Effects of competition on acute phase proteins and lymphocyte subpopulations – oxidative stress markers in eventing horses

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Effects of competition on acute phase proteins, lymphocyte subpopulations, oxidative stress markers in eventing horses.

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Running head: Acute phase response in exercising horses

Summary

The aim of the study was to evaluate markers of the acute phase response (APR) in eventing horses by measuring acute phase proteins (APP) (haptoglobin, Hp, and serum amyloid A, SAA), lysozyme, protein adducts like pentosidine-like adducts (PENT), malondyaldeyde adducts (MDA), hydroxynonenal adducts (HNE) and total advanced glycation/glycoxidation end products (AGEs), complete blood count and lymphocyte subpopulations (CD4+, CD8+ and CD21+) both at rest and at the end of an eventing competition. Blood samples were collected from 8 Warmblood horses (medium age 10 ± 3) during an official national two-day event competition at rest (R) and 10 minutes after arrival of the country-cross test on the second day. Exercise caused a significant increase in red blood cell number, haemoglobin, packed cell volume, neutrophils, white blood cell and lymphocyte number, however these values remained within the normal range. The CD4+ and CD8+ cells significantly increased, whereas the CD21+ lymphocytes decreased; a significant increase in serum SAA, lysozyme and protein carbonyl derivates was also observed. Two day event causes significant changes in APR markers such as lysozyme, protein carbonyl derivates (HNE, AGEs, PENT) and lymphocyte subpopulations. Data support the hypothesis that two-day event may alter significantly APR markers. Limitations of the study were the relatively small sample size and sampling time conditioned by the official regulations of the event. Therefore further studies are needed to investigate the time required for recovery to basal values in order to define the possible effects on the immune function of the athlete horse.

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Introduction

The study of the extent and nature of the acute phase response (APR) to exercise of different intensity and duration is crucial to optimize horse performance and to preserve animal welfare. The APR is an immune-based reaction to several non-specific stimuli including exercise, characterized by oxidative stress and the release of acute phase proteins (APPs) (Scopetta et al., 2012; Fallon, 2001). Among different exercise where horses are involved, eventing, that represents a demanding discipline for horses, has received little attention within the scientific available literature. Nevertheless, the response elicited by this kind of physical stress could have important modulatory effects on immunocyte dynamics and possibly on immune function. These effects are mediated by different factors including exercise-induced release of pro-inflammatory cytokines, classical stress hormones and hemodynamic effects leading to cell redistribution (Pedersen and Hoffman-Goets, 2000). Particularly, during exercise adrenaline and glucocorticoids modulate the immune system. Immune cells support production of potent circulating inflammatory mediators, i.e. interleukin (IL)-6, IL-1 and tumour necrosis factor (TNF)-α (Donovan et al., 2007; Holbrook et al., 2010).

The response pattern of the APR could be species specific especially for APPs with the exception of serum albumin which decreases by 10–30% to several non specific stimuli in all mammalian species (Suffredini et al., 1999). In horses, serum amyloid A (SAA) is defined as the major APP and haptoglobin (Hp) as a moderate APP (Cywinska et al., 2012). Blood SAA concentrations markedly increase in 24 hours in response to infections and surgical trauma as well as to a long distance rides of 120-160 km (Cywinska et al., 2012; Hulten et al., 1999). Therefore exercise can act as stressor promoting an APR, however no data are available concerning the APP levels following two day event competition.

Several reports describe exercise-induced changes in subsets of blood mononuclear cells (McCarthy and Dale, 1988; Pedersen and Hoffman-Goets, 2000; Pedersen and Nieman, 1998). Specific populations within the circulating leucocyte pool vary significantly with exercise and there is some evidence that in humans the CD4+/CD8+ lymphocyte ratio may become significantly reduced (Keast

75 et al., 1988). According to Malinowski and coworkers (2006) a simulated race test causes changes in

76 the CD4+:CD8+ ratio in Standardbred horses. Nevertheless, few data are available concerning the

77 effects of exercise on lymphocyte subpopulations in horses.

