# Salivary Cortisol and Infrared Thermographic Ocular Temperature Use as Biomarkers during Endurance Competitions

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# Abstract

Objective quantification of effort and distress during endurance rides through biomarkers could help manage competitions more effectively and monitor horse welfare through an evidence-based approach. This study aimed to determine if salivary cortisol (SC) and ocular temperature measured by infrared thermography ($IRT^{OT}$) are related to the outcome in endurance competitions. Saliva was collected and $IRT^{OT}$ measured from 61 and 14 horses, respectively, competing at qualifier 40km and 80km rides at Pre-Inspection (PI) and Vet Gates (VG). The variation of the baseline SC at the PI (median±IQR=0.27ng/dl±0.36) into VG1 was abrupt (93-256% rise) and in the next VGs either decreased or rose at a very modest level. Less experienced horses in the 40km ride showed a significantly ($p<0.05$) higher $IRT^{OT}$ (median±IQR=35.7ºC±1.4) at the PI, than their counterparts in the 80Km ride (median±IQR=35. ºC ±1.5). Horses classifying in the Top5, in the 40 km ride category had significantly ($p=0.05$) higher SC levels (median±IQR=0.90ng/ml ±0.61) at the PI, than horses positioned from 10th position on (median±IQR = 0.16ng/ml ±0.40). A lower $IRT^{OT}$ in the PI was correlated with a better placement ($p>0.05$) and those in the Top5 (median±IQR = 33.9ºC ±0.0) had a higher variation (+10.65%) into the last VG. A 62% predictive value for elimination (80% sensibility and 82% specificity) where SC is higher than 0.23ng/ml is advanced. SC and $IRT^{OT}$ can be potentially used in association to characterise physical effort and emotional stress in endurance competitions, but its significance to performance has to be put in context with the competition level.


# Introduction

Endurance ride competitions are long distance races of 40 to 160 km against the clock in phases that consist of a minimum of 16 to a maximum of 40 km, followed by a mandatory rest period, at least equal in minutes to the distance in km of the competition [1]. A pre-inspection (PI) before the event and a veterinary inspection after each phase are compulsorily, and performed by a veterinary commission on every competing horse in an assigned area called the vet gate (VG). Upon examination of the heart rate recovery, metabolic status and regularity of gait, it is the veterinary commission that ultimately decides if a competing horse proceeds to the next phase and, after completion of the last phase, if the competitor merits the qualification and the finish line classification [2]. Despite having the highest elimination rates of all equestrian disciplines and the introduction of stricter Fédération Equestre Internationale (FEI) rules, the recurrence of catastrophic injuries in endurance, particularly musculoskeletal [3–5], frustrates not only competitors, but also veterinarians. Moreover, the ongoing social license debate centred on the health and growing welfare concerns with equine athletes arising from the public and society [6, 7], largely reflected on social media [8], are jeopardizing not only horseracing, but equestrian sport in general, and endurance in particular.

As a result, there is a current quest for solutions to objectively quantify stress in horses during exercise. Biomarkers can be defined as a characteristic, substance or process which can objectively be measured and evaluated as an indicator of normal biologic and/or pathogenic processes and as a predictor of the outcome [9]. Their usefulness in sports consists of providing accurate measurement about the response of an athlete to exercise undertaken. This is particularly important in equine athletes, because they cannot vocalize distress or pain as humans and cannot take decisions for themselves [10]. However, biomarker testing poses some challenges in exercise physiology, i.e. limited sensitivity and specificity of single biomarkers to detect injury risk, interindividual variance in absolute values and relative changes. Results/ reliability are also dependent upon the context, such as previous training level and experience and type of exercise, for example an agility type exercise such as dressage versus and an effort type of exercise like racing. This can result in poorly defined reference ranges for athletes [11]. Exercise is naturally a stressor and, as such, induces a biologic response to exercise that can be either an enhancer or a limiting factor for the sporting ability of an athlete and, therefore, determine the performance obtained [12]. During competition, horses face a mixture of stressors including transportation [13], veterinary examinations [14], rider's ability [15] a new and a noisy environment [16], separation from stable mates [17] and, specifically in endurance, exposure to large conglomerations of unfamiliar horses in large starts, and musculoskeletal pain from an injury that might arise [18]. This complicates the interpretation of the levels of some stress biomarkers because it is hard to separate the impact of the different stressors on the welfare and performance of horses.

