Evaluation of Oxidative Stress Parameters in Healthy Saddle Horses in Relation to Housing Conditions, Presence of Stereotypies, Age, Sex and Breed

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Keywords: stereotypy, behavior, free radicals, redox status, horse

Abstract:

Oxidative stress plays an important role in the development of many horse diseases and it has been shown that housing has important implications for the psychophysical well-being of horses. The aim of this study is to determine if there are any differences between the redox status in horses in relation to housing conditions. The four housing conditions analyzed were: single box, without external access and without contact (Cat A), single box with external access and possibility of partial contact (Cat B), group housing with box and large paddock (Cat C), pasture with more than 7 horses and the possibility of green forage for the whole year (Cat D). A group of 117 healthy horses were selected in several private stables in Northern Italy. All subjects treated with any type of drug were excluded. At the end of the enrollment, the 117 selected horses were divided into the four housing categories. Stereotypies were highest in the group of horses in single box, without external access and without contact (Cat A). Oxidative stress was evaluated by testing plasma or serum samples for the following parameters: superoxide anion (WST), nitric oxide (NO), reactive oxygen species (d-ROMs), ferric reducing ability of plasma (FRAP), and the activity of superoxide dismutase (SOD). Simultaneously with the blood sampling, the owners completed a questionnaire with all the management aspects of the horse (signaling, feeding, equestrian activity, vaccinations, foot management etc.). The statistical evaluation was carried out based on the categories previously described, on the presence and absence of stereotypies and on some signaling data obtained from the questionnaire. There were no significant differences in the parameters analyzed between the categories. No significant redox status differences were detected based on the presence or absence of stereotypies. Interestingly, when the age was introduced as selection (14 years old) parameter inside the categories, statistical significance was observed for some of the stress markers considered. Finally, independently of the housing conditions, the horses of the most two represented breeds exhibited different values of FRAP. All these aspects are commented in the discussion.

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Evaluation of Oxidative Stress Parameters in Healthy Saddle Horses in Relation to Housing Conditions, Presence of Stereotypies, Age, Sex and Breed

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Abstract: Oxidative stress plays an important role in the development of many horse diseases and it has been shown that housing has important implications for the psychophysical well-being of horses. The aim of this study is to determine if there are any differences between the redox status in horses in relation to housing conditions. The four housing conditions analyzed were: single box, without external access and without contact (Cat A), single box with external access and possibility of partial contact (Cat B), group housing with box and large paddock (Cat C), pasture with more than 7 horses and the possibility of green forage for the whole year (Cat D). A group of 117 healthy horses were selected in several private stables in Northern Italy. All subjects treated with any type of drug were excluded. At the end of the enrollment, the 117 selected horses were divided into the four housing categories. Stereotypies were highest in the group of horses in single box, without external access and without contact (Cat A). Oxidative stress was evaluated by testing plasma or serum samples for the following parameters: superoxide anion (WST), nitric oxide (NO), reactive oxygen species (d-ROMs), ferric reducing ability of plasma (FRAP), and the activity of superoxide dismutase (SOD). Simultaneously with the blood sampling, the owners completed a questionnaire with all the management aspects of the horse (signaling, feeding, equestrian activity, vaccinations, foot management etc.). The statistical evaluation was carried out based on the categories previously described, on the presence and absence of stereotypies and on some signaling data obtained from the questionnaire. There were no significant differences in the parameters analyzed between the categories. No significant redox status differences were detected based on the presence or absence of stereotypies. Interestingly, when the age was introduced as selection (<14 and >14 years old) parameter inside the categories, statistical significance was observed for some of the stress markers considered. Finally, independently of the housing conditions, the horses of the most two represented breeds exhibited different values of FRAP. All these aspects are commented in the discussion.

