup>12,30,41</sup>. Instead we used the exercise test that allows to investigate the changes in post exercise LA concentrations and the decreases after 40-min. restitution. Compared to Sato et al.<sup>32</sup> findings, in our study the antioxidant effect of astaxanthin supplementation was observed earlier, after 4 weeks of supplementation and then remained at the similar level till the end of the observation period.

Changes of hematological measurements and LA concentration confirmed that the training session examined in this study posed a significant effort for horses, required for the training progress, however, the effort did not exceed exercise capacity of the horses, as indicated by the decreases after 40-min. restitution. The measurements of oxidative stress changed after single training session and during whole training period and the effect of astaxanthin supplementation was significant.

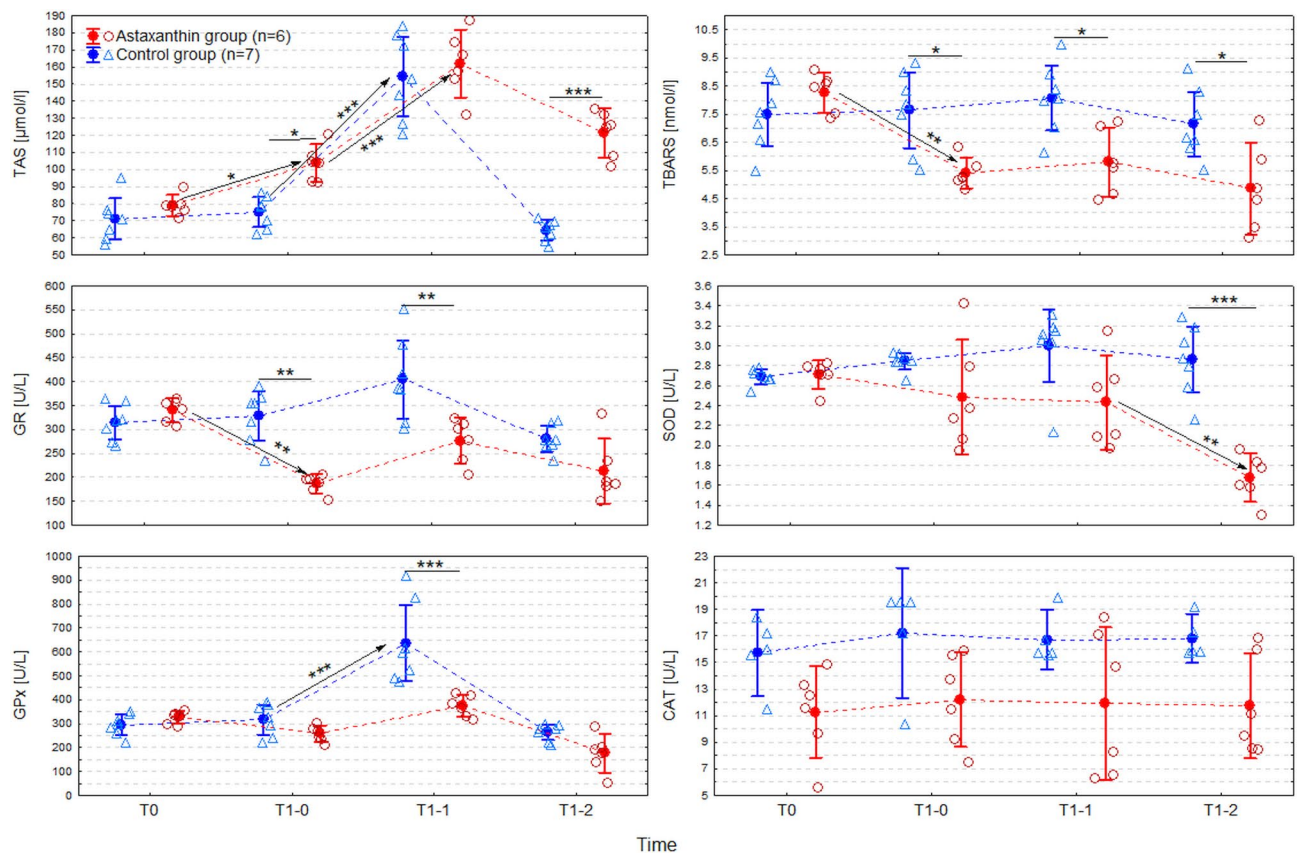
Higher TAS values observed one month after astaxanthin supplementation accompanied by lower TBARS indicate the improvement in ROS removal, and diminished level of lipid peroxidation. The oxidant/antioxidant imbalance phenomenon has been described in race horses as occurring during a 3-month race period<sup>27</sup>. The design of the present study covered this period and provided more details regarding the changes related to training and supplementation.