Cortisol has been studied exhaustedly in horses to determine stress levels and the response to different types, intensities and durations of exercise in sport and racehorses, including endurance. See the reviews from Hyyppä [19] and König v. Borstel et al. [20] for further information. Cortisol is the end result of the activation of the hypothalamic-pituitary-adrenal (HPA) axis as a response to any psychological or physical stressor. This response is influenced by intrinsic factors (age, gender, breed, inherited temperament, experience) and environmental extrinsic factors (competition setting, noise, type of imposed exercise, weather) [12]. The first cortisol studies were performed using plasma, but the identification of free circulating, i.e. the truly biologically active component of blood cortisol in saliva, and its validation in horses by Peeters et al. (2011), made the collection of this biologic fluid, specially due to its non-invasiveness, much more popular. A circadian rhythm was demonstrated for salivary cortisol although a correlation with blood cortisol seems not to be proportional, most likely due to shifts between the free active component, the sole present in saliva, and the inactive bound component [21]. Cortisol has been studied mostly in controlled environments, but also during endurance [22–24], show jumping [16, 25, 26] and dressage competitions [14, 27, 28]. In all studies effort induced the activation of HPA activity. Cortisol in saliva shows greater variations than in plasma [29, 30]. The highest increases from pre-exercise levels were registered in endurance (up to 1000%) [22] followed by eventing (240%) [31], showjumping (150–340%) and dressage (200%)[27] competitions.

The changes in circulation associated with the HPA axis activation induce periorbital warming that can be quantified by thermal imaging cameras [20]. The use of hairless vascularised areas such as the lacrimal caruncle to measure temperature minimises interferences of skin and coat colour, and environmental conditions [32]. The rise in ocular temperature measured by infrared thermography ($IRT^{OT}$) has been reported as a reliable indicator of short-term stress in animals and is often studied together with salivary cortisol measurements [33]. IRT has identified the levels of stress induced by certain equestrian practices such as neck hyperflexion [15] or a tight noseband [34]. More recently, $IRT^{OT}$ has been also studied in showjumping [35, 36] and dressage competitions [37], in Standardbred harness races [38] and in flat race Arabian and Thoroughbred horses in training [39]. It has been generally accepted that the rise in ocular temperature represents an emotional response to stressors, including exercise [38], as opposed to a physiological response to physical demand of exercise, as proposed recently [40]. $IRT^{OT}$ may represent a measure of emotive reactivity to effort, that can have a beneficial or detrimental effect on performance [15, 38]. For this reason it has recently been proposed as a selection tool to help identify emotional reactivity as a desirable, or undesirable, trait to performance according to the intended use of the horse [37, 38]. The complimentary use of salivary cortisol and $IRT^{OT}$ as non-invasive biomarkers of stress during endurance competitions could help characterise distress and physiological response to effort of endurance horses to endurance exercise in competition.

To our knowledge $IRT^{OT}$ alone or concomitantly with SC has not been studied before during endurance rides. This study aimed to determine trends in salivary cortisol (SC) and ocular temperature measured by infrared thermography ($IRT^{OT}$), and its variation before and during endurance competitions in relation to the outcome and performance of competing horses.