78 A heavy acute exercise is also known to induce oxidative stress (OS) (Vider et al., 2001). In this

79 condition a high concentration of free radicals can damage molecules such as proteins. Specifically,

80 protein oxidative modification can be due to direct damage by reactive oxygen species or indirectly

81 by reaction of secondary by-products of OS (Dalle et al., 2003a). In this case, for example, lipid

82 peroxidation breakdown products such as hydroxynonenal (HNE) and malondialdehyde (MDA) or

83 advanced glycation/glycoxidation end products (AGEs) such as pentosidine (PENT) (Valle et al.,

84 2012) are able to link to protein aminoacid residues leading to the formation of protein carbonyl

85 derivates (Dalle Donne et al., 2003; Traverso et al., 1998). These compounds may be used as measure

86 of protein damage (Odetti et al., 1999) and markers of oxidative stress. In fact, they are stable adducts

87 produced early and circulate in the blood for long periods (Dalle Donne et al., 2003 b). In human

88 plasma and serum samples many of these adducts have been effectively quantified thanks to their

89 fluorescent properties attributable to double bonds and ring structures in a number of moieties using

90 fluorometry (Forbes et al., 2005). In humans there are some evidence demonstrating changes in AGEs

91 following physical activity (Kotani et al., 2011); however, no data are available on sport horses.

92 On the basis of these premises, the aim of the study was to evaluate possible markers of the APR in

93 eventing horses by measuring APPs (SAA, Hp), lysozime, protein adducts (HNE, MDA, AGEs,

94 PENT), complete blood count (CBC) and lymphocyte subpopulations (CD4+, CD8+, CD21+) both

95 at rest and at the end of a 2-day event competition.

96

97 **Materials and methods**

98 Animals

4

99   Eight experienced Warmblood horses (5 gelding, one stallion and 2 females), medium age 10

100   ± 3 (mean ± standard deviation (s.d.)), mean body weight 580±41 kg were included in the study. All

101   horses were clinically healthy and had not received any medication during the last 15 days before the

102   beginning of the study. The animals were kept in the same stable and fed with first cut meadow hay

103   and commercial cereal-mix feed for sport horses (crude protein 12.5%, crude fat 5.2%, crude ash

104   8.5%, crude fiber 8.5%, Vitamin E 200 UI/kg and Selenium 0.35 mg/kg) in a ratio forage:concentrate

105   corresponding to 60:40. They were trained six times a week with a typical conditioning program for

106   eventing horses: flat work (50 min/day, 3 days/wk.), flat work and jump (50 min/day, 2 days/wk.)

107   and conditioning (warm up and gallop at 450-480 m/min for 5-8 minutes once a week).

108

109   Competition feature

110   Data were collected during an official national two-day event competition organized by the

111   Italian Equestrian Federation at Cameri sport club (Italy). The competition was held in May, the

112   weather conditions remained relatively constant throughout the two days of competition

113   (environmental temperature 19°C, relative humidity 66 %). The ground conditions made up of grass

114   were similar for all horses, irrespective of starting order. On day 1, horses performed the dressage

115   (marks given for medium walk, working-medium-extended trot, working canter, working-medium-

116   collected canter, shoulder in at trot and rein back) and the show jumping phase (maximum fence 1.20

117   m; speed 350 m/min). On day 2, animals performed the cross country test on a distance of 3100 m

118   (maximum distance) (minimum speed 530 m/min and fence 1.10 m maximum height). All horses

119   competed between 8:30 am and 11:30 am.

120

121   Blood samples collection

122   Blood samples were collected by jugular venipuncture at rest (R), between 8:00 and 9:00 a.m.,

123   in the original barn, before being moved to the competition site. Horses were moved the day before

124 the competition with a travel of about 50 minutes. A second blood sample was performed within 10

125 minutes after the end (PC) of the cross country phase on day 2. This time frame was influenced by

126 the official regulations of the competition. Samples for the determination of APPs and lysozyme were

127 placed into 10 mL vacutainer tube contain clot activator, allowed to clot at room temperature,

128 centrifuged at 2500 g for 10 minutes and aliquots of the obtained sera stored at -80°C until analysis.

129 Blood for the determination of complete blood cell count (CBC), lymphocyte subpopulations was

130 placed into a 10 mL EDTA vacutainer tube, refrigerated and immediately transferred to the laboratory

131 for analysis. For protein adducts plasma aliquots were obtained by centrifugation (2500 g for 10

132 minutes at 4°C) immediately after collection and then stored at -80° C until analysis.