TAS or total antioxidant capacity (TAC) provide the information regarding overall antioxidant status, involving all the antioxidants in the body, including the ones not recognized yet or difficult to measure. It increased by 31.5% after one month of astaxanthin supplementation, together with the decrease in TBARS by 34.5%. The change was significant only in the 1st month, however, in both astaxanthin and control groups of horses a tendency to increase in TAS during 4 months of intensive training was seen with higher values in the supplemented group. Thus, long term changes related to the race training were proved, however, such changes were not detected in the horses that began moderate training<sup>42</sup>. It is expectable that supplementation with antioxidants should increase TAS and it was confirmed in our study. However, it has also been reported that 2 weeks of supplementation with vitamin E alone or in combination with coenzyme Q<sub>10</sub> did not affect either TAS at rest or after exercise in leisure horses<sup>29</sup>.



Oxidative status measurements	Group	Time points at which oxidative status measurements were taken					Mauchly's test for sphericity (p-value)	Comparison between			Interaction between time and group			
		Time points (T0, T1-0, T1-1, T1-2)						Groups (A, C)		p-value	F-statistic (df <sub>1</sub> ,df <sub>2</sub> )	p-value	F-statistic (df <sub>1</sub> ,df <sub>2</sub> )	p-value
		T0	T1-0	T1-1	T1-2	T1-2		F-statistic (df <sub>1</sub> ,df <sub>2</sub> )	p-value					
TAS [μmol/l]	A	78.9 (69.6, 88.2)	104 (94.8, 113)	162 (142, 182)	122 (112, 131)		0.043*	99.8 (1.80,19.8) <sup>a</sup>	28.6 (1.11)	<0.001*	9.95 (1.80,19.8)	0.001*	9.95 (1.80,19.8)	0.001*
	C	71.0 (62.4, 79.6)	74.9 (66.7, 83.2)	154 (136, 173)	64.4 (55.9, 73.0)									
	RSE	10.33	9.93	22.59	10.30									
TBARS [nmol/l]	A	8.27 (7.37, 9.18)	5.42 (4.40, 6.44)	5.81 (4.72, 6.90)	4.86 (3.60, 6.11)		0.344	5.86 (3.33)	19.0 (1.11)	0.003*	5.34 (3.33)	0.004*	5.34 (3.33)	0.004*
	C	7.49 (6.65, 8.33)	7.64 (6.70, 8.58)	8.07 (7.06, 9.08)	7.14 (5.98, 8.30)									
	RSE	1.006	1.132	1.214	1.394									
GR [U/l]	A	341 (312, 370)	186 (148, 225)	277 (212, 341)	213 (169, 257)		0.593	10.2 (3.33)	48.4 (1.11)	<0.001*	6.44 (3.33)	0.002*	6.44 (3.33)	0.002*
	C	315 (288, 342)	328 (293, 364)	405 (345, 465)	280 (240, 321)									
	RSE	32.17	42.71	72.16	48.57									
GPx [U/l]	A	326 (292, 360)	258 (209, 307)	375 (257, 493)	177 (124, 229)		0.028*	31.2 (1.68,18.4) <sup>a</sup>	18.0 (1.11)	0.001*	7.72 (1.68,18.4)	0.001*	7.72 (1.68,18.4)	0.001*
	C	296 (264, 327)	317 (273, 363)	636 (527, 745)	264 (216, 313)									
	RSE	37.96	54.77	131.2	58.14									
SOD [U/l]	A	2.71 (2.61, 2.81)	2.48 (2.14, 2.82)	2.43 (2.05, 2.81)	1.68 (1.40, 1.95)		0.273	5.44 (3.33)	34.0 (1.11)	0.004*	7.72 (3.33)	0.001*	7.72 (3.33)	0.001*
	C	2.69 (2.60, 2.78)	2.85 (2.53, 3.16)	3.00 (2.65, 3.35)	2.86 (2.61, 3.12)									
	RSE	0.108	0.376	0.420	0.307									
CAT [U/l]	A	11.3 (8.5, 14.0)	12.2 (8.9, 15.6)	11.9 (8.0, 15.8)	11.7 (9.0, 14.5)		0.196	0.55 (3.33)	7.60 (1.11)	0.655	0.04 (3.33)	0.989	0.04 (3.33)	0.989
	C	15.7 (12.7, 18.8)	17.2 (13.5, 20.9)	16.7 (12.4, 21)	16.8 (13.8, 19.8)									
	RSE	2.99	3.65	4.27	2.97									