Keywords: horse; redox status; free radicals; behavior; stereotypy

1. Introduction

Over the past few decades, the oxidative stress has been increasingly investigated by the scientific community [1–3] pointing out the following players: the oxidizing agents and the antioxidant agents [4]. Free radicals are produced during physiological cellular processes, i.e., as cell messengers, but when they are produced in excess they can damage cells and tissues. Oxidative stress is defined as an

imbalance between oxidants and antioxidants in favor of the oxidants [1]. Numerous studies have shown that it has a fundamental role in the etiopathogenesis of several acute and chronic diseases. Oxidative stress has been deeply investigated in human, while in equine veterinary medicine the data are yet less fragmented. In particular, the role of oxidative stress during recurrent airway obstruction has been investigated [5–9]. Moreover, the knowledge on redox status has been deepened in pathologies such as Equine Motor Neuron Disease [10–13], as well as in many orthopedic pathologies [14–16], Cushing's syndrome [17,18], endometritis [19,20], gastrointestinal diseases [21,22], during Rhodococcus equi pneumonia, or Equine Infectious Anemia (EIA) infection [23,24] and parasitic infestations [25–29]. These studies show that oxidative stress can also play an important role as a therapeutic target [5,9,30], as well as a prognostic index during serious pathologies [31,32]. Moreover, oxidative stress aids to shed light on several aspects of exercise physiology [33–37]. Equine welfare is fundamental in maintaining correct health and a good level of mental and physical well-being of the animal [38–43]. Several studies correlate the animal's living conditions with the development of pathologies and behavioral problems such as stereotypies [44]. In particular, social interaction might reduce both the stress of the single subjects and occurrence of stereotyped behaviors [45]. Stereotypies are also related to different orthopedic pathologies during the weaving or colic and dental problems in horses that shows crib-biting [46–48]. On this basis, our study aims to investigate if oxidative stress markers are affected by horses living conditions, and/or the presence of stereotyped behaviors. In particular, we assayed superoxide anion (WST test), nitric oxide (Griess test), hydroperoxides (d-ROMs test), superoxide dismutase activity (SOD test) and non-enzymatic scavenger activities (FRAP test).

2. Materials and Methods

2.1. Study Protocol and Selection of Subjects

The protocol of this study was submitted to the Committee for Animal Ethics of the University of Parma (approval number PROT. 04B-CE209 31/01/2020), and the experiments were conducted in accordance with the approved guidelines, in particular the AWIN (Animal Welfare Indicators) welfare assessment protocol for horses [49]. The research was carried out in the field by enrolling horses housed in five different private stables in northern Italy. 117 healthy horses of 22 different breeds, sex (62 gelding, 55 females, 1 male) and ages were selected. Equine athlets were not included. All the animals enrolled had been housed in the same conditions for over 6 months. Horses exhibiting stereotyped behaviors were admitted to the study:

- A: Single box with no possibility of interaction with other horses (N = 27)
- B: Single box with access to the outside and possibility of partial interaction with others horses (N = 30)
- C: Paddock with shelter and direct interaction between groups of 2 to 5 horses (N = 30)
- D: Pasture with available green grass, shelter, and pack of minimum 7 horses (N = 30)

The 32 horses exhibiting stereotyped behaviors admitted to the study were distributed in all categories. For the comparison between the different breeds, we selected "Thoroughbred" and "Italian Saddle," that were those with the highest numerosity of subjects (24 and 23, respectively). To assess the redox status as a function of the age, the horses were divided into two groups. For the comparison between the different breeds, the horses were divided into two groups based on a cutoff of the age at 14 years old. At rest, for each horse a blood sample was taken from the left jugular vein using a syringe with an 18 G needle. Afterwards the blood was divided into two test tubes, one containing a coagulation activator for the serum and the second containing ethylenediamine tetraacetic acid (EDTA) for the plasma. The samples were stored at 4 °C and were delivered to the laboratory within 12 h of collection. After a centrifugation at 5500 rpm for 4 min serum and plasma were then divided into aliquots and stored in a deep refrigerator at −18 °C.

2.2. WST Measurement

The WST-1 is a colorimetric test that is currently employed for the quantification of the superoxide anion levels present in the sample [6]. To carry out the test, the reagent for the WST test produced by the Roche company (Mannheim, Germany) was used according to published procedures [50–52]. The WST levels in the graphs are reported as absorbance units.

2.3. Nitric Oxide Measurement

The nitric oxide assay was carried out in serum through the Griess test accordingly to previously published procedure [53,54]. NO levels in the graphs are expressed as molarity.