## Material And Methods

# Animals

The study took place during two endurance events in Portugal at two different sites, the first at Polo da Mitra of the University of Evora (MI) in June and the second at Torre de Palma Resort in Monforte (TP) in November. Competitors and/or persons responsible for horses competing in either a 40 km or a 80 km controlled speed (up to 16 km/h) qualifier endurance rides, or a CEN (Concours d'Endurance National)1* 80 km competition, were invited to participate in the research. After being informed about the methods and aim of the study in detail through informed consent, 61 out of a total 110 competitors volunteered for the study. Of these, 34 and 25 were competing in 40 and 80 km qualifier rides, respectively, and two in a CEN*. For data processing competitors were grouped under 40 and 80K categories. Both 40 km qualifier rides were composed of two phases of 20 km each; the cumulative distance at VG1 was 20 km (VG1@20 Km) and at VG2 40 km (VG2@40K). The 80 km rides had different layouts according to the site of the competition. At MI (80 km-A), the 80 km rides consisted in 3 phases of 40 km (VG1@40 Km), 20 km (VG2@60 Km), and 20 km (VG3@80K), whereas at TP (80 km-B) they consisted in 3 phases of 30 km (VG1@30 Km), 30 km (VG2@60 Km) and 20 km (VG2@80K). Thus, at VG1 in MI, horses had performed 40 km and at TP, 30 km. The remaining cumulative distance was the same at both sites, i.e. at VG2 it was 60 km and at VG3 it was 80 km. Saliva for free cortisol determination was collected from 23 horses at MI and, simultaneously with $IRT^{OT}$ measurements, from 38 horses at TP. The PI commenced in both sites at 7:00 AM and starts into the track took place in a staggered manner from 8:00 AM for the 80 km and 9:00 AM for the 40 km rides. The competitions finished around 3:00 PM. All horses were transported to the competition sites the same day of the competition. Transportation time ranged from 15 minutes to 2.5 hours.

$IRT^{OT}$ measurements and/or saliva collections (in that order) were made immediately after the horses had passed the vet gate of the PI (same day), intermediary and final veterinary inspections. If the horse failed to meet the heart rate criteria, the collections were made after the heart rate reinspection. Requested or compulsory reinspections data were collected.

Additionally, the saliva of 14 horses (8 starters for the 40K and 6 for the 80K category) was collected 22 to 24 h before the start of the competitions of the first event at Home. This group was used for comparisons between the SC levels at Home and PI. Furthermore, as control and identification of a circadian rhythm, saliva was collected every two hours starting at 8:00 AM and finishing at 2:00 PM after measuring the $IRT^{OT}$ at home from four horses, six weeks after the second event. Horses measured at home, were selected according to number of horses in a training centre and participating in the competitions in order to minimise environmental variation factors.

Age, breed, gender and previous total km in competitions were obtained from the Portuguese National Federation on-line database. Information from the veterinary inspections, such as first (HR1) and second heart rate (HR2), mucous membranes (MM) and capillary refill time (CRT), skin tent (ST) and trot-up assessment (TR) were obtained from the vet cards, as well as final outcome (Classified versus Fail to Qualify), and performance details (speed, recovery time and classification) were obtained from the timing system. For analysis purposes, groups were created according to final position: Top5 (1 to 5th ), G2 (from 6th to 10$^{th)}$ and G3 (from 11th ). Those that failed to qualify were grouped under FTQ.

# Collection of saliva

A Salivette® (Starsted) synthetic swab was held on a metal clamp and maintained in the horse's mouth for 30–40 s, over and under the tongue, as described before by Peeters et al. [41], and then placed into the Salivette® (Starsted) tube to be stored at 4 °C. At the end of each day, the Salivettes were centrifuged for 10 min at 1500 *g* for saliva extraction and stored at -28 °C until assayed. After thawing the samples, free cortisol was determined using a double-antibody immunoassay kit (Cortisol ELISA, IBL International GMBH, Germany).

# Infrared Thermographic Ocular Temperature ($IRT^{OT}$)

Ocular temperature was measured using a portable infrared thermography camera (Thermal Imaging Camera, E60BX, FLIR Systems AB, Sweden) with 320 × 240 pixels set to an emissivity of 0.98. In order to calibrate the camera results, environmental air temperature and relative humidity were measured with a digital thermohygrometer (MR77, FLIR systems AB) at each collection. The left eye was scanned at a 90º angle at a distance of 1 m, as described previously [36], and several images were obtained. Subsequently, an image analysis software (ThermaCam Researcher Pro, FLIR systems AB) was used to measure the maximal temperature within an oval area traced around the inner canthus of the eye, including the lacrimal caruncle at ~ 1 cm around the outside of the eyelids [42].

