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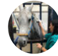

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Changes in saliva biomarkers during a standardized increasing intensity field exercise test in endurance horses

M.D. Contreras-Aguilar [a], J.J. Cerón [a], A. Muñoz [b,1], I. Ayala [c,*]

[a] Interdisciplinary Laboratory of Clinical Analysis of the University of Murcia (Interlab-UMU), Veterinary School, Regional Campus of International Excellence 'Campus Mare Nostrum', University of Murcia, Campus de Espinardo, 30100 Espinardo, Murcia, Spain

[b] Department of Animal Medicine & Surgery, Veterinary School, Campus Univ Rabanales, University of Córdoba, E-14004 Córdoba, Spain

[c] Department of Animal Medicine & Surgery, Veterinary School, Regional Campus of International Excellence 'Campus Mare Nostrum', University of Murcia, Campus de Espinardo, 30100 Espinardo, Murcia, Spain

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Salivary biomarkers could be useful to evaluate stress, fitness level, and skeletal muscle damage associated to exercise in horses in an easy and non-painful way. Therefore, this study aims to evaluate if cortisol in saliva (**sCor**), salivary alpha-amylase (**sAMY**) and butyrylcholinesterase (**sBChE**) and lactate (**sLA**) and creatine kinase (**sCK**) in saliva of horses can show changes during a standardized exercise test, and if they are related to heart rate variability (**HRV**) parameters related to sympathetic and parasympathetic tone, fitness level or skeletal muscle damage. For this purpose, ten endurance horses were submitted to a standardized exercise test in field conditions. Saliva and blood were obtained at basal time (**TB**), after the seven bouts of velocity (T + 01 to T + 07), and 5, 15, 30, and 45 min later (T + 5, T + 15, T + 30, and T + 45). Five endurance horses in resting condition (control group) were also enrolled. HRV and fitness level parameters, and plasma CK as a marker of muscle damage were also evaluated. Salivaryalpha-amylase increased at T + 30 (P = 0.03), sBChE at T + 5 (P = 008), and sCK at T + 07 (P = 0.009) after the exercise test, with significant differences between the exercise and control groups' results. The sCor did not show significant changes during the exercise test in the exercise group but higher concentration compared to the control horses (P < 0.001) were observed. sCor, sAMY, sBChE, and sCK showed a positive correlation (r values between 0.47 and 0.64) with the sympathetic tone and a negative correlation (r values between −0.37 and −0.56) with the parasympathetic tone. In conclusion, sAMY, sBChE, and sCK showed significant increases in ten endurance horses after an increasing intensity velocity exercise. Values of sCor, sAMY, sBChE, and sCK were associated with HRV, which is used to evaluate stress, and therefore, they could be potentially used to assess the exercise-related stress after a physical effort.

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Implications

The evaluation of stress associated to a physical effort is of importance not only in endurance horses, but also in all horses from other disciplines. In this study, we demonstrated that some saliva analytes such as salivary alpha-amylase, butyrylcholinesterase, and creatine kinase increase in a physical effort and could be potentially used to assess the stress associated to this effort. Although this should be considered a pilot study, these results open a new window for the possible application of saliva analytes to evaluate the changes associated with exercise in horses.

Introduction

The assessment of the fitness level and stress in horses from different exercise disciplines during exercise test, in field conditions or using treadmills, is a topic of interest (Couroucé, 1999; Rietmann et al., 2004; Davie and Evans, 2006; Fraipont et al., 2011; Lorello et al., 2019). The fitness level in horses is commonly evaluated from blood lactate (**bLA**) accumulation and heart rate response to defined velocities during an increasing intensity exercise test. Velocities, at which bLA concentrations of 4 mmol/L and heart rate of 160 beats/min are reached (V4 and V160, respectively), are calculated and higher values of these parameters have been associated with a better fitness level in horses competing in submaximal exercises (Davie and Evans, 2006; Munsters et al., 2014; Fisher et al., 2016). Evaluation of stress can be assessed by the heart rate variability (**HRV**)

* Corresponding author.
E-mail address: iayape@um.es (I. Ayala).

[1] Present address: Equine Sport Med CEMEDE, veterinary School, Campus Univ Rabanales, University of Córdoba, E-14004 Córdoba, Spain.

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recorded by electrocardiogram, which quantifies the sympathovagal balance during exercise in horses (Rietmann et al., 2004; Borell et al., 2007), and by cortisol concentration, which increases after the hypothalamic–pituitaryadrenal axis activation due to a physical effort (Desmecht et al., 1996; Marc et al., 2000). Additionally, plasma creatine kinase (**pCK**) concentrations have been recently associated with the exercise intensity and training level, being able to reflect the level of skeletal muscle damage in horses during exercise in response to the stress exerted on the muscles associated with repetitive exercise bouts (Muñoz et al., 2002; Fraipont et al., 2011; Fisher et al., 2016).

Nowadays, there is a tendency in the use of saliva as a diagnostic fluid (Franco-Martínez et al., 2020). In particular, in horses, the studies for evaluating the adaptability to exercises of different intensities have been focused on cortisol as a marker for exercise-related stress (Kedzierski et al., 2013; Kang and Lee, 2016; Lorello et al., 2017), and LA as a marker of aerobic capacity (Lindner et al., 2000) in saliva. However, other salivary biomarkers such as the salivary alpha-amylase (**sAMY**) and the butyrylcholinesterase (**sBChE**) have recently been proposed as possible stress biomarkers in horses related to the autonomic nervous system (Fuentes-Rubio et al., 2015; Contreras-Aguilar et al., 2019c; 2019a), or CK in saliva (**sCK**) as possible marker of skeletal muscle damage in dogs (Tvarijonaviciute et al., 2017). Therefore, it could be hypothesized that biomarkers in saliva such as cortisol (**sCor**), sAMY, sBChE, sCK, and LA in saliva (**sLA**) can change during a physical effort, and also that they can be related to physiological variables that are used for the assessment of the fitness level or the autonomic nervous system activation related to stress (HRV parameters) (Schlosser, 1996).

Consequently, this study aims to evaluate (1) the possible changes in saliva analytes related to stress (sCor, sAMY and sBChE), sLA and sCK during a standardized exercise test with increasing intensity at field condition in endurance horses; and (2) the relationship with physiological variables for assessing fitness level (V_4 and V_{160}), exercise-related stress (HRV parameters) and skeletal muscle damage (pCK) in horses.