2.4. d-ROMs Measurement

For the evaluation of hydroperoxides, the d-ROMs test kit (DIACRON International, Grosseto, Italy) was used on the serum samples [55]. The test is based on the reaction that occurs between hydroperoxides and the iron released from the endogenous proteins in consequence of the acidic pH of the R2 reagent of the kit, that following Fenton reaction mechanism, give rise to peroxyl and alkoxyl radicals (ROOH); these compounds, finally develop a pink color into the R1 reagent of the test, through the reaction with an alkyl-substituted aromatic amine. Briefly, 2 µL of chromogenic substrate (R1) and 200 µL of buffer, pH 4.8 (R2), were mixed with 2 µL of plasma in each well of a microplate. A blank reagent, obtained by replacing the plasma with distilled water and a standard "calibrator" sample, containing known amounts of ROOH (provided by the manufacturer), were included for each assay. After 20 min of incubation at 37 °C, the absorbance was measured at 540 nm by Multilabel Counter Victor3 (Perkin Elmer, Groningen, The Netherlands). The results were expressed in arbitrary units called "Carratelli Units" (CARR U) according to the following formula:

$$CARR\ U = Absorbance\ sample \div Absorbance\ calibrator \times [calibrator] \quad (1)$$

2.5. SOD Activity Measurement

Superoxide dismutase activity (SOD) was assayed by means of SOD assay kit (Sigma-Aldrich, Mannheim, Germany) [55] in equine serum samples. SOD levels were evaluated as enzymatic activity (U/mL).

2.6. FRAP Measurement

The FRAP (ferric reducing ability of plasma) method assesses the ability of the antioxidants present in the sample to reduce the Fe^{3+}/trifyridyltriazine complex under acid pH conditions. The reduction of the ferric ion (Fe^{3+}) to the ferrous ion (Fe^{2+}) takes place according to a colorimetric reaction evaluable by spectrophotometer at 593 nm [56]. FRAP levels were determined as molarity, based on a standard curve of $FeCl_2$ solutions at known concentrations.

2.7. Statistical Analysis

Continuous variables are expressed as mean ± standard deviation or median and interquartile range (IQR) and categorical variables as frequencies. For continuous and normally distributed variables, comparison among groups was tested by means of Analysis of Variance (ANOVA). Normality was assessed by means of the Kolmogorov–Smirnov test. For variables not normally distributed Mann–Whitney and Kruskal–Wallis test were used to assess the differences among groups. The chi-square test was performed for categorical variables. Spearman correlation test was performed to assess correlation among parameters. The software used was SPSS v.26 (IBM SPSS Statistics, Milano, Italia) with a significance level set at 5%.

3. Results

Table 1 reports for each of the four housing conditions (category A–D) both the mean values of the ages of the horses enrolled, the numerosity of female and gelding subjects. Since only one stallion was enrolled for the study, male subjects could not be considered for the following statistical analysis.

Table 1. Summary of the ages and sex of horses included in the different housing categories.

	Mean Age ± SD (Years)	Geldings	Females	Stallions
category A	16.6 ± 5.2	16	11	0
category B	14.7 ± 6.9	15	14	1
category C	14.3 ± 4.9	12	18	0
category D	14.3 ± 5.2	20	10	0

Regarding the distribution of subjects, our analysis showed that the population of subjects included in this study was very homogenous, including age ($p = 0.34$) and gender ($p = 0.2$).

Figure 1 reports the number of the subjects with stereotypies for the different housing conditions. Interestingly, there was a significant prevalence of stereotypies in category A, that include the oldest subjects, compared to the others ($p = 0.034$).