# Air Temperature, Relative Humidity and Wind Speed

The air temperature and relative humidity were collected from the local weather station at MI and from the digital thermohygrometer of the thermographic camera at TP. The wind speed was obtained from Weather Underground (www.wunderground.com) at the nearest weather stations (Viana do Alentejo and Badajoz Airport).

# Data Analysis

SPSS® version 22 software (Armonk, NY: IBM Corp.) was used for descriptive analysis and inferential statistics.

Variations in SC (ΔSC) were calculated as percent of variation from one moment of collection to the following, according to the following formula:

$$\Delta \mathrm{SC} = \frac{Mean\ SC\ (t+1) - Mean\ SC(t)}{Mean\ SC(t)}\ x\ 100$$

Where t is a determined moment of collection and t + 1 the following moment of collection. Variations in $IRT^{OT}$ ($\Delta IRT^{OT}$) were calculated in the same manner. Since data did not assume a normal distribution, a series of Kruskal Wallis analyses with post hoc Mann Whitney U tests identified if significant differences occurred between the variables recorded across ride categories, site, breed and gender. A Wilcoxon signed-rank test assessed if cortisol and $IRT^{OT}$ or variation of each measure were significantly different between the moments of collection. Where significance was found, post-hoc Bonferroni t-tests were used for pairwise multiple comparisons. Spearman correlations analysed if cortisol and $IRT^{OT}$ were impacted by age, gender, air temperature, relative humidity, speed and classification of the horses. Analysis significance was set at $P < 0.05$. The predictive ability of cortisol to determine race outcome (classification versus elimination) was investigated using receiver operating characteristic (ROC) curve analysis [43].

## Results

# Descriptive characterisation

Of the 61 horses collected for SC quantification, 34 and 27 horses were competing in the 40K and 80K ride categories, respectively. $IRT^{OT}$ was measured in 38 horses only during TP event, of which 24 were in the 40K and 14 in the 80K category. The MI competition took place in June with temperatures ranging from 16 to 26ºC and a relative humidity interval between 26 and 77%. The TP competition occurred in November with temperatures ranging from 15 to 23ºC for a relative humidity of 58 to 60%. The wind speed was in MI between 7 (morning) and 27 Km/h (afternoon), and at TP between 7 and 19 Km/h.

Across all competitions, 11 horses (18%) failed to qualify: 6 for irregular gait, 2 for metabolic reasons and the remaining 3 for other reasons. There were 30 Arabian, 27 Anglo-Arabian and Part-Arabian horses, and 4 were other breeds of horses. The median age (± IQR) was 6 years (± 3.0), 24 were geldings, 29 mares and eight males. The age of the horses was not significantly different ($p > 0.05$) between the 40K (median ± IQR = 6.0 ± 1.5) and 80K categories (median ± IQR = 6.0 ± 3.0). There was a significant difference in previous experience between the two categories, i.e., horses in the 40 km category had less km in competitions (median ± IQR = 40 ± 30, min = 0, max 120) than horses in the 80 km category (median ± IQR = 80 ± 40, min = 80, max = 240). The speed median (± IQR) was 14.9 km/h(± 2.5) and 15.7 km/h(± 1.0) for the 40 and 80K categories, respectively.Means and medians of SC and $IRT^{OT}$ of all individuals collected at different moments (previous and competition day) are displayed in Supplementary Fig. 1.

The saliva samples were subjectively judged to have less volume with the progression of the ride. Also, many samples were contaminated with food particles that horses kept in the mouth during the ride phases.

## Baseline SC and $IRT^{OT}$ and evolution along the competitions

Despite the reduced subset of data used for comparisons among moments of collection and ride categories or site, it was possible to infer some conclusions among variables.