Material and methods

Animals

Fifteen endurance horses were enrolled in this study and two groups of horses was performed. Ten trained horses (11.4 ± 2.99 years, six geldings and four females; body condition score = 2.9 ± 0.15) (Carroll and Huntington, 1988) in competing endurance races between 80 km (n = 5) and 120 km (n = 5) were selected for the exercise group, while five horses (10.5 ± 4.51 years, two geldings and three females; body condition score = 3.1 ± 0.20) trained in races between 80 km (n = 3) and 120 km (n = 2) for a control group. The population was composed of six Arabian and nine Crossbreds including Arabian bred with a percentage higher than 50%. All of them were checked by a veterinary specialist (**IA**) showing no clinical signs of pain or discomfort after a physical examination, heart and respiratory rates within normal limits (32.3 ± 5.40 and 12.5 ± 2.23, respectively). Animals showed also no hematological (complete blood count including hematocrit value, hemoglobin concentration, erythrocyte indices, platelet count, white blood cell and differential white blood cell counts) or biochemical (chemistry profile including proteins as total proteins and acute phase proteins, hepatic enzymes, metabolites as creatinine, urea, total bilirubin, cholesterol, triglycerides, and glucose; and minerals as phosphorus and total calcium) abnormalities when the blood samples at Tb (Fig. 1) were analyzed.

Horses were fed with a commercial fodder (2–4 kg according to the amount of work and reproductive activity) (Supplementary Table S1) and hay ad libitum (at least 10–12 kg per day). They were fed twice a day, in the morning after exercise and in the early evening.

Experimental design

The exercise group was subjected to a field standardized exercise test (Fig. 1), performed on two different lineal-shape sand tracks, depending on the horse, where they were usually trained. Both tracks had an inclination of 0% and with a similar flat sand surface. The external environmental conditions were noted (Thermo-/Hygrometer National Geographic™, Bresser GmbH, Rhede, Germany), obtaining temperatures of 21.4 ± 3.39 °C and relative humidity of 46.3 ± 10%. All tests were carried out between the months of March and June.

The horses were transported from their facilities to the tracks (16.3 ± 2.5 min) where the exercise test was carried out at least 15 min later. The standardized exercise test was preceded by a warm-up period consisted of 10 min at walk (approximately at 6 km/h) and 10 min trotting at 12 km/h. After, the exercise test started and consisted of seven bouts of progressive intensity exercise, increasing by 3 km/h at every bout at velocities of 15, 18, 21, 24, 27, 30 and 33 km/h (i.e., 4.17, 5.00, 5.83, 6.67, 7.50, 8.33 and

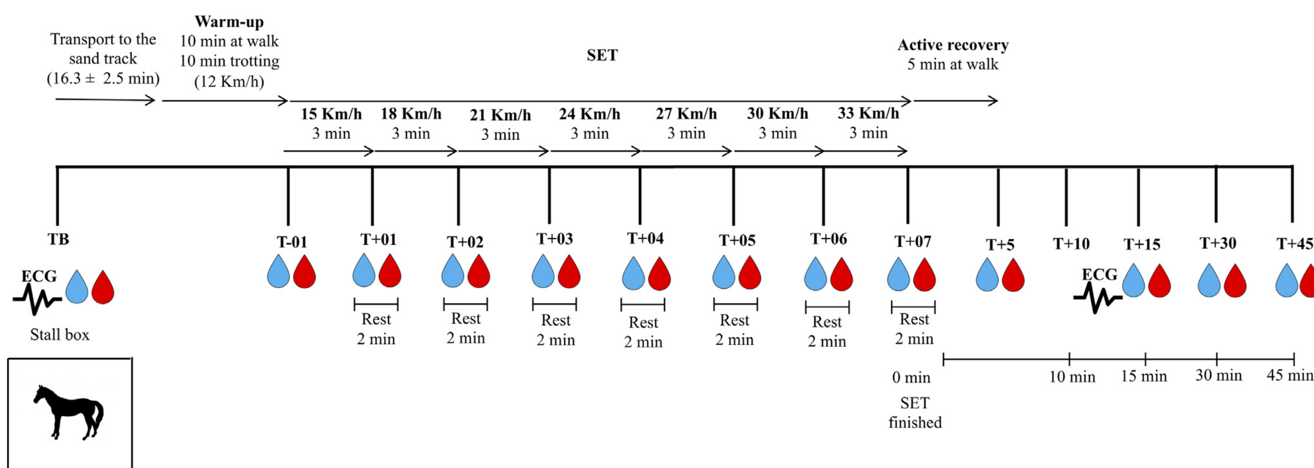

Fig. 1. Scheme of the standardized exercise test (SET) at field conditions in increasing intensity velocity, electrocardiogram (ECG) recordings, and samplings performed in endurance horses.

9.17 m/s), and with a duration of 3 min every bout. The horses were given a 2 min rest period between each bout, where blood and saliva were obtained, always during the first minute after exercise. A 5 min of active recovery at walk was performed immediately after the exercise test. The velocity at each bout was controlled by the horse rider provided with a Polar monitor (Polar® M430, Polar Electro, Kempele, Finland).

The control group was completely evaluated in their origin stable following the same sampling procedure than in the exercise group (Fig. 1) but always in resting states. This control group was sampled in February (18.3 ± 3.21 °C and 32.3 ± 0.4% of relative humidity).

Saliva and blood samplings

The sample collection is outlined in Fig. 1. Basal time (**TB**) in the exercise group was always obtained before traveling to the track in the stable of origin from each horse in calm weather. Saliva was obtained by using a propylene sponge (Esponja Marina, La Griega E. Koronis, Madrid, Spain) as described in Contreras-Aguilar et al., (2020) until sponge was soaked with saliva. Then, the sponge was placed in collection devices (Salivette, Sarstedt, Aktiengesellschaft & Co, Nümbrecht, Germany). At TB and T-01 (Fig. 1), horses' mouth was previously washed using a manual suction pump as reported (Contreras-Aguilar et al., 2020), and horses were not allowed to take any feed until samplings were finished. Blood was always collected after saliva sampling by jugular venipuncture, each time varying the side of the neck and the extraction point from the bottom to the top of the jugular to avoid risks associated with trauma to the sampled vessel. Just after, blood (2.5 mL approximately) was placed into Li-heparinized tubes (Li Heparin, Aquisel®, Barcelona, Spain), where one drop was reserved for the immediate blood lactate measurement. Both saliva and blood samples were kept in refrigeration on ice until arrival at the laboratory (less than 3 h). Once within the laboratory, the tubes were centrifuged at 3 000*g* for 10 min at 4 °C to obtain saliva and plasma specimens, which were stored at −80 °C until analysis (less than 1 month). No blood or food contamination in saliva samples or plasma with hemolysis was observed.

Always the same researchers collected saliva and blood, respectively. In addition, horses were previously made accustomed to the procedure of saliva and blood collection by earlier contact with the researchers. The samplings ranged from 8:30 to 14:00 in both groups.