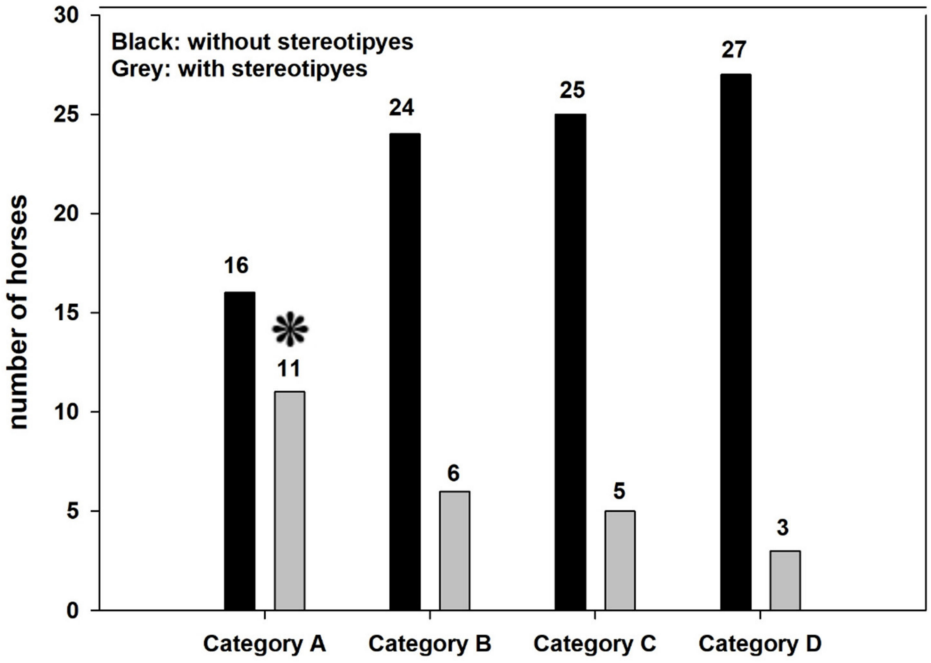

Figure 1. Distribution of horses with and without stereotypes in the various housing conditions. * means $p < 0.05$.

As regards the assessment of oxidative stress levels in these animals, there were no significant differences for the blood parameters in presence or absence of stereotypy (see below Figure 2A–E, yellow boxes).

With regard to the blood parameters, a preliminary evaluation by Kolmogorov–Smirnov test showed that values of the analytical measurements were not normally distributed, therefore the differences between the groups of data were assessed either by Mann–Whitney or Kruskal–Wallis tests.

The first statistical analysis that aimed to evaluate the impact of the housing conditions on the redox status of the all 117 horses enrolled, did not reveal any significant differences between the levels of the blood oxidative stress markers in the different categories (Figure 2A–E, green boxes).