**Table 4.** Analysis of oxidative status measurements using repeated-measure analysis of variance. Oxidative status measurements are presented as the estimated marginal means with 95% confidence intervals (CI 95%) in parentheses and residual standard error (RSE). Oxidative status measurements were performed right after the training session involving fast gallop (T1-1), and after 40-min. restitution in the horse walker (T1-2). A – astaxanthin group, C – control group, TAS – total antioxidant status, TBARS – thiobarbituric acid-reactive substances, GR – glutathione reductases, GPx – glutathione peroxidases, SOD – superoxide dismutases, CAT – catalases, RSE – residual standard error, df<sub>1</sub> – degrees of freedom associated with the among-group sum of squares, df<sub>2</sub> – degrees of freedom associated with the within-group (error) sum of squares. <sup>a</sup> Greenhouse–Geisser correction for sphericity applied. \*Significant at α = 0.05.



**Fig. 4.** The effect of astaxanthin supplementation on oxidative status during a single training session. Statistical significance is indicated above the lines as follows: \*  $0.01 \leq p < 0.5$ , \*\*  $0.001 \leq p < 0.01$ , \*\*\*  $p < 0.001$ .

Major antioxidant defense and so that antioxidant status is enzymatic. Antioxidant enzymes orchestrate in removing free radicals and in turn prevent lipid peroxidation. GR and GPx act as a system for reduction and peroxidation of glutathione disulfide, which allows to utilize hydrogen peroxide ( $H_2O_2$ ), and other organic hydroperoxides with the electron from glutathione. SOD acts synergistically with GPx by catalyzing the dismutation of the superoxide radical ( $O_2^-$ ) into molecular oxygen ( $O_2$ ) and  $H_2O_2$ , then converted by CAT into oxygen and water.

Results presented in this study show that GR activity significantly decreased by 45.4% one month after astaxanthin supplementation and remained lower than in control group by the end of the observation. GPx, SOD, and CAT activities also tended to be lower in astaxanthin-supplemented horses, but the differences were not significant. Studies report various patterns of the changes in antioxidant activities of enzymes in response to antioxidant supplementation, but the increases were interpreted as beneficial<sup>27</sup>. However, astaxanthin's mechanism of action is more complex than supplements tested in equine studies. Astaxanthin is a ROS scavenger acting by both donating electrons and by bounding free radicals and so that form a non-reactive product. Additionally, due to a unique chemical structure with a series of conjugated bounds in non-polar region, the molecule can remove free radicals by transporting them along its own carbon chain outside the cell where other antioxidant can neutralize them<sup>43</sup>. It seems that astaxanthin supplementation posed an additional background for removing free radicals and increasing the redox capacity, leading to higher TAS values. Training itself does not seem to affect the activities of antioxidant enzymes, except SOD which increased in July (after 4 months of training) in control group but not the group supplemented with astaxanthin. The patterns of changes in the activities of antioxidant enzymes in response to training in horses vary across the literature. The decreases in SOD and GPx activities in Thoroughbred horses have been reported in 6th and 12th weeks of training but GPx increased in the horses supplemented with antioxidant vitamins and trace elements<sup>27</sup>. In leisure horses subjected to training of moderate intensity SOD and GPx levels did not change<sup>29</sup> or varied, mostly decreased at the beginning of training and increased in 2nd (GPx) and 8th (SOD) weeks of exercise<sup>42</sup>. Human studies have shown that training, understood as repeating exposure to increased ROS levels leads to the upregulation of antioxidant defense including increasing SOD and GPx activities at rest<sup>44,45</sup>. Similar phenomenon may be expected in horses, however, it is likely dependent on the time and intensity of training. It should be strongly indicated that the training loads recommended for horses are much lower than recommended for humans, which can also influence the type, level and time of changes in antioxidant enzymes' activities.