Heart rate and heart rate variability

The heart rate recorded for each sampling time was obtained with a commercially available heart rate meter (Equine Polar® H7, Polar Electro, Kempele, Finland) at 5 sec intervals. Although the heart rate ideally should be measured by assessing the R-R intervals by a telemetry electrocardiogram system, the Bluetooth system's range from the telemetry unit used for evaluating the HRV parameters (see below) does not exceed 100 meters away. Therefore, it could not be employed during the standardized exercise test. Nevertheless, it is reported a good agreement for heart rate between the heart rate meter and R-R intervals in endurance horses (Lenoir et al., 2017). The sensors were placed on the skin attached to the left thorax wall from the horse according to the manufacturer's instructions and fixed with a stable girth. Gel between the sensor and the skin was applied to improve the conductivity (Electro-gel, Transonic®, The Netherlands). Horses are trained with the heart rate meter and therefore are accustomed to it. Then, the data recorded by the Polar® monitor for each velocity bout (distance, altitude, duration, heart rate and velocity) were synchronized with the Polar FlowSync program (Polar FlowSync program 3.0, Polar®, Polar Electro, Kempele, Finland) to a computer. The heart rate at each bout was considered as the mean heart rate after heart rate stabilization.

The electrocardiogram for the HRV parameter evaluation was recorded continuously at least 5 min (Borell et al., 2007) using a telemetry unit (Televet® 100, Kruuse, Denmark) with a sampling rate of 1 000 Hz and the R-peaks stored in milliseconds. Each R-R interval was checked, and if errors (premature complexes-R-R interval 20% shorter than the previous R-R intervals, artefacts, or second degree auricular-ventricular block) were observed, they were excluded from the analysis. Electrodes (Kruuse ECG, Denmark) were placed as recommended by the manufacturer and underneath a girth to ensure appropriate contact between electrode and skin. A conductive gel (Electro-gel, Transonic®, The Netherlands) was also used. These R-R intervals recorded were down-loaded to a portable computer (Toshiba, Satellite 2450, Spain) and analyzed with Kubius HRV system (Kubios HRV software version 3.1, Biosignal Analysis and Medical Imaging Group). The HRV parameters analyzed are abbreviated and described in Table 1. Frequency domain variables were evaluated using Fast Fourier transformation (Borell et al., 2007; Lorello et al., 2017). The LF and HF power were measured with frequency band thresholds of 0.01–0.07 Hz and 0.07–0.60 Hz, respectively, to be species-specific (Kuwahara et al., 1999; Borell et al., 2007; Stucke et al., 2015; Lorello et al., 2017). The electrocardiogram was recorded at Tb and T + 10 (Fig. 1) since it has been described that well-trained horses have lower heart rate 10 min postexercise compared to horses with a lower training level (Fraipont et al., 2011; Lorello et al., 2017). The movement of horse was always avoided during the electrocardiogram recording and it was performed before saliva and blood samplings in order to minimize stress.

Analyte's measurements

Salivary alpha-amylase, sBChE, sLA, sCK, and pCK were measured using an automated chemistry analyzer (Olympus Diagnostica GmbH AU 400, Beckman Coulter, Ennis, Ireland). Salivary cortisol was assessed by an automated chemiluminescence immunoassay system (Immulite 1 000, Siemens Healthcare Diagnostic, Deerfields, IL). All the assays used in our study have been

Table 1
Time-varying, time-domain and frequency-domain variables of heart rate variability assessed in endurance horses during a standardized exercise test (Borell et al., 2007; Sahoo et al., 2019).

Variable	Units	Description
Time-varying methods		
Stress index	index	Parameter calculated by the formula from Baevsky's stress index.
PNS index	index	Parasympathetic nervous system index calculated based on mean R-R, RMSSD and HF power (n.u)
SNS index	index	Sympathetic nervous system index calculated based on mean heart rate, Baevsky's stress index and LF power (n. u).
Time domain analysis		
SDNN	ms	SD of all normal R-R intervals of the data set.
RMSSD	ms	The square root of the mean of the sum of the squares of differences between successive R-R intervals.
Frequency domain analysis		
LF power	n.u	Low frequency band which integrates both vagal (parasympathetic) and sympathetic influences.
HF power	n.u	High frequency band which depends mainly on vagal (parasympathetic) influences.
LF:HF ratio	ratio	LF power value divided by the HF power value which represents the sympathovagal balance

Abbreviations: R-R = interval between R wave peaks (two consecutive heart beats) in the electrocardiogram; n.u = normalized *unit, as percentage or proportion of total power (LF/total power × 100 or HF/ total power × 100);* ms = milliseconds.

analytically validated (Fuentes-Rubio et al., 2015; Tecles et al., 2016; Contreras-Aguilar et al., 2018; 2019a), with lower limits of quantification of 1.6 IU/L, 4.27 nmol/mL/min, 0.20 mmol/L, 2.5 IU/L, and 0.197 μg/dL, respectively. Manufacturer's control solutions of two different values were used for the quality control analysis of LA, CK and alpha-amylase (Beckman Coulter, Ennis, Ireland, Lot 1043D, and Lot 1044P), while an external control from the ESVE Veterinary Endocrinology External Quality Assessment was used as quality control for cortisol measurements. In all cases, the values of the quality control solutions gave values below 2 SD of the expected value. Blood LA was determined using a calibrated hand-held LA analyzer (SensLabGmbH, Lepzig, Germany), which has been previously validated in horses (Castagnetti et al., 2010), with an analytical range of 0.5–25 mmol/L.

Statistical analysis

The V_4 was extrapolated from the curve of bLA calculated by an exponential line of best fit, while V_{160} from the line regression of heart rate on the velocity bouts (T + 01–T + 07) (Davie and Evans, 2006; Fraipont et al., 2011; Munsters et al., 2014). Both parameters were calculated using a spread-sheet software (Excel 16.35, Microsoft Corporation, Redmond, WA, USA).

Data were checked for normality by the Shapiro-Wilk normality test. Those which showed non-normal distribution (sCor, sAMY, sLA, SDNN, Stress index, PNS index, SNS index, and LF:HF ratio) were previously transformed by log applying the formula $\ln x = \ln (x + 1)$ (Rohleder and Nater, 2009), which restored normality, before the statistical analysis to assess whether or not the different data changed at different times. The two-way ANOVA followed by Tukey's multiple comparison test was performed for the salivary and blood analytes; and followed by Bonferroni pairwise comparison test for the HRV parameters. Additionally, the power $(1 - \beta)$ obtained from the previous statistical analysis were calculated by a posthoc analysis by the G-Power program (Faul et al., 2007) to evaluate if a type I error has been incurred with the number of horses evaluated.