133

134 Complete blood count

135   Complete blood count was performed by using an automated laser cell counter hematology

136 System (Advia 120, Siemens Diagnostics Inc., Tarrytown, USA) to assess the following parameters:

137 white blood cell count (WBC, 10^3/µL), neutrophils (NEUT, 10^3/µL), lymphocytes (LYM, 10^3/µL),

138 red blood cells (RBC, 10^6/µL), hemoglobin (Hb, g/dL), packed cell volumes (PCV, %), platelets (PLT

139 10^3/µL).

140

141 Lymphocyte subpopulations

142   Lymphocyte subpopulations were characterized by flow cytometry using monoclonal

143 antibodies against the following antigens: CD21+ (CA2.1D6, Serotec, B cells, Serotec, Kidlington,

144 UK), CD4+ (CVS4, Serotec, T-helper, Serotec, Kidlington, UK), CD8+ (CVS8, Serotec, T-

145 cytotoxic/suppressor, Serotec, Kidlington, UK). For surface staining, 100 µL of whole blood were

146 incubated with monoclonal antibodies at the appropriate dilution for 30 min at 4 °C. After two washes

147 with phosphate-buffered saline (PBS), cells were incubated with 10 µL of 1:5 diluted fluorescein

148 isothiocyanate (FITC)-labeled goat anti mouse immunoglobulin F (ab') 2 fragment (SouthernBiotech,

149   Birmingham, USA) for 30 min at 4 °C. Negative controls consisted of cells incubated with

150   fluoresceinated goat anti mouse antibody alone. Two milliliters of lysing solution (ammonium

151   chloride) were added and left for 20 min at room temperature. Cells were then washed twice and

152   resuspended in 0.5 mL of PBS.

153   A minimum of 10000 events from a gated lymphocyte population was acquired and analyzed on a

154   Epics XL-MCL flow cytometer (Coulter, cCoulter® Epics® XL-MCLTM Miami, USA), thus

155   obtaining percentages of the different subpopulations. Absolute lymphocyte subset counts were

156   calculated as the product of the lymphocyte absolute count and the lymphocyte subset percentage.

157

158   Protein carbonyl adducts

159   Delipidated plasma samples were analyzed for fluorescence modification with

160   spectrophotofluorometer at wave-length of 370-nm excitation (EX)/440-nm emission (EM) for total

161   AGE adducts (AGEs), 335-nm EX/385-nm EM for pentosidine-like adducts (PENT), 390-nm

162   EX/460-nm EM for malondyaldeyde adducts (MDA), 356-nm EX/460-nm EM for hydroxynonenal

163   adducts (HNE) and 280EX/390-nm EM for triptophane (Trip).

164   Control of the absorbance of the protein solution was performed in order to rule out any possible

165   interference with the evaluation of fluorescence: the absorbance of the protein solution at the

166   wavelengths used for fluorescence excitation never exceeded 0.1; therefore absorbance should not

167   have been significant as possible interference on the evaluation of fluorescence. Fluorescence

168   intensity was expressed in arbitrary units (AU) of fluorescence per g of total proteins.

169

170   Acute phase proteins and lysozyme

171   Serum SAA and Hp concentrations were measured using a commercial assay kits (Phase, Tridelta

172   Ltd. Ireland). Serum lysozyme was assessed by the lyso-plate assay method according to Osserman

173   and Lawlor (1966); briefly, serum samples were reacted with a suspension of *Micrococcus*

174   *lysodeikticus* in agar gel in 10-cm Petri dishes. Under these conditions, lysozyme concentration

175   (µg/mL) is proportional to the diameter of lysis areas and is determined from a standard curve created

176   with a standard lysozyme preparation (Sigma-Aldrich SRL, Milano).

177

178   Protein content determination

179   Plasma protein content was determined using the BCA protein assay kit according to the

180   manufacturer's instructions (Pierce, BCA Protein Assay).