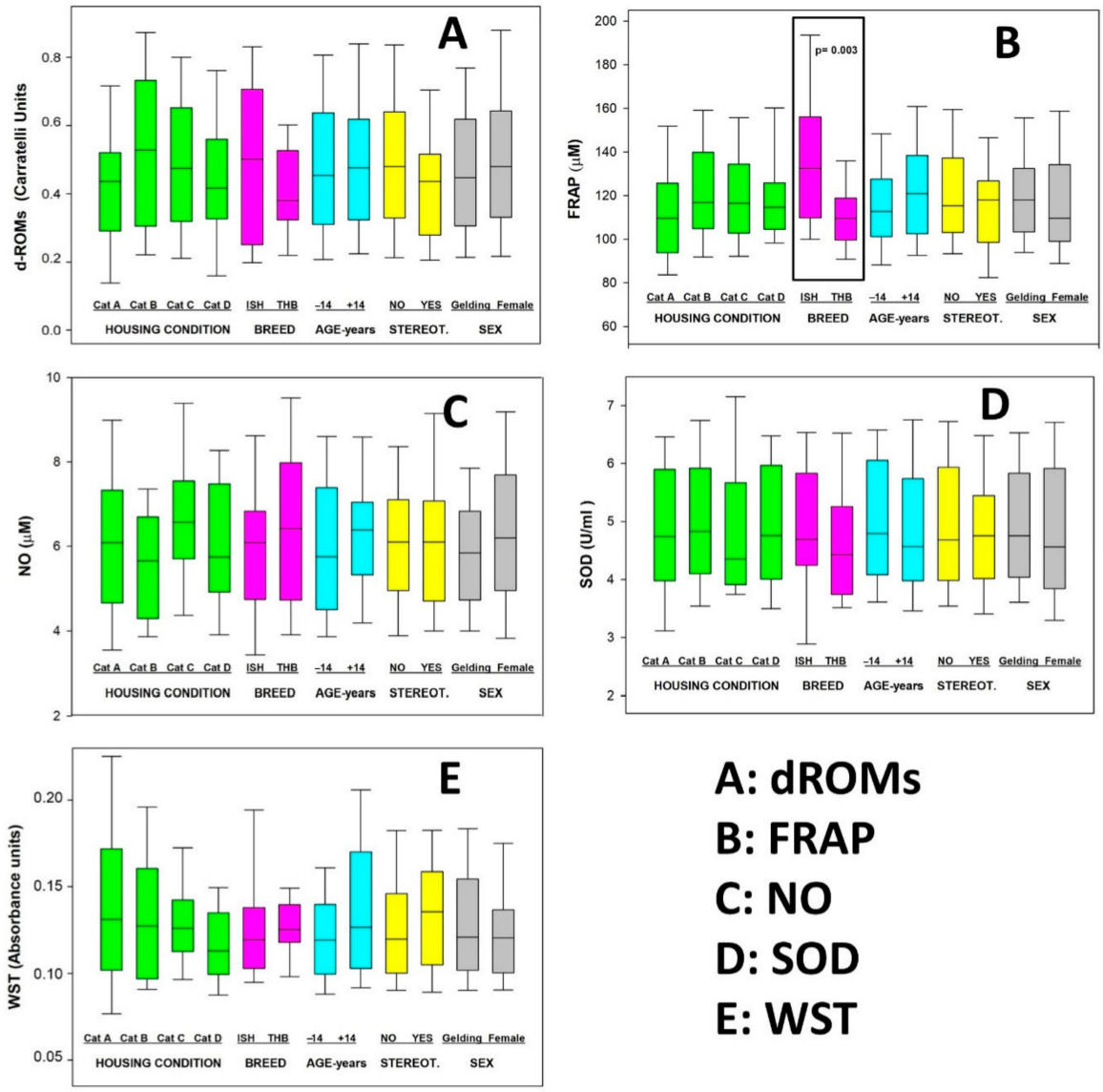

Figure 2. Box plots of the redox markers of the 117 horses enrolled, considering the different housing conditions (green), breed (pink), age (cyan), stereotypy (yellow) and gender. The data were analyzed by Kruskal–Wallis test.

For the statistical evaluation based on gender, only females and geldings were taken into consideration since only one stallion was present in the study. The statistical analysis showed no significant differences in blood parameters between females and geldings (Figure 2A–E, cyan boxes). For the statistical evaluation based on age, the horses were divided into two groups based on the value of the median of the parameter: respectively younger and older than 14 years, thus two numerically homogeneous populations were obtained. As reported in Figure 1 (Figure 2A–E, grey boxes) no significant differences were found in blood parameters for the two groups. Nevertheless a further statistical analysis restricted to the single housing conditions, showed significant differences for FRAP and WST values in the horses belonging to the housing category A (Figure 3), that were higher for the older subjects (>14).

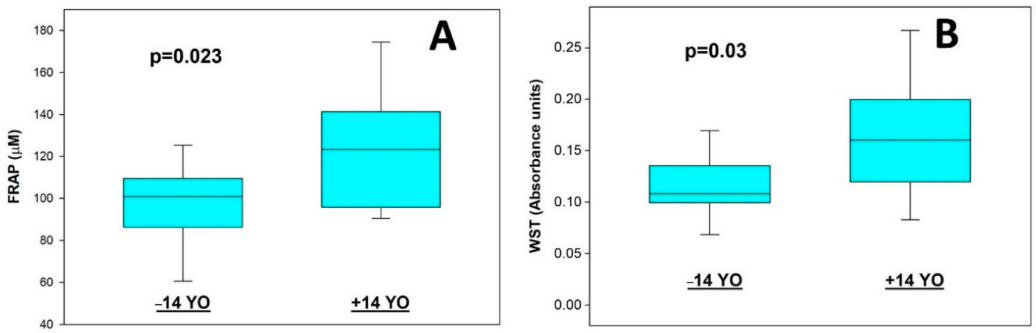

Figure 3. Box plots of the ferric reducing ability of plasma (FRAP) (**A**) and superoxide anion (WST) (**B**) values of the <14 and >14-years-old horses belonging to housing category A. The data have been analyzed by Mann–Whitney test.

Moreover, significant differences of FRAP could be found between the <14-years-old horses of category A and those of the other categories (Figure 4A). With regard to the age, finally d-ROMs levels were significantly different for the >14-years-old subjects of category B with respect to those of categories A, C and D (Figure 4B).