Spearman r was calculated between the fitness level parameters (V_4 and V_{160}) at each time, salivary and blood analytes, and HRV parameters (Milton, 2013). An r value between 0.90 and 1 was considered to have very high correlation, 0.70–0.89 high correlation, 0.50–0.69 moderate correlation, 0.30–0.49 low correlation and less than 0.30 little if any correlation, following the Rule of Thumb (Hinkle et al., 2003). The statistical analyses were performed using the commercial statistics package Graph PadPrism (GraphPad Prism 8 Software Inc., La Jolla, California, USA). A P-value less than 0.05 was selected to indicate significance for all analyses.

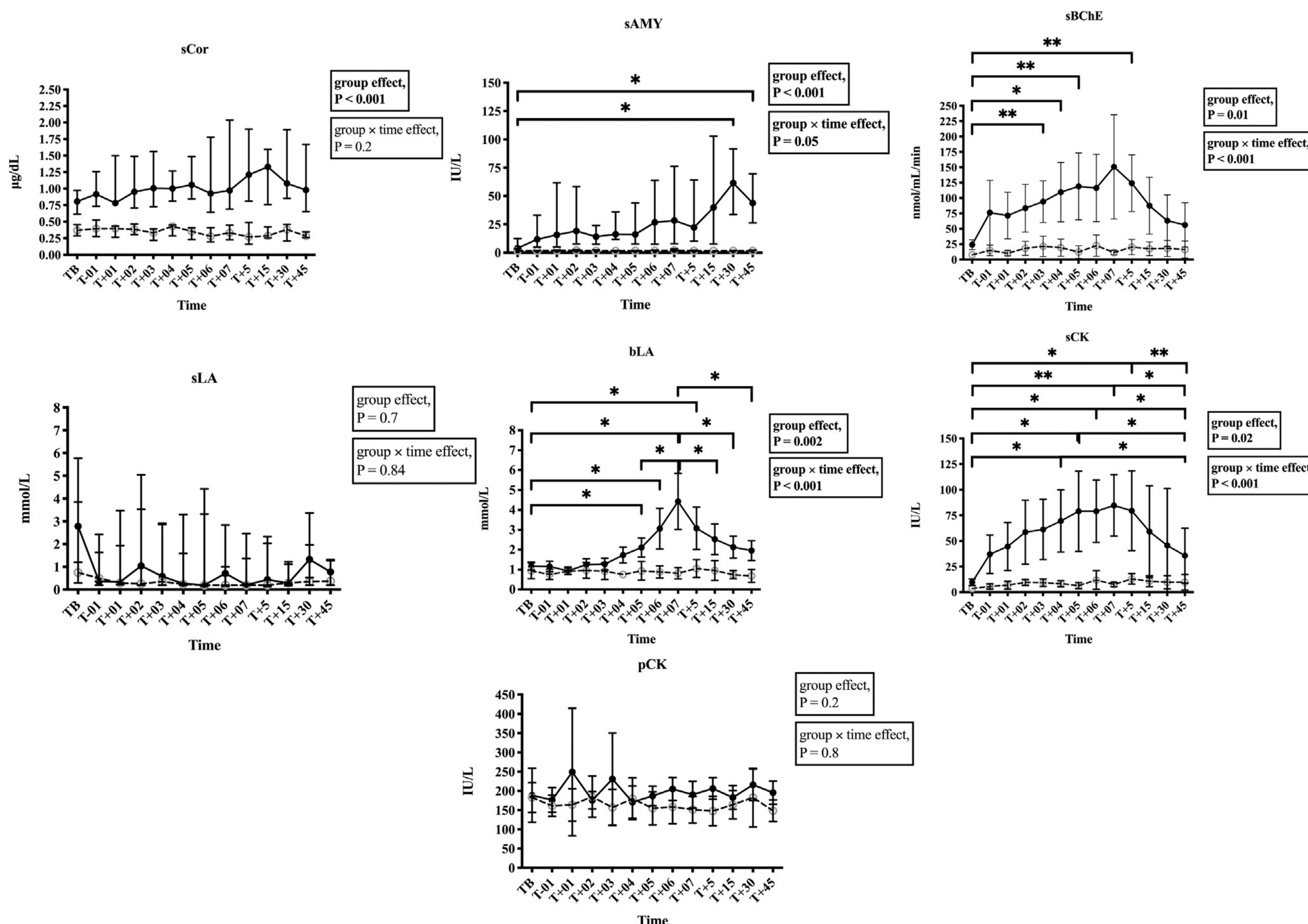

Fig. 2. Results of cortisol in saliva (sCor), salivary alpha-amylase (sAMY), butyrylcholinesterase in saliva (sBChE), lactate in saliva (sLA), blood lactate (bLA), creatine kinase in saliva (sCK), and plasma creatine kinase (pCK) in ten endurance horses during a standardized exercise test at increasing intensity velocity at field condition and in five endurance horses at control conditions. Saliva and blood samplings from each exercised horse (black points and solid lines) were collected at basal level (TB), after a warm-up period (T-01), seven velocity bouts (T + 01 to T + 07), and after 5, 15, 30 and 45 min (T + 5, T + 15, T + 30; T + 45, respectively) of finishing the standardized exercise test; and the same sampling times in the control horses' stable of origin (white points and dot lines). The plots show median (sCor, sAMY and sLA) with interquartile range, or means (sBChE, bLA, sCK, and pCK) with 95% confidence interval. Asterisks indicate statistically significant difference (*$P < 0.05$, **$P < 0.01$) between times. The line under asterisks indicates the times that show significant differences with respect to TB or with a post-time after a Tukey's multiple comparison test.

Results

Changes during the standardized exercise test

The endurance horses enrolled in this study had mean values of V_4 of 34.3 ± 3.92 km/h (9.5 ± 1.09 m/s) and V_{160} of 29.0 ± 5.14 km/h (8.1 ± 1.43 m/s) (Supplementary Table S2).

Salivary and blood analytes results are shown in Fig. 2. Changes along the time in the exercise group and between the exercise and control group were observed in sAMY (P = 0.05), sBChE (P < 0.001), bLA (P < 0.001) and sCK (P < 0.001). Specifically, the higher increases were observed at T + 30 in sAMY (P = 0.03), at T + 5 in sBChE (P = 0.008), at T + 07 in bLA (P = 0.04), and at T + 07 in sCK (P = 0.009) compared to TB. The sCor concentrations showed higher values in the exercise group than in the control group, but no changes occurred during the standardized exercise test.

The HRV parameters which showed changes at T + 10 with respect to TB (Table 2) in the exercise group were the stress index (P = 0.02), PNS index (P < 0.001), SNS index (P < 0.001), SDNN (P = 0.03), and RMSSD (P = 0.03). Differences between the exercise and control group were only observed with the PNS (P < 0.05) and SNS (P < 0.01) indexes.

The salivary and blood analytes and the HRV parameters which reached a power higher than 80% were sCor (99%), sAMY (92%), sBChE (99%), bLA (100%), sCK (85%), PNS index (99%), and SNS index (99%).