The intensive training leads to ROS production which in turn may result in lipid peroxidation<sup>3</sup>. TBARS assay gives an insight into overall levels of oxidative stress due to the fact that it allows for the measurement

of many products of lipid peroxidation. Thiobarbituric acid (TBA) used in the assay bounds malondialdehyde (MDA), the most commonly measured product of lipid peroxidation but also other molecules derived from this process<sup>46</sup>. In the present study, TBARS was not affected by the training process, but significantly influenced by astaxanthin supplementation. It cannot be excluded that training related changes in lipid peroxidation occur earlier as it was shown in the 2nd week of moderate training in the horses that were not trained before<sup>42</sup>. The astaxanthin effect occurred after one month of administration when TBARS level was significantly lower by 34.5% in the horses supplemented with astaxanthin, then remained lower until the end of observation. Other studies regarding antioxidant supplementation have reported lower or no differences. After 30 days of vitamin E supplementation, MDA production did not change in Thoroughbred horses undergoing low-intensity exercise and interval training<sup>30</sup>. Ergothioneine administration for 4 weeks in Arabian stallions resulted in the reduction of MDA concentrations by 5%<sup>31</sup>. Unfortunately, singularly assessed MDA production cannot be directly compared to TBARS measurements and even TBARS assay results should not be compared across different laboratories due to possible pre-analytical differences and assay conditions that do not allow even minor variations in the protocol<sup>46</sup>.

Marked increase in TAS accompanied by marked decrease in TBARS (both by over 30%) proved the beneficial effect of astaxanthin on the antioxidant defense in the examined horses. Decrease in TBARS indicates also lower level of lipid peroxidation, which likely deals also with the oxidative damage of the muscles. Such high level of changes has not been reported before in the literature regarding dietary antioxidants for horses.

The training session produced significant increases in TAS immediately after exercise in all horses regardless of the supplementation, indicating that antioxidant defense was triggered by exercise. However, the values before and 40 min after exercise were higher in astaxanthin supplemented horses. Thus, the relative increase was lower in supplemented horses (1.6-fold vs. 2-fold in the control group). Similar TAS increases (1.6-fold and 1.7-fold) have been reported in Arabian horses after 1250- and 1400- meters races<sup>9</sup>. In contrast, moderate exercise in sedentary horses produced only a slight increase (by 7.5%) and only in the 1st days of training<sup>42</sup>. It confirms that TAS changes in response to exercise depend on the intensity of effort, but in the present study they were also influenced by astaxanthin supplementation.

Both GPx and GR activities tended to increase immediately after training session and decrease after 40-min. restitution. Still, only the increase in GPx activity in the control group was significant. The increases, visible although not significant, may show the compensation triggered by increased ROS production during an effort, while the depletion after restitution may be related to already reduced ROS amount. Lower activities of these enzymes in the supplemented group of horses seem to mirror lower pool of ROS that must be removed and a better antioxidant state, resulting from the presence of astaxanthin that poses an additional antioxidant.

The differences in SOD activities were slight and insignificant in the C group and the only significant difference was the decrease after 40-min. restitution in the horses supplemented with astaxanthin additionally suggesting its synergistic action with endogenous antioxidant system. Various effects of astaxanthin on the activities of antioxidant enzymes have been presented in the literature. The increases in SOD, CAT, and GPx activities have been reported in cell lines subjected to oxidative stress<sup>47</sup>. However, in mice undergoing moderate intensity exercise the decreases have been observed<sup>48</sup>. Similarly, various results have been reported regarding the activities of antioxidant enzymes in response to the effort.

Slight, insignificant changes in the activities of SOD and GPx have occurred after moderate exercise in leisure horses<sup>29,42</sup>. Slight but significant decreases in GPx activities have been reported in Arabians after 1250- and 1400- meters races<sup>9</sup>. In contrast, significant post exercise increases dealing with all antioxidant enzymes (SOD, GPx, GR, CAT) activities have been reported in Arabians after 30 km training session and have been markedly amplified by ergothioneine supplementation<sup>31</sup>.