Baseline SC levels collected at Home and at the PI were not significantly different between ride categories (Supplementary Fig. 1). The SC variation between Home and next day PI was only significant ($p = 0.017$) in the 80K ride category with a 122% rise. An association of SC baseline values, and their variations, with outcome could not be established. At the PI, $IRT^{OT}$ was, however, significantly higher ($p = 0.007$) in the horses competing in the 40K (median ± IQR = 35.7 ± 1.4) when compared to those in the 80K (median ± IQR = 35.0 ± 1.5) category ride (Table 1).

Table 1
Significant correlations of SC (Salivary Cortisol) and OT (Ocular Temperature) measured by Infrared Thermography) with performance data in the 40 and 80 km ride categories.

| **SPEED ρ** | | **N** | **Sig. (2-tailed)** | **Ride Cat** |
|---|---|---|---|---|
| PHASE 1 | | | | |
| ΔSC Home-PI | − 0,7* | 10 | 0,023 | 40 km |
| OT PI | − 0,6 | 13 | 0,024 | 80 km |
| ΔOT PI-VG60km | + 0,6* | 12 | 0,027 | 80 km |
| ΔOT VG60- VG80km | + 0,6* | 12 | 0,037 | 80 km |
| PHASE 2 | | | | |
| ΔSC PI-VG30km | + 0,6 | 13 | 0,047 | 80 km |
| OT VG3 | + 0,6 | 12 | 0,045 | 80 km |
| ΔOT PI -VG60km | + 0,7* | 12 | 0,015 | 80 km |
| ΔOT VG30 -VG60km | + 0,7* | 10 | 0,033 | 80 km |
| PHASE 3 | | | | |
| OT VG3 | + 0,6* | 12 | 0,12 | 80 km |
| ΔOT PI-VG60km | + 0,7** | 12 | 0,009 | 80 km |
| ΔOT PI-VG80 | + 0,7* | 12 | 0,033 | 80 km |
| AVERAGE | | | | |
| OT PI | − 0,6* | 11 | 0,038 | 80 km |
| OT 20 km | − 0,5* | 22 | 0,024 | 40 km |
| OT 80 km | + 0,7* | 11 | 0,024 | 80 km |
| ΔOT PI-VG60km | + 0,8** | 11 | 0,006 | 80 km |
| ΔOT PI-VG80km | + 0,7* | 11 | 0,035 | 80 km |
| RECOVERY TIME | | | | |
| VG1 | | | | |
| OT 60 km | − 0,6* | 12 | 0,034 | 80 km |
| ΔOT VG0-VG80 | + 0,6* | 12 | 0,036 | 80 km |
| VG3 | | | | |

| SPEED ρ | | N | Sig. (2-tailed) | Ride Cat |
|---|---|---|---|---|
| ΔSC VG0-VG30km | − 0,6* | 13 | 0,047 | 80 km |
| ΔOT VG30-VG80km | − 0,8** | 10 | 0,009 | 80 km |
| ΔOT VG60-VG80km | − 0,6* | 12 | 0,028 | 80 km |
| QUALIFICATION | | | | |
| CL vs FTQ | | | | |
| OT PI | -0,5* | 24 | 0,026 | 40 km |
| POSITION | | | | |
| SC PI | -0,5* | 25** | 0,025 | 40 km |
| OT VG80km | -0,7* | 12 | 0,011 | 80 km |
| ΔOT PI-VG60km | -0,8** | 12 | 0,005 | 80 km |
| ΔOT PI-VG80 | -0,6* | 12 | 0,034 | 80 km |
| * (p < 0,05) **(p < 0,001). Pre-Insp (Pre-Inspection ), VG (Vet Gate); 20, 30, 60 and 80 (distance covered in km. Δ (variation between moments of collection) | | | | |

The lowest SC and $IRT^{OT}$ levels were registered in all categories at Home and in PI. The highest SC levels were registered at VG2@40K and VG1@30/40K for the horses in the 40K and the 80K ride categories, respectively. The highest $IRT^{OT}$ was obtained in VG2 in both ride categories, independently of the covered distance (Supplementary Fig. 1).