Correlation between the fitness level parameters, salivary and blood analytes, and heart rate variability parameters

In the exercise group, the bLA results correlated negatively and highly or very highly with the V_4 and V_{160} values at T + 05, T + 06, T + 07, and T + 10. The V_{160} value correlated positively and highly with the PNS index and negatively and highly with the SNS index results at T + 10. In addition, the V_4 value and bLA results correlated negatively and highly at T + 04, at T + 5, and at T + 30. However, the fitness level parameters did not correlate at any time with any of the salivary analytes evaluated.

The correlation matrix between the salivary and blood analytes, and the HRV parameters are shown in Table 3. Positive correlations were observed between sCor and the SNS index (low); between sAMY and sBChE with the stress index (moderate and low) and the SNS index (moderate), respectively; between sCK and the stress index (low), and the SNS index (moderate); and between sLA and the PNS index (low). In addition, sCor, sAMY, sBChE, and sCK showed a low and moderate negative correlation with the PNS index, and the sAMY and sCK a low negative correlation with the RMSSD. Finally, sCK values showed low positive correlation with the pCK values. No correlation between sLA and bLA was observed.

Discussion

The standardized exercise test performed in this study was applied according to the conditions previously reported, including ambient temperature and humidity, track and surface, duration and incremental velocity of each bout, recovery period between bout, the constancy of velocity, method of saliva and blood collection, and handling and analyzing samples (Couroucé, 1999; Munsters et al., 2014). In addition, the majority of horses (seven of the ten) reached the threshold of 4 mmol/L of bLA during the exercise test (Supplementary Table S1). Also, a significant increase in bLA concentration was reached when the last velocity was run by the horses. These data would indicate that the model of standardized exercise test performed in this study produced the physiological changes expected associated to a physical effort.

The saliva sampling procedure used in this study was based on the previously validated method in horses (Contreras-Aguilar et al., 2020), avoiding food contaminated saliva by cleaning the horses' mouth before the collection, and using a polypropylene sponge instead of a cotton roll to avoid possible interferences (Contreras-Aguilar et al., 2020). All the salivary analytes studied, except the sLA, showed higher values in the exercise group than in the control horses. In humans, sAMY activity increases immediately after an intense physical effort (Koibuchi and Suzuki, 2014). It is known that sAMY is released by salivary glands mainly by the β1-receptors excitation (Contreras-Aguilar and Gómez-García, 2020) due to the bindings with catecholamines secreted by the postganglionic sympathetic neurons (autonomic nervous system) and the adrenal medulla (sympathetic adrenal medullary) within a rapid activation (Hyyppä, 2005). However, horses showed sAMY increases only 30 and 45 min after the standardized exercise test in our study. The possible decrease in the sympathetic activity and return of the parasympathetic dominance during those times could be the reason of the delayed sAMY activity increase since this enzyme is also secreted due to parasympathetic stimulation (Contreras-Aguilar and Gómez-García, 2020).

Although the sBChE function is unknown, there are evidences reporting an overexpression of cholinesterase in the nervous tissue after acute stress (Kaufer et al., 1998; Meshorer et al., 2002). Cholinesterase in saliva has been related to the autonomic nervous system activity (Fedorova et al., 2015), and increases after acute

Table 2
Heart rate variability (HRV) parameters' mean (95% confidence interval) or median [Interquartile range, 25th–75th percentiles] obtained from ten endurance horses during a standardized exercise test at increasing intensity velocity at field conditions and five endurance horses at control conditions. *In the exercise group, the TB (basal time) was recorded in the stable of origin and T + 10 at 10 minutes of finishing the standardized exercise test. The control group was evaluated entirely in their stable of origin following the same recording procedure than in the exercise group always in resting states.*

HRV parameters	Exercise group		Control group		Group × time effect (P-value)
	TB	T + 10	TB	T + 10	
Stress index	2.6 [1.97–5.75]	6.9 [5.08–11.30][a]	2.6 [1.65–3.85]	3.9 [2.63–8.20]	0.5
PNS index	9.0 [5.61–15.85]	2.5 [1.79–2.80][b]	11.0 [4.43–13.80]	7.0 [4.9–10.7]	**0.02**
SNS index	−3.5 [−3.73 to −2.74]	−1.5 [−1.66 to −1.32][b]	−3.7 [−3.76-−2.54]	−3.4 [−3.64-−2.61]	**0.005**
SDNN	128.4 [49.40–286.10]	44.0 [25.23–72.30][a]	137.6 [54.55–233.80]	86.7 [44.55–151.80]	0.3
RMSSD	299.3 (89.36–509.3)	65.6 (34.22–97.00)[a]	223.6 (60.10–471.8)	144.6 (40.90–337.70)	0.3
LF power	31.6 (20.84–42.27)	33.0 (23.83–42.17)	27.3 (12.27–44.49)	32.2 (9.62–68.15)	0.8
HF power	68.6 (57.79–79.32)	67.0 (57.83–76.17)	72.7 (55.37–87.83)	67.8 (23.77–10.63)	0.8
LF:HF ratio	0.55 [0.231–0.727]	0.44 [0.276–0.693]	0.31 [0.161–0.745]	0.36 [0.137–1.439]	0.6

Abbreviations: PNS = parasympathetic nervous system; SNS = sympathetic nervous system; SDNN = SD of all normal R-R intervals (interval between R wave peaks, two consecutive heart beats, in the electrocardiogram) of the data set; RMSSD = the square root of the mean of the sum of the squares of differences between successive R-R intervals; LF = low frequency; HF = high frequency.

[a,b] Letters show statistical analysis results between times in Bonferroni pairwise comparison; a: P < 0.001; b: P < 0.05.

Table 3
Correlation coefficients between the selected salivary and blood analytes, and the heart rate variability parameters in ten endurance horses during a standardized exercise test at increasing intensity velocity at field conditions and in five endurance horses at control conditions. *The correlation study included data from all the times sampled. Values in bold were considerate with statistic correlation following the Rule of Thumb.*

Parameters	sCor	sAMY	sBChE	sLA	sCK
sCor		**0.73*****	**0.79*****	0.10	**0.62*****
sAMY	**0.73*****		**0.67*****	0.12	**0.55*****
sBChE	**0.79*****	**0.67*****		−0.11	**0.88*****
sLA	0.10	0.12	−0.11		−0.15
bLA	**0.57*****	0.61***	0.61***	−0.08	**0.50*****
sCK	**0.62*****	**0.55*****	**0.88*****	−0.15	
pCK	0.10	0.18	0.20	−0.05	**0.39*****
Stress index	0.24	**0.53****	**0.36***	−0.21	**0.42***
PNS index	−**0.37***	−**0.56****	−**0.51****	**0.38***	−**0.53****
SNS index	**0.47***	**0.64*****	**0.56****	−0.31	**0.61*****
SDNN	−0.15	−**0.44***	−0.27	0.18	−**0.35***
RMSSD	−0.19	−**0.46***	−0.30	0.21	−**0.40***
LF power	0.14	0.22	0.12	−0.21	0.22
HF power	−0.14	−0.22	−0.12	0.21	−0.22
LF:HF ratio	0.14	0.22	0.12	−0.21	0.22