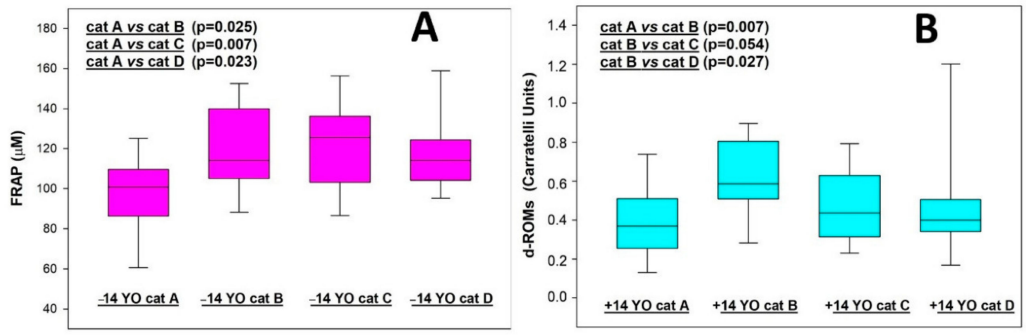

Figure 4. (**A**): Box plots of the FRAP values of the <14-years-old horses of the different housing categories. (**B**): Box plot of the WST values of the >14 years old horses belonging to the different housing conditions. The comparisons were realized by Mann-Whitney test.

Taking into account the two prevalent breeds within the study (Italian saddle horses—ISH and Throughbred—THB) it was possible to verify that the FRAP levels were significantly lower in Italian saddle horses than in Throughbred (Figure 4B).

4. Discussion

The primary objective of the work was to evaluate if different housing conditions imbalance the redox status in the horse. As reported in Figure 2 (green boxes) the statistical analysis of the experimental data disproved the hypothesis that lower oxidative stress levels should have been assessed in grazing horses compared to those housed in box stables; moreover, no difference could be observed by comparing the different types of stables. These data suggest that changing of the redox status be rather determined by acute and/or chronic pathological conditions of different origins, eventually accompanied by the insurgence of systemic inflammatory states. Taking into account the limitations of our study that has been carried out in the field, aiming to exclude the influence of other variables on the reliability of the statistical analysis concerning comparison of the horses in different housing conditions, the effects of other variables like age, gender, breed and presence of stereotypies on the values of the oxidative stress markers considered, were also assessed. Interestingly as reported in Figure 2, the statistical analysis of the blood parameters of all the horses enrolled did not reveal any significant difference in the various groups, with the only exception of FRAP, but limited to the comparison of

the two most represented breed, i.e., Thoroughbreds and Italian saddle horses. Actually, there are no data in the literature that reveal variations of this parameter in relation to the breed, while the SOD data, which did not reveal statistical significance between the two breeds, agree with a previous study [57] which, however, compared thoroughbreds with jumping horses. The latter category may be comparable to that of Italian saddle horses, confirming what was previously stated. The difference in the FRAP levels, might indicate a peculiar response of the two breeds to the oxidative stress on a genetic basis. Further investigations comparing groups composed of high and homogeneous numbers of horses of different breed, might aid to shed light on the general relevance of this result. As regards the gender, in agreement with a previous study [58] there were no significant differences in oxidative stress parameters between females and geldings. Another research [57] considered sex as a discriminant to evaluate differences in oxidative stress, and a significant difference in SOD levels between females and geldings was assessed, contrary to our work. A recent study carried out on Thoroughbred horses aged between 2 and 5 years has identified significantly higher values of d-ROMs in females compared to males in this highly selected population, while the biological antioxidant potential and the oxidative stress index were significantly lower in females than in males [59]. Our data agree with those of Mendoza-Nunez et al. [60] which did not found differences between the levels of oxidative stress on the basis of gender in the human species. On the contrary, Brunelli et al. [61] showed a significant increase in the levels of d-ROMs in women compared to men, while no significant changes were observed in the levels of total antioxidants. This suggests that, not only in horses, but also in human, consensus is lacking on the influence of sex on oxidative stress. In human, it has been repeatedly hypothesized that there is a greater risk of developing oxidative stress with advancing age, even if the results of studies are sometimes controversial. Within our study, no significant variations were identified in the oxidative stress parameters in relation to age considering all the horses enrolled when grouped into <14 and >14 years old. Nevertheless, as reported in Figure 3, there was a significant increase in the FRAP and WST values in horses stabled in category A over 14 years of age. These data suggest that even if the housing condition does not generally affect the redox status of the horses, some difference can be found when the variable age is introduced for comparison inside the single categories. Considering the lack of information, further investigation is required to better understand this point. Kirschvink et al. [57] identified a significant decrease in SOD levels in horses older than 6 years. In human, it has been showed a decrease in the levels of FRAP in elderly subjects, thus suggesting a risk to develop oxidative stress in these subjects [62], while another more detailed study [60] showed that with increasing age there is an increase in lipoperoxides associated with a decrease in the total antioxidant capacity and in the activity of glutathione peroxidase, while SOD activity was unchanged, as also shown by our present study; on the contrary, in human, a decrease in the SOD activity in elderly subjects has been demonstrated, leaving the controversy still open [63]. According to Mendoza-Nunez et al. [60], it could be useful to verify a potential increase in the risk of oxidative stress with increasing age, in order to provide for a preventive integration with specific antioxidants in older horses.