181

182   Statistical analysis

183   Statistical analyses were performed with SPSS software package (SPSS, Chicago, Illinois, USA).

184   After checking the normality, a paired sample t-test was used to compare the values for lymphocyte

185   subpopulation data, protein carbonyl adducts data, acute phase proteins and lysozyme and complete

186   blood count data at rest and after exercise. Statistical significance was set at $p \leq 0.05$. All measurements

187   were performed in triplicate and data are expressed as mean $\pm$ SD

188

189   **Results**

190   Complete blood cell count

191   Exercise caused a significant increase in red blood cell number, haemoglobin concentration, packed

192   cell volume percentage, neutrophils, white blood cells numbers, and lymphocytes and a decrease in

193   platelet number (Table 1). However all the values were in the reference range values according to

194   the Laboratory of the Department of Veterinary Sciences, University of Turin

195

196   Lymphocyte subpopulation

197   The absolute number of CD4+ cells was significantly higher after exercise ($1650 \pm 110 \times 10^3$ cell/µL,

198   $p = 0.01$) compared to basal values ($1260 \pm 80 \times 10^3$ cell/µL), while the relative percentage with respect

199   to the total lymphocyte population was decreased (54.51 ± 6.05 % vs 60.73 ± 5.50 %, p = 0.002)

200   (Fig 1a-1d). Similar results were obtained also for the CD8+ cell subpopulation (300 ± 30 10^3 cell/µL,

201   R, vs 620 ± 90 10^3 cell/µL, PC, p = 0.007) (Fig 1b). However, the relative percentage was increased

202   after competition (20.66 ± 7.90 % vs 13.71 ± 2.28 %, p=0.02) (Fig 1d). The CD4+:CD8+ ratio was

203   significantly (p = 0.047) decreased at the end of the event compared to its value at rest (3.03 ± 0.6 vs

204   4.62 ± 0.4, p = 0.047). From rest to after the event, the CD21+ subpopulation significantly decreased

205   (270 ± 30 10^3 cell/µL, R, vs.160 ± 20 10^3 cell/µL, PC, p = 0.01, Figure 1c) and represented a smaller

206   portion of lymphocytes in percentage (12.80 ± 4.27 % vs. 5.25 ± 1.49 %, p = 0.002; Figure 1d).

207

208   Acute phase proteins and lysozyme

209   After exercise, a significant increase in serum SAA (1.96 ± 0.55 µg/mL, R, vs 3.91 ± 0.73 µg/mL,

210   PC; p = 0.001) and lysozyme (3.35 ± 0.85 µg/mL vs 4.52 ± 1.57 µg/mL; p = 0.05) was observed.

211   No significant changes of HP concentration was observed (0.91±0.42 mg/mL vs. 1.29±0.55 mg/mL;

212   p = 0.169)

213

214   Protein carbonyl adducts

215   Exercise caused a significant (p < 0.05) increase of blood concentration in total AGE adducts,

216   pentosidine-like adducts, hydroxynonenal adducts and tryptophan. For MDA adducts, besides a trend

217   suggesting an increase at the end of competition, no significant differences were observed (p = 0.058)

218   (Fig 2).

219

220   **4. Discussion**

221   To our knowledge, this is the first study investigating the effects of exercise on acute phase response

222   in eventing horses. The modifications observed in complete blood cell count may be considered as

223   typical expected exercise-induced responses in performance horse (Jumping Endurance and

9

224 Standardbred horses) (Cuniberti et al., 2012). White blood cells and neutrophils increase during and

225 after exercise, whereas lymphocyte concentrations increase during acute exercise and fall below basal

226 values after long-duration physical exercise (Pedersen and Hoffman-Goets, 2000). In our study, both

227 neutrophil and lymphocyte number increase after exercise. However, according to Malinowski et al.,

228 (2006) in the horse the magnitude of lymphocyte number modifications due to exercise depends on

229 age and training. The increase in the total number of red blood cells, packed cell volume and

230 haemoglobin concentration can be ascribed both to spleen contraction regulated by catecholamine

231 and to sweating that causes fluids loss (Cuniberti et al., 2012).

232 In our in-field study, for practical reasons, we did not measure horse body weight at the end of

233 competition, therefore we did not estimate the possible dehydration percentage. However, protein

234 concentration before and at the end of competition, that could reflect dehydration and fluid loss (Rose

235 and Hodgson, 2005) is not significant and minimal (5.75 ± 0.38 vs 6.14 ± 0.45). For this reason we can

236 assume that the effect of dehydration is negligible and the variation of the observed parameters is not

237 masked by this effect. This is also confirmed by the fact that the lymphocyte undergo to a real

238 redistribution. In fact interestingly, we found a significant increase in the number of CD4+ and CD8+

239 cells after exercise. It has been postulated that the activation of the sympathetic system and the

240 increased levels of circulating catecholamines during exercise leads to increased lymphocyte

241 concentration due to the recruitment of lymphocyte subpopulations to the vascular compartment