Abbreviations: sCor = cortisol in saliva; sAMY = salivary alpha-amylase; sBChE = butyrylcholinesterase in saliva; sLA = lactate in saliva; bLA = blood lactate; sCK = creatine kinase in saliva; pCK = plasma creatine kinase; PNS = parasympathetic nervous system; SNS = sympathetic nervous system; SDNN = standard deviation of all normal R-R intervals (interval between R wave peaks, two consecutive heart beats, in the electrocardiogram) of the data set; RMSSD = the square root of the mean of the sum of the squares of differences between successive R-R intervals; LF = low frequency; HF = high frequency.
Asterisks indicate significant correlations: *P < 0.05, **P < 0.01, ***P < 0.01.

stress in horses, pigs, sheep, and humans (Yamanouchi et al., 2015; Tecles et al., 2016; Contreras-Aguilar et al., 2019c; 2019b). In this line, our study also showed a progressive increase in the sBChE activity while the physical effort increased, with a positive and negative correlation between the sympathetic and parasympathetic nervous system levels, respectively. The tetrameric form of Acetylcholinesterase type T, which also exists for BChE, is located surrounding the neuromuscular junctions, and it can increase after muscle exercise (Massoulié, 2002). Therefore, it could be possible that a local production of BChE in salivary glands could be the reason for the BChE increases after exercise found in our study since there are myoepithelial cells innerved by the autonomic nervous system surrounding the glandular acini (Tamarin, 1966).

An increase in sCK during the standardized exercise test was observed, while no changes were observed in pCK during the increasing intensity exercise test, as it was also described in a previous report (Muñoz et al., 2002). These divergences between sCK and pCK would indicate that salivary glands could directly synthetize CK. Differences between saliva and plasma CK response with a moderate correlation between both of them have been also previously reported in dogs (Tvarijonaviciute et al., 2017).

It is widely known that exercise activates the hypothalamic–pituitary–adrenal axis in horses (Hyyppä, 2005; von Lewinski et al., 2013), with plasma cortisol increases immediately after the exercise (Kedzierski et al., 2014), and 20–30 min after finishing the physical effort (Marc et al., 2000; Kedzierski et al., 2013). However, in our study, although there was a sCor increase tendency after the exercise test, these changes were not significant. There could be various hypotheses to explain this. One could be that our velocity was not so high (less than 33 km/h) compared to other studies that reached 55 km/h and reported increases in cortisol (Kedzierski et al., 2013). Another hypothesis could be that the higher basal values found in the horses before doing exercise compared to the control group, probably due to a pre-excitability state before traveling to the track as previously reported (Schmidt et al., 2010), could have made the sCor changes after exercise less evident. Lactate in saliva also showed no change after exercise with no correlation with bLA, as it was also reported in a previous study (Lindner et al., 2000).

The HRV parameters were evaluated from ten to fifteen minutes after the end of the effort, since a cardiac recovery time, defined as the time differences between the end of the exercise and the instantaneous heart rate value lower or equal to 64 beats/min, between 10.5 and 15.1 min in endurance horses was reported (Younes et al., 2016), being considered this recovery time a reliable index of training level and may provide a useful marker of fatigue in horses. In the present study, decreases in the parasympathetic tone (PNS index, SDNN, and RMSSD) after exercise were observed as previously reported (Yamamoto et al., 1991; Voss et al., 2002). In addition, similar values of SDNN and RMSSD to those found in our study with our control horses have been reported (Lorello et al., 2017). Furthermore, similar V_4 and V_{160} values in analogous standardized exercise test conditions than those used in our study were obtained in other reports assessing endurance horses (Fraipont et al., 2011). There was a positive and negative high correlation between the fitness level parameter V_{160} and the parasympathetic and sympathetic nervous system after exercise, respectively, ten minutes after exercise. Therefore, the horses with a better fitness level would have a higher parasympathetic tone during the recovery associated to a faster cardiac recovery rate as has been previously reported in the endurance horse (Younes et al., 2016).

Some salivary analytes such as sCor, sAMY sBChE and sCK correlated positively with the HRV parameters associated with the sympathetic nervous system and negatively with those associated to the parasympathetic nervous system, being these correlations higher for the sAMY levels. Also, sAMY and sCK were the only analytes that negatively correlate with the RMSSD, a vagal component, reported as a possible reliable index of postexercise cardiac recovery in endurance horses (Younes et al., 2016). Thus, sAMY and sCK could be more related to the perception of intensity in exercise, and therefore the exercise-related stress.

This study has some limitations. The statistical power was adequate for the most salivary analytes, the HRV parameters evaluated, and the bLA results. Nonetheless, future studies assessing different intensity and duration exercises and including a larger population grouping according to different fitness, breeds, and age should be performed to corroborate the results of this study, and to fully evaluate the potential of these salivary analytes as welfare biomarkers in horses to discriminate stress or overtraining states during exercise. Ideally, the protein concentration and the saliva volume obtained should have been controlled to determine whether the salivary biomarkers' increased levels found in our study, particularly the sBChE and sCK, could be due to increases in salivary production/releasing or to lower fluid into the horse's mouth. However, this effect would not fully explain these higher

analyte concentrations during the increasing intensity exercise, since no similar increases were observed at these times in other analytes that could also be affected by the concentration of saliva due to exercise such as sLA. The salivary analytes' data correction by protein concentration or salivary flow produces contradictory interpretations (Contreras-Aguilar et al., 2017). Additionally, the HRV parameter evaluation during the exercise and though after but not only in the late recovery phase will help to better understand the relationship between the salivary analytes measured in this study with the sympathetic/parasympathetic balance while the exercise in horses.

Conclusions

In our experimental conditions, sAMY, sBChE, and sCK can increase in endurance horses submitted to a standardized exercise test based on an increasing intensity velocity at field conditions. Additionally, these salivary analyte changes were associated positively and negatively with the sympathetic and parasympathetic tone, respectively, but not with the fitness level of the horses. Therefore, these salivary biomarkers could be potentially used as additional tools to assess the exercise-related stress after a physical effort. Although this should be considered a pilot study, these results open a new window for the possible application of saliva analytes to evaluate the changes associated with exercise in horses.

Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.animal.2021.100236.

Ethics approval

This project has been approved by the Murcia University Ethics Committee with the number CEEA 288/2017. Owners gave informed consent for their animals' inclusion in the study and they were informed about all the procedure and potential risks associated to the exercise test with increasing intensity velocity.