As it has been mentioned, a certain ROS concentration is beneficial for proper adaptation to the workload. Low concentrations of ROS increase  $\text{Ca}^{2+}$  release and force production, but further increase leading to high amounts of ROS result in the drop in force output<sup>49</sup>. Moreover, adaptation occurs only when the stimulus (ROS concentrations) exceeds minimal threshold, overloading the system<sup>45</sup>. Thus, the dose of antioxidant should be carefully chosen to allow proper adaptation resulting from the training sessions including the upregulation of the body's antioxidant defense. The dose selected in the present study allowed for contribution to the antioxidant defense, thus it seems that the production of ROS necessary to induce adaptive changes was maintained.

TBARS values did not change significantly in response to the exercise in this study, however, the levels in the horses supplemented with astaxanthin were lower in all sampling points. There are reports showing increased or unchanged lipid peroxidation rate measured as MDA production in response to exercise. Pronounced increases have been found in endurance horses. In Arabian stallions after 30 km long training MDA production increased 1.8-fold, but at lower extent in the horses supplemented with ergothioneine<sup>31</sup>. An increase in TBARS has been reported also after 160 km endurance ride together with the decrease in TAS in the halfway<sup>6</sup>. Shorter exercise sessions generally produced less or no increases. Chiaradia et al.<sup>11</sup> have shown the slight but significant increase in MDA production after training session in Maremmana race stallions. Similarly, in untrained leisure horses subjected to moderate exercise MDA levels increased by 47%, but remained unchanged when vitamin E was supplemented<sup>29</sup>. Other studies regarding single, short time training sessions have reported no changes in MDA concentrations after exercise. Such results have been obtained in Standardbreds conditioned for 8 weeks before the examined training session<sup>12</sup> and in Arabians after 1250- and 1400- meters races, regardless of the distance<sup>9</sup>.

The results presented in this study confirmed the beneficial effects of astaxanthin supplementation on the antioxidant status of Arabian horses that begin their race training. Astaxanthin seems to counterbalance the training-related oxidative stress partially. The selected dosing seems proper to shift the redox status towards more reducing environment, which is undoubtedly beneficial for the health and welfare of a horse. At the same time, the effects of training sessions which trigger ROS production and, in this manner, contribute to training progress are maintained.

## Methods

### Yeast biomass cultivation, astaxanthin extraction and micellization

The *P. rhodozyma* NCYC 874 strain (National Yeast Culture Collection, Great Britain) was cultured for 72 h at 21 °C in a shaker at 160 rpm (LS 500 POL-EKO Apparatus, Wodzisław Śląski, Poland) on YPD medium (Sigma Aldrich; Poznań, Poland). Cells were harvested by centrifugation at  $3200 \times g$  for 5 min. at 4 °C.

Ten g of the collected biomass was extracted with continuous shaking (160 rpm, 30 °C, 2 h) with 200 ml of acetone (Sigma Aldrich; Poznań, Poland). Then, the cells were centrifuged at  $3200 \times g$  for 5 min. at 4 °C. The acetone was evaporated in a ventilated incubator at 35 °C in the dark. The astaxanthin concentration and total carotenoid content (as  $\beta$ -carotene) in the sample was determined in an external laboratory (ChemProf Doradztwo Chemiczne s.c. Olsztyn, Poland) using the HPLC/UV-VIS method.

To ensure the stability of astaxanthin, the micellization process was carried out in a high-pressure homogenizer (NS1001L2K, Niro-Savi; Italy) at a pressure of 200 bar. 0.5% (w/w) methylcellulose (Certeck; Poland), 16.6% (w/w) rapeseed oil (Kruszwica; Poland) and 83.4% (w/w) astaxanthin suspended in water were used to prepare micelles. Emulsion stability was assessed visually using the ZOE™ Fluorescent Cell Imager (Bio-Rad) after one month of storage. To improve the organoleptic properties, astaxanthin micelles were mixed with water and 10% (w/w) malt.

### Horses and training

Sixteen privately owned 3 years old Arabian horses, 11 stallions and 5 mares, in regular race training were enrolled in the study. All horses were stabled in one racing facility (Służewiec Race Track in Warsaw) and trained by the same trainer. The animals were selected on the basis of similar advancement in training. Clinical examination by qualified veterinarian and hematological analysis were performed to exclude pathological conditions. All horses were dewormed and vaccinated according to the routine schedule, not earlier than 3 weeks before the onset of the study. They were housed in the same environment and fed the diet of hay, oats, and concentrate which maintained the recommendation for racing Arabian horses, on average, digestible energy of 0.27 MJ/kg BW per day and protein of 2.5 g/kg BW per day, divided into 3 meals. The diet was adjusted individually to each horse, depending on the training progress and so was the workload. Salt and water were available *ad libitum*. At the beginning of the study (in April), the horses were randomly allocated into the astaxanthin (A) group (5 stallions and 2 mares) and control (C) group (6 stallion and 3 mares). The A group was supplemented daily with astaxanthin at a dose of 250 mg per a horse which corresponded to the dose of 0.52–0.58 mg/kg BW. The C group did not receive any supplementation.