242 (Gannon et al., 2001). Although the number of lymphocyte subpopulation increases, the percentage

243 of CD4+ decreases probably due to the fact that NK cells increase more than other lymphocyte

244 subpopulations such as the helper CD4+ (Fry et al., 1992). As a consequence, in our study the

245 CD4+:CD8+ ratio significantly decreased in response to exercise. This finding agrees with the results

246 obtained by Malinowski and coll., (2006) where the acute response to exercise influence the percent

247 CD4+ and CD8+ both in unfit and trained Standardbred mares.

As far as CD21+ lymphocytes are concerned, in our study a significant exercise-induced reduction was observed. In humans several reports have documented either increased or unchanged B lymphocyte numbers after exercise (Tvede et al., 1989; Pedersen and Hoffman-Goets, 2000). No data concerning CD21+ lymphocyte modifications related to exercise in horses are available in literature. According to Stull and coll. (2008), in horses long-distance road transport induces a decline in CD21+ lymphocyte subpopulation. Both during transport and exercise the redistribution of lymphocyte subpopulations may represent a cortisol-mediated stress response.

According to our results, eventing causes a significant increase in SAA and lysozime levels. It has been shown that during the acute phase response, SAA increases within 4 hours reaching the maximum serum levels 24-48 hours after the initiating stimulus (Petersen et al., 2004). Although the precise biological function of increased SAA expression has not been fully elucidated, it has been suggested that SAA is involved in chemotaxis of leucocytes and T cells, inhibition of platelet activation, and induction of calcium mobilization by monocytes (Zimlichman et al., 1990; Badolato et al., 1995). A 10-fold increased in circulating SAA concentrations has been observed in horses after long-distance ride (Cywinska et al., 2012). In our study, at the end of competition a 2-fold increase in SAA concentrations was found compared to pre-race values. It should be considered that in humans and horses individual variations in SAA levels have been described (Hulten et al., 1999, 32). Moreover, while the effects of endurance exercise on APPs have been extensively documented and the hypothesis that strenuous exercise is analogous to the APR elicited by tissue injury has been confirmed, no data concerning eventing horses are available. In humans, for example, an acute phase response does not seem to occur in a variety of field and court sports, whereas in ultramarathon runners acute phase reactants are significantly affected by exercise (Fallon, 2001, Fallon et al., 2001). Therefore, our results confirm that SAA is the most sensitive APP in horses although eventing seems to elicit a mild response compared with long-distance endurance races.

Studies of the exercise/stress influence on lysozyme concentrations in horses are scarce. According to Sotirov and coworkers (2004) exercise has no negative effects on equine innate immune factors. In our study a significant increase of circulating lysozyme was found at the end of competition (Sotirov et al., 2004). It has been demonstrated that increased levels of glucocorticoids and catecholamines during exercise stimulate the secretion of different neutrophil granule proteins such as lysozyme in blood (Morozow et al., 2003). In swimming rats and treadmill runner athletes the plasma lysozyme content is increased by 41% following intensive exercise load. In humans, several studies have documented the effects of exercise on salivary lysozyme concentration (Papacosta and Nassis 2011). It has been suggested that the lysozyme response to exercise depends on exercise intensity with a significant increase following acute bouts of intense exercise and no changes at lower workload (West et al., 2010).

The main biological functions of Hp are represented by binding hemoglobin and preventing iron loss, bacteriostatic and immunomodulatory effects and regulation of lipid metabolism (Murata et al., 1993). In horses fluctuations in blood Hp concentrations have been observed in case of natural diseases, experimental-induced inflammation and after surgery (Pollock et al., 2005). In unhealthy horses the circulating Hp levels increase slowly after surgery and they are still elevated by 72 hours after the initial stimulus (Pollock et al., 2005). Controversial data have been published on Hp levels in horse after physical exercise (Pellegrini Masini et al., 2003; Fazio et al., 2010). According to Fazio and coworkers (2010), serum Hp concentrations significantly increase during a typical thoroughbreds training. By contrast, in endurance horses neither limited nor long distance rides seem to significantly affect peripheral Hp concentrations (Cywinska et al., 2012). In another study, Scoppetta et al., (2012) found that Hp is highly reduced immediately after the race. The decreased Hp levels may be related to its binding with hemoglobin released into circulation following exercise-induced haemolysis. Haptoglobin levels in our study are in line with those measured by Cywinska and coworkers (Cywinska et al., 2012) and do not seem to be significantly affected by eventing exercise.