Software and data repository resources

None of the data were deposited in an official repository. The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author ORCIDs

M.D. Contreras-Aguilar: https://orcid.org/0000-0003-2898-0410.
J.J. Cerón: https://orcid.org/0000-0002-8654-1793.
A. Muñoz: https://orcid.org/0000-0001-8613-8059.
I. Ayala: https://orcid.org/0000-0002-0335-482X.

Author contributions

M.D Contreras-Aguilar: Conceptualization. Methodology. Validation. Formal analysis. Investigation. Resources. Writing - original draft. Writing - Review & editing. Visualization. Funding acquisition.
J.J. Cerón: Conceptualization. Investigation. Resources. Writing - Review & editing. Supervision. Project administration.
A. Muñoz: Methodology. Resources. Writing - Review & editing.
I. Ayala: Conceptualization. Methodology. Investigation. Resources. Writing - Review & editing. Supervision. Project administration.

Declaration of interest

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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References

Borell, E.V., Langbein, J., Després, G., Hansen, S., Leterrier, C., Marchant-forde, J., Marchant-forde, R., Minero, M., Mohr, E., Prunier, A., Valance, D., Veissier, I., 2007. Heart rate variability as a measure of autonomic regulation of cardiac activity for assessing stress and welfare in farm animals — A review. Physiology & Behavior 92, 293–316.
Carroll, C.L., Huntington, P.J., 1988. Body condition scoring and weight estimation of horses. Equine Veterinary Journal 20, 41–45.
Castagnetti, C., Pirrone, A., Mariella, J., Mari, G., 2010. Venous blood lactate evaluation in equine neonatal intensive care. Theriogenology 73, 343–357.
Contreras-Aguilar, M., Escribano, D., Martín-Cuervo, M., Tecles, F., Cerón, J.J., 2018. Salivary alpha-amylase activity and cortisol in horses with acute abdominal disease: a pilot study. BMC Veterinary Research 14, 1–7.
Contreras-Aguilar, M., Escribano, D., Martínez-Subiela, S., Martín-Cuervo, M., Lamy, E., Tecles, F., Cerón, J.J., 2019a. Changes in saliva analytes in equine acute abdominal disease: a sialochemistry approach. BMC Veterinary Research 15, 1–9.
Contreras-Aguilar, M.D., Escribano, D., Martínez-Subiela, S., Martínez-Miró, S., Rubio, M., Tvarijonaviciute, A., Tecles, F., Cerón, J.J., 2017. Influence of the way of reporting alpha- Amylase values in saliva in different naturalistic situations: A pilot study. PLoS ONE 12, 1–13.
Contreras-Aguilar, M., Escribano, D., Quiles, A., López-Arjona, M., Cerón, J., Martínez-Subiela, S., Hevia, M.L., Tecles, F., 2019b. Evaluation of new biomarkers of stress in saliva of sheep. Animal 13, 1278–1286.
Contreras-Aguilar, M.D., Gómez-García, F., 2020. Salivary glands' anatomy and physiology. In: Tvarijonaviciute, A., Martínez-Subiela, S., López-Jornet, P., Lamy, E. (Eds.), Saliva in Health and Disease: The Present and Future of a Unique Sample for Diagnosis. Springer International Publishing, Cham, Switzerland, pp. 3–21.
Contreras-Aguilar, M., Henry, S., Coste, C., Tecles, F., Escribano, D., Cerón, J., Hausberger, M., 2019c. Changes in Saliva analytes correlate with horses' behavioural reactions to an acute stressor: A pilot study. Animals 9, 993.
Contreras-Aguilar, M.D., Hevia, M.L., Escribano, D., Lamy, E., Tecles, F., Cerón, J.J., 2020. Effect of food contamination and collection material in the measurement of biomarkers in saliva of horses. Research in Veterinary Science 129, 90–95.
Couroucé, A., 1999. Field exercise testing for assessing fitness in French standardbred trotters. Veterinary Journal 157, 112–122.
Davie, A.J., Evans, D.L., 2006. Blood Lactate Responses to Submaximal Field Exercise. The Veterinary Journal 159, 252–258.
Desmecht, D., Linden, A., Amory, H., Art, T., Lekeux, P., 1996. Relationship of plasma lactate production to cortisol release following completion of different types of sporting events in horses. Veterinary Research Communications 20, 371–379.
Faul, F., Erdfelder, E., Lang, A., Buchner, A., 2007. G*Power: A flexible statistical power analysis program for the social, behavioral, and biomedical sciences. Behavior Research Methods 39, 175–191.
Fedorova, T., Knudsen, C.S., Mouridsen, K., Nexo, E., Borghammer, P., 2015. Salivary acetylcholinesterase activity is increased in Parkinson's disease: A potential marker of parasympathetic dysfunction. Parkinsons Disease 2015, 1–7.
Fisher, A.L., Greene, H.M., Talmadge, R.J., Nout-Lomas, Y.S., 2016. Lactate-guided conditioning program using variable exercise intensities improves fitness and