Stallions and mares trained together in a mixed group with the same intensity at Służewiec Race Track in Warsaw according to the exercise schedule involving 2 intensive training sessions every week. The intensive training sessions included warm-up walking and trotting with the rider (about 15 min.), followed by cantering and fast gallop (45–50 km/h) for 800 m, and 40 min. of exercise in a horse walker.

### Blood samples

Blood samples were collected by jugular venipuncture in the time points presented at Fig. 2. During the sampling procedure the horses were handled by their usual riders to minimize stress, as recommended by the Ethical Committee guidelines. Samples were collected using 0.8 mm needle into 3 vacuum tubes: EDTA tube for hematological tests, heparinized tube for oxidative status measurements, and plain tube for the analysis of lactic acid (lactate, LA) concentration. LA concentration was determined immediately after blood collection by ejecting a drop of blood onto a single-use lactate strip (Accusport, Roche). EDTA and heparinized samples were transported to the laboratory at +4 °C and analyzed within 6 h after collection. In EDTA-blood, the following hematological measurements were performed using an automated hematology analyzer: red blood cell count (RBC), hemoglobin concentration (HGB), hematocrit (HCT), white blood cell count (WBC), neutrophil count (NEU), lymphocyte count (LYM), monocyte count (MON), eosinophil count (EOS), basophil count (BAS), and platelet count (PLT). The measurements were performed in a certified laboratory and the methods were validated according to certification standards. The high-quality peripheral blood smears stained with the May-Grünwald-Giemsa reagent were assessed in the light microscope (Primo Star, Zeiss, Germany) under 1000 $\times$  magnification (with standard leukocyte differential count). The leukocyte differential count was performed by classifying one hundred leukocytes into five subpopulations: neutrophils, eosinophils, basophils, monocytes, and lymphocytes. One ml of blood from the heparinized tube was transferred into another tube, while the rest was centrifuged and plasma was harvested. Then, both heparinized blood and plasma samples were immediately frozen and kept at -80 °C until the analysis of oxidative status.

All the procedures of blood sampling were performed as part of routine health examination and exercise test and thus, according to the European directive EU/2010/63<sup>50</sup> and Polish regulations regarding experiments in animal there was no need for the approval of Ethics Committee for the described procedures, qualified as non-experimental clinical veterinary practices, excluded from the directive. The trainer granted a written informed consent for the use of blood for scientific analyses.

### Oxidative status measurements

TAS as well as GR and CAT activities were analyzed in plasma samples using dedicated commercial kits (Randox Laboratories Ltd., Crumlin, Co. Antrim, UK and Sigma Aldrich for CAT) by either colorimetric or UV methods according to the protocols supplied by the manufacturer. GPx and SOD activities were quantified in the whole blood using the Randox assay kits (RANSEL and RANSOD kit; Randox Laboratories Ltd., Crumlin, Co. Antrim, UK) according to the manufacturer's manuals. The measurements were performed spectrophotometrically using Biochrom Anthos Zenyth 200 spectrophotometer (Cambridge, UK) at a wavelength of 505 nm for the enzymes activity and 600 nm for TAS. According to the manufacturer's declaration, the TAS assay was linear up to 2.5