Regarding the age, the data reported in Figure 4, finally show that taking into account either <14 or >14-years-old subjects, the redox status can be affected at a different extent in the different housing conditions. In particular, FRAP levels were lower for the <14-years-old subjects of category A with respect to the horses of the same age belonging to other categories. Horses as wild animals, live in groups of consistent numerosity, therefore the complete lack of interactions with other subjects may have a negative effect on their attitude of living as part of a community. It is plausible to hypothesize that the lack of contacts might have a more negative impact on the young subjects who have not yet developed their social behaviors. Further studies, considering more restrictive age categories, might aid to provide indications on this point. More cryptical is the evidence of the data reported in the figure for the >14-years-old horses, that show a peak of the d-ROMs levels for the horses of housing category B. Nevertheless, also this finding supports the indication came out from this study that the redox status of horses of different ages can be influenced by the housing condition.

Another aim of the study was to evaluate whether oxidative stress parameters could be modified in horses that exhibited stereotyped behaviors compared to horses that did not exhibit these alterations. It should be noted that in our enrolled horses, the prevalent stereotype (21 subjects out of 25 total subjects) is crib biting, followed by weaving (2 subjects) and finally by the circling (1 subjects) according to previous data [44]. Moreover, from our present study emerges a significant prevalence of subjects with stereotypy within category A thus suggesting that the type of housing condition could significantly affect the manifestation of stereotypies. The analysis of oxidative stress parameters in relation to the presence or absence of stereotyped behaviors needs to be investigated since no conclusive results have been reached at present. Omidi et al. [64] evaluated the basal levels of oxidative stress and antioxidant capacity in horses with stereotypies compared to control horses and on horses with stereotypies compared the values in basal conditions and following a prolonged episode of crib biting. Since our study only took into account the basal levels of oxidative stress in horses with stereotypy, the comparison can only be made on these data. For the evaluation of oxidizing agents in the previous study, malondialdehyde (MDA) was used, in contrast to our study where NO, WST and d-ROMs were used. Omidi et al. [64] found no significant differences in baseline MDA values between healthy and stereotyped horses. However, although using different parameters to investigate similar aspect, it can be said that our data are in agreement with the data reported, having not detected significant differences in the levels of NO, WST and d-ROMs. As regards to the evaluation of antioxidants, the data reported by Omidi et al. [64] show a significant decrease in the total antioxidant capacity (TAC) and in the activity of the SOD, CAT and GPx enzymes both in the basal levels and following an intense episode of stereotyped behavior. Another study [65], found in the crib biters a significant drop in selenium levels, a trace element closely linked to the antioxidant capacity. Our measurements regarding the antioxidant component were based on the evaluation of the FRAP and the activity of the SOD, which however gave results that disagreed with the previous studies as there were no significant changes in these two parameters. For this reason, further investigations would be necessary, in order to better clarify these aspects. The different types of housing can interfere with the horse's state of mental and physical well-being. Oxidative stress seems to play an important role in the onset of numerous pathologies. Therefore, it is possible to hypothesize a role also in the determination of some stereotypies. However, the analysis of the data obtained did not reveal relevant changes of the redox status in relation to the management conditions taken into consideration. Some impact was assessed only for horses of different breeds and when the horses, within the different housing condition categories, where grouped on the basis of the age. Nevertheless, from what emerged from this study, there are no risk factors that can negatively influence the redox status related to the conditions in which horses are kept in Italy, even if these data are not to be considered completely exhaustive. Therefore, also in light of the partial disagreement highlighted between our and previous studies on the redox status in the course of stereotypy, it would be appropriate to carry out studies aimed at evaluating these aspects in the course of anomalous behavior on a more consistent animal population.

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