alters muscle enzyme activity but not inflammatory response in horses. Comparative Exercise Physiology 12, 183–192.
Fraipont, A., Van Erck, E., Ramery, E., Richard, E., Denoix, J.M., Lekeux, P., Art, T., 2011. Subclinical diseases underlying poor performance in endurance horses: Diagnostic methods and predictive tests. Veterinary Record 169, 154.
Franco-Martínez, L., Rubio, C.P., Contreras-Aguilar, M.D., 2020. Methodology assays for the salivary biomarkers' identification and measurement. In: Tvarijonaviciute, A., Martínez-Subiela, S., López-Jornet, P., Lamy, E. (Eds.), Saliva in Health and Disease: The Present and Future of a Unique Sample for Diagnosis. Springer International Publishing, Cham, Switzerland, pp. 67–95.
Fuentes-Rubio, M., Fuentes, F., Otal, J., Quiles, A., Tecles, F., Cerón, J.J., Hevia, M.L., 2015. Measurements of salivary alpha-amylase in horse: Comparison of 2 different assays. Journal of Veterinary Behavior: Clinical Applications and Research 10, 122–127.
Hinkle, D.E., Wiersma, W., Jurs, S.G., 2003. Applied statistics for the behavioral sciences. Houghton Mifflin, London, UK.
Hyyppä, S., 2005. Endocrinal responses in exercising horses. Livestock Production Science 92, 113–121.
Kang, O., Lee, W., 2016. Changes in salivary cortisol concentration in horses during different types of exercise. Asian Australasian Journal Animal Science 29, 747–752.
Kaufer, D., Friedman, A., Seidman, S., Soreq, H., 1998. Acute stress facilitates long-lasting changes in cholinergic gene expression. Nature 393, 373–377.
Kedzierski, W., Cywińska, A., Strzelec, K., Kowalik, S., 2014. Changes in salivary and plasma cortisol levels in Purebred Arabian horses during race training session. Animal Science Journal 85, 313–317.
Kedzierski, W., Strzelec, K., Cywinska, A., Kowalik, S., 2013. Salivary cortisol concentration in exercised thoroughbred horses. Journal of Equine Veterinary Science 33, 1106–1109.
Koibuchi, E., Suzuki, Y., 2014. Exercise upregulates salivary amylase in humans. Experimental and Therapeutic Medicine 7, 773–777.
Kuwahara, M., Hiraga, A., Kai, M., Tsubone, H., Sugano, S., 1999. Influence of training on autonomic nervous function in horses: evaluation by power spectral analysis of heart rate variability. Equine Veterinary Journal 30 (Suppl.), 178–180.
Lenoir, A., Trachsel, D.S., Younes, M., Barrey, E., Robert, C., 2017. Agreement between electrocardiogram and heart rate meter is low for the measurement of heart rate variability during exercise in young endurance horses. Frontiers in Veterinary Science 4, 170.
von Lewinski, M., Biau, S., Erber, R., Ille, N., Aurich, J., Faure, J.M., Möstl, E., Aurich, C., 2013. Cortisol release, heart rate and heart rate variability in the horse and its rider: Different responses to training and performance. Veterinary Journal 197, 229–232.
Lindner, A., Marx, S., Kissenbeck, S., Mosen, H., 2000. Saliva collection and relationship between lactate concentration in blood and saliva of exercising horses. Journal of Equine Veterinary Science 20, 52–54.
Lorello, O., Ramseyer, A., Burger, D., Gerber, V., 2019. Cardiovascular variables in eventing and endurance horses over a season. Journal of Veterinary Cardiology 21, 67–78.
Lorello, O., Ramseyer, A., Burger, D., Gerber, V., Bruckmaier, R.M., Kolk, J.H.V.D., Navas, C., Solis, D., 2017. Repeated measurements of markers of autonomic tone over a training season in eventing horses. Journal of Equine Veterinary Science 53, 38–44.
Marc, M., Parvizi, N., Ellendorff, F., Kallweit, E., Elsaesser, F., 2000. Plasma cortisol and ACTH concentrations in the warmblood horse in response to a standardized treadmill exercise test as physiological markers for evaluation of training status. Journal of Animal Science 78, 1936–1946.
Massoulié, J., 2002. The origin of the molecular diversity and functional anchoring of cholinesterases. Neurosignals 11, 130–143.
Meshorer, E., Erb, C., Gazit, R., Pavlovsky, L., Kaufer, D., Glick, D., Ben-arie, N., Soreq, H., 2002. Alternative splicing and neuritic mRNA translocation under long-term neuronal hypersensitivity. Science 295, 508–512.
Milton, J.S., 2013. Statistical Methods in the Biological and Health Sciences. McGraw-HILL, Radford, VA, USA.
Muñoz, A., Riber, C., Santisteban, R., Lucas, R.G., Castejón, F.M., 2002. Effect of training duration and exercise on blood-borne substrates, plasma lactate and enzyme concentrations in Andalusian, Anglo-Arabian and Arabian breeds. Equine Veterinary Journal 34 (Suppl.), 245–251.
Munsters, C.C.B.M., Iwaarden, A. Van, Weeren, R. Van, Oldruitenborgh-Oosterbaan, M.M.S. Van, 2014. Exercise testing in Warmblood sport horses under field conditions. The Veterinary Journal 202, 11–19.
Rietmann, T.R., Stuart, A.E.A., Bernasconi, P., Stauffacher, M., Auer, J.A., Weishaupt, M.A., 2004. Assessment of mental stress in warmblood horses: heart rate variability in comparison to heart rate and selected behavioural parameters. Applied Animal Behaviour Science 88, 121–136.
Rohleder, N., Nater, U.M., 2009. Determinants of salivary α-amylase in humans and methodological considerations. Psychoneuroendocrinology 34, 469–485.
Sahoo, T., Mahapatra, A., Ruban, N., 2019. Stress index calculation and analysis based on heart rate variability of ECG signal with arrhythmia. In: Proceedings of the Innovations in Power and Advanced Computing Technologies (i-PACT), 22-23 March 2019, Vellore, India, pp. 1–7.
Schlosser, J., 1996. Heart rate variability. Standards of measurement, physiological interpretation, and clinical use. European Heart Journal 17, 354–381.
Schmidt, A., Biau, S., Möstl, E., Becker-Birck, M., Morillon, B., Aurich, J., Faure, J.M., Aurich, C., 2010. Changes in cortisol release and heart rate variability in sport horses during long-distance road transport. Domestic Animal Endocrinology 38, 179–189.
Stucke, D., Große, Ruse M., Lebelt, D., 2015. Measuring heart rate variability in horses to investigate the autonomic nervous system activity - Pros and cons of different methods. Applied Animal Behaviour Science 166, 1–10.
Tamarin, A., 1966. Myoepithelium of rat submaxillary gland. Journal. Journal of Ultrastructure Research 16, 320-.
Tecles, F., Escribano, D., Martínez-Miró, S., Hernández, F., Contreras, M.D., Cerón, J.J., 2016. Cholinesterase in porcine saliva: Analytical characterization and behavior after experimental stress. Research in Veterinary Science 106, 23–28.
Tvarijonaviciute, A., Barranco, T., Rubio, M., Carrillo, J.M., Martinez-Subiela, S., Tecles, F., Carrillo, J.D., Cerón, J.J., 2017. Measurement of Creatine kinase and Aspartate aminotransferase in saliva of dogs: a pilot study. BMC Veterinary Research 13, 168.
Voss, B., Mohr, E., Krzywanek, H., 2002. Effects of aqua-treadmill exercise on selected blood parameters and on heart-rate variability of horses. Journal of Veterinary Medicine Series A: Physiology Pathology Clinical Medicine 49, 137–143.
Yamamoto, Y., Hughson, R.L., Peterson, J.C., 1991. Autonomic control of heart rate during exercise studied by heart rate variability spectral analysis. Journal of Applied Physiology 71, 1136–1142.
Yamanouchi, K., Hayashida, N., Kuba, S., Sakimura, C., Kuroki, T., Togo, M., Katayama, N., Takamura, N., Eguchi, S., 2015. Increase in operator's sympathetic nerve activity during complicated hepatobiliary surgery: evidence for surgeons' mental stress. Tohoku Journal of Experimental Medicine 237, 157–162.
Younes, M., Robert, C., Barrey, E., Cottin, F., 2016. Effects of age, exercise duration, and test conditions on heart rate variability in young endurance horses. Frontiers in Physiology 7, 155.

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