In the first phase, horses in the 40K covered 20 km at a significantly (p = 0.006) slower speed (median ± IQR = 14.0 km/h ± 1.8), when compared with those in the 80K ride category, that covered either 30 or 40 km (median ± IQR = 15.1 km/h ± 0.9). Subsequently at VG1, the 40K horses had a significantly lower SC (p = 0.006), but a significantly higher $IRT^{OT}$ (p = 0.023), when compared with the 80K horses.

When comparing the same covered distance among ride categories, horses in the 40K having performed two phases of 20 km, with a rest period in-between, showed in VG2 a significantly (p = 0.001) lower SC, when compared with those in the 80K ride that had raced uninterruptedly 40 km in one phase and were at VG1.

The magnitude of variations in SC levels and $IRT^{OT}$ between Home, PI and Vet Gates of different ride categories and its significance is shown in Supplementary Fig. 2. SC variation was highest between PI and VG1, but only significant, in the 80K ride category with a 216% and 256% in 80K-A and 80K-B, respectively. $IRT^{OT}$ variation was also positive, but only significant when values were compared across more than one Vet Gate.

# Analysis of SC and $IRT^{OT}$ measurements regarding performance

Significant correlations between SC or $IRT^{OT}$ and its variations, and performance data (speed, recovery time, final position) are presented in Table 1. Correlations between SC and $IRT^{OT}$ were scarce (Table 2).

Table 2
– Significant correlations between SC (Salivary Cortisol) and (Ocular Temperature measured by Infrared Thermography) in the 80 km category.

| | ρ | N | Sig<br>(2-tailed) |
|---|---|---|---|
| SC 40 km | | | |
| OT 60 km | – 0,9* | 5 | 0,037 |
| Δ SC VG30-VG60 | | | |
| ΔOT PI-VG30km | – 0,8* | 8 | 0,032 |
| ΔOT PI -VG80km | – 0,8* | 9 | 0,012 |
| ΔSC VG40-VG80 | | | |
| ΔOT VG60-VG80km | + 0,8* | 6 | 0,050 |
| ΔSC VG60-VG80 | | | |
| ΔOT VG60-VG80km | – 0,8* | 6 | 0,050 |

* (p < 0,05) **(p < 0,001). PI (Pre-Inspection ), VG (Vet Gate); 20, 30, 60 and 80 (distance covered in Km. Δ (variation between moments of collection)

There were no significant differences in SC or $IRT^{OT}$ measurements between the horses that completed the ride and those that failed to qualify, in none of the moments evaluated. However, a 62% predictive value for failing to qualify (sensitivity of 80% and a sensibility of 83%) where SC was higher than 0.23 ng/ml was found.

The SC or $IRT^{OT}$ levels according to classification groups (Top 5, G2, G3 and FTQ) and its evolution across Vet Gates in both ride categories, can be visualised in Fig. 1.

Regarding comparisons among these groups, the most important findings were that horses classifying in the Top5, in the 40 km ride category, had significantly ($p = 0.05$) higher SC levels (median ± IQR = 0.90 ng/ml ± 0.61) at the PI, than horses positioned in G3 (median ± IQR = 0.16 ng/ml ± 0.40); horses classifying in the Top5 in the 80 km ride category had significantly ($p = 0.05$) lower SC levels (median ± IQR = 0.70 ng/ml ± 1.00) at VG2, than horses positioned in G2 (median ± IQR = 1.88 ng/ml ± 1.00), and significantly ($p = 0.053$) higher $IRT^{OT}$ (median ± IQR = 37.60ºC ± 0.00) at the final Vet Gate (VG3), than horses positioned in G3 (median ± IQR = 35.70ºC ± 1.00). Additionally, a classification in the Top5 of the 40 km ride category was significantly ($p < 0.05$) associated with an $IRT^{OT}$ decrease from PI to VG2. On the other hand, in the 80 km ride category, a lower $IRT^{OT}$ at the PI was significantly ($p > 0.05$) associated with a faster speed in phase 1, overall average speed and completion. Also, the higher the $IRT^{OT}$ variation from PI into VG2 and VG3 was associated with a better placement (Fig. 3). Horses classified in the Top5 (median ± IQR = 33.9 ± 0.0) and in G3 (median ± IQR = 35.3 ± 1.0) had a variation of 10.65% and 1.78% from the PI to VG3, respectively.