mmol/l, which allowed for the detection of clinically important results without sample dilution. The intra-assay TAS variability (expressed as CV%) was 4.8% at 100  $\mu$ mol/l. The detection limit of GR assay was 10 U/l and the linearity was up to 387 U/l. The detection limit of CAT assay was 1  $\mu$ U of CAT activity and the linearity was ensured by testing the undiluted samples and the samples diluted 1:5 and 1:10. The inter- and intra-assay variability (CV%) was <9% in all cases. The detection limit of SOD assay was 1.25 U/l. The biological role of SOD is dismutation of superoxide radicals, and the measurement of its activity is based on the degree of inhibition of this reaction (one unit of SOD corresponds to 50% inhibition). The measurements were provided within 30% and 60% of the inhibition the sample diluent rate. The intra- and interassay variables for RANSOD assay declared by the manufacturer were 4.6% and 7.1%, respectively. The detection limit of GPx was 75 U/l and the method ensured linearity up to 925 U/l. The intra- and interassay variables for RANSEL assay declared by the manufacturer were 4.9% and 7.3%, respectively. The protocol described by Ohkawa et al.<sup>51</sup> was used to measure TBARS. Briefly, plasma samples were incubated with reaction mixture containing 0.8% Sodium Dodecyl Sulfate (SDS), 10% acetic acid, and 0.17% thiobarbituric acid (TBA) for 1 h at 100 °C. Then, the samples were centrifuged and absorbance values were read at 530 nm using a spectrophotometer (Biochrom Anthos Zenyth 200 spectrophotometer, Cambridge, UK). Values were referred to a calibration curve of 1,1,3,3-tetraethoxypropane. The detection limit of TBARS was 1.25 nmol/l. The intra-assay variability (CV%) was 6.8% at 5 nmol/l TBARS.

### Statistical analysis

Numerical variables and residuals (errors) were shown to be normally distributed according to the normal probability quantile-quantile (Q-Q) plots and Shapiro-Wilk W test. Hematological measurements and oxidative status measurements were compared between time points (within-subject factor) and groups (between-subject factor) using the mixed linear model (MLM) if the number of horses differed between timepoints (analysis of the long-term effect of astaxanthin supplementation on baseline oxidative status i.e. comparison between T0, T1-0, T2-0, T3-0 timepoints) or using the repeated-measure analysis of variance (RM-ANOVA) with the post-hoc Tukey's test for unequal groups if the number of horses remained stable (analysis of the effect of astaxanthin supplementation on oxidative status during a single training session i.e. comparison between T0, T1-0, T1-1, T1-2 timepoints). In RM-ANOVA, the sphericity assumption was verified using the Mauchly's test of sphericity and, if violated, the Greenhouse-Geisser correction of degrees of freedom was applied. In MLM, a horse was fitted as the random effect and the time and group as the fixed effects and pairwise comparisons were performed using the paired and unpaired Student's t-test with the Bonferroni-Holm correction for multiple comparisons. Results were presented as the estimated marginal means with 95% confidence intervals (CI 95%) in parentheses in each group in subsequent timepoints with the residual standard error (RSE) for each timepoint. RSE was calculated as the square root of the mean square residual (error). On graphs, the oxidative status measurements were presented as the arithmetic mean, standard deviation ( $\pm$  SD), and individual measurements in each group in subsequent timepoints. Categorical data were presented as counts and proportions in groups and compared between groups with the Fisher exact test. All tests were 2-tailed. A significance level ( $\alpha$ ) was set at 0.05. The analysis was performed in TIBCO Statistica 13.3 (TIBCO Software Inc., Palo Alto, CA) and IBM SPSS Statistics 29 (IBM Corp., Armonk, NY).

### Data availability

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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## Author contributions

Conceptualization – B.G.H., A.C. Investigation – B.G.H, M.K., M.C., E.S., K.S., A.G., S.G., A.H., M.M., A.C. Methodology – M.K., J.W., E.S., K.S., A.G., S.G., A.H., M.M. Formal analysis – B.G.H., M.C., J.W., A.C. Writing—Original Draft – B.G.H., K.M., M.C., A.C. Writing—Review and Editing – M.K., M.C., J.W., E.S., K.S., K.M., A.C. Supervision – K.M., A.C. All authors contributed to the interpretations of the results, the discussion and prepared the final manuscript. All authors read and approved the final manuscript.

## Declarations

### Competing interests

The authors declare no competing interests.

### Ethical approval and consent to participate

All the procedures of blood sampling were performed as part of routine health examination and exercise test and thus, according to the European directive EU/2010/63 and Polish regulations regarding experiments in animal there was no need for the approval of Ethics Committee for the described procedures, qualified as non-experimental clinical veterinary practices, excluded from the directive. The trainer granted a written informed consent for the use of blood for scientific analyses. The authors complied with the ARRIVE guidelines (<https://arriveguidelines.org>).

### Additional information

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