12

Even though horses can be considered as excellent models for studying reactive species production due to their high aerobic capacity, data on exercise-induced oxidative stress in horses are heterogeneous and mainly concern racehorses. In the present research an increase in the fluorescence related to lipoxidation (HNE adducts) and glycoxidation adducts (total AGE and PENT) was observed. Exercise-induced protein carbonylation, has previously been described both in Thoroughbred (Duberstein et al., 2009) and Standardbred horses (Kinnunen et al., 2005). Kinnunen and coworkers 2005 showed that an increased concentration of plasma and muscle protein carbonyls can be detected after a single session of moderate intensity exercise. The increased protein carbonyl derivates levels are detectable immediately after exercise and are still elevated after 4 h of recovery, returning to pre-exercise values within 24 h of recovery. MDA adducts in our study tended to increase after exercise, although the modification was not statistically significant. We therefore hypothesize that lipid peroxidation plays a minor role in protein damage and/or that the generation of protein carbonyl derivates from this pathways occurs later. The increase in tryptophan fluorescence observed at the end of competition could be ascribed to the alteration in protein organization which leads the exposition of tryptophan residues due to the bound of the aldehyde in other positions in the protein (Traverso et al. 2004). Our results suggest that competition causes increased protein carbonyl derivates that may predispose eventing horses to tissue damage and inflammation. However, the life span of the stress response in terms of oxidation end products should be further investigated to estimate the quantity of dietary antioxidants required by horses especially during the competition season.

5. Conclusions

Our data support the hypothesis that two-day event may alter significantly APR markers such as acute phase proteins, lysozime, protein carbonyl derivatives, complete blood count and lymphocyte subpopulations. These preliminary findings were obtained during an official competition on a

restricted number of horses and the variation of parameters studied could be influenced by sampling time, that was conditioned by the official regulations of the event. Further studies are needed to investigate the time required for recovery to basal values in order to define the possible effects on the immune function of the athlete horse.

Conflict of interest statement

All authors declare they have no financial or commercial conflicts of interest.

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461 **Table 1**

462 Complete blood cell count in eventing horses at rest and after competition

	Rest	After competition	p*	Reference values*[1]
WBC (x10^3 cell/μL)	5.76 ± 0.74	7.74 ± 0 .74	0.002	4.30-14.80
Neutrophils (x10^3 cell/μL)	3.21 ± 0.52	4.30 ± 1.02	0.005	2.20-8.10

Lymphocytes (x10^3 cell/µL)	2.12 ± 0.38	3.02 ± 0.52	0.002	1.70-5.80
RBC (x10^6 cell/µL)	8.05 ± 0.76	10.01 ± 0.77	0.003	4.45-12.0
Hemoglobin (g/dL)	13.88 ± 1.08	17.56 ± 1.54	0.002	11.30-18.90
PCV %	34.94 ± 2.77	45.24 ± 4.93	0.003	31.10-50.50
Platelets (x10^3 cell/µL)	131.75 ± 23.63	89.25 ± 36.63	0.003	69.90-250.80

463

464 Results are mean values ± sd. WBC, white blood cell count; lymphocytes; RBC, red blood cells;

465 PCV, Packed Cell Volume;

466 *Statistical analysis performed by paired sample *t* test.

467 *[1] Reference range values according to the Laboratory of the Department of Veterinary Sciences,

468 University of Turin

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470

471 **Fig. 1**

472   a) Number of CD4+ cells at rest (R, dot filled bar) and after competition (PC, line filled bar) (**p*

473   ≤ 0.05)

474   b) Number of CD8+ cells at rest (R) and after competition (PC) (*p ≤ 0.05)

475   c) Number of CD21+ cells at rest (R) and after competition (PC) (*p ≤ 0.05)

476   d) Lymphocyte subpopulation percentage at rest (R) and after competition (PC) (*p ≤0.05)

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478

479 **Fig 2**

480 Mean ± SEM values for protein carbonyl adducts (AGEs, total AGE adducts; PENT, pentosidine-like

481 adducts; MDA, malondyaldeyde adducts; HNE, hydroxynonenal adducts; Trip, triptophane)

482 before (R, dot filled bar) and after competition (PC, line filled bar) (*p ≤ 0.05)

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