# Age, gender and environmental impact in SC and $IRT^{OT}$

No significant differences or correlations were identified for SC or $IRT^{OT}$ with age or gender, except for mares that showed a significantly higher SC ($p = 0.037$) at VG3@80K in the 80K-B ride. An association between air temperature or relative humidity and $IRT^{OT}$ could not be found at any point-in-time.

# Circadian Rhythm

Within the control group to assess a circadian rhythm during the time of day frame corresponding to competition time of the 40K category, three from four horses recorded the highest SC at 10:00 AM at Home and at 2:00 PM (VG2) in the competition. No significant differences were found for SC (Supplementary Fig. 3) or $IRT^{OT}$ measurements from control horses to the competition site.

## Discussion

Endurance riding evolved in the last two decades from an amateur activity into a highly professionalised sport. Better training techniques and more specialised breeding allowed the creation of equine endurance super-athletes, capable of achieving a sustained high speed along with a fast-cardiac recovery capacity. This preliminary study aimed to contribute to determine the way salivary cortisol (SC) and ocular temperature measured by infrared thermography ($IRT^{OT}$), and their variations before and during endurance competitions were related to the outcome and performance of competing horses, and their potential usefulness in depicting compromised horses.

# Behaviour of SC and $IRT^{OT}$ during competitions

Various factors inherent to competitions, such as accustoming to a novel environment [44] and a new group of horses [45] or undergoing a veterinary examination [46] are described as potential stressors to horses. Yet, transportation is considered a major stressor capable of generating greater SC rises than exercise [14]. Even in short distances such as 1 hour, a 4-fold SC increase was previously reported [13]. All horses in our study were transported to the venue the same morning of the competition, arriving typically near the time of the PI and the estimated transportation time ranged between 10 minutes and no longer than 2 hours. Yet, the overall 65% SC increase from the baseline values at Home to the first collection performed at the competition venue immediately after the PI, was modest and less than the rise generated by the competition itself.

Higher cortisol rest levels [14, 26], as well as $IRT^{OT}$ [36, 38] were previously reported in younger and/or less experienced horses. However, basal levels of cortisol were also reported to be similar in a competition setting between horses with different experience levels [47]. In our case, there a significant difference in SC levels at Home or the PI among ride categories. However, $IRT^{OT}$ was higher in the less experienced horses participating in the 40K ride in the PI. Both SC and $IRT^{OT}$ have been used as indicators of distress in non-exercised horses [48]. Yet, ocular temperature is considered a more immediate stress indicator than cortisol reported to take at least 15 minutes to increase after exposure to a stressor [20]. Since $IRT^{OT}$ was measured immediately after exiting the VG this could be a reflex of a higher distress of the 40K horses exposed to the veterinary examination and, often, being separated from their mates at the PI.

Our study corroborates that both intensity and duration, if uninterrupted, contribute to SC increase [49]. Indeed, those horses in the 80K category that performed a straight 40 km phase into VG1 at a higher speed showed a 3-fold higher cortisol level than horses in the 40 km ride, that raced two 20 km phases with a rest period in-between. As expected, both SC and $IRT^{OT}$ minimum values were registered at Home and at the PI. However, regarding the maximum values there was a difference between ride categories. In the 40K ride the maximum SC and $IRT^{OT}$ were registered in the final vet gate, as opposed to the 80K rides, where they were obtained at mid-distance in VG1 and VG2, respectively but not in the final vet gate

Independently of the covered distance and registered levels, it was in VG1 that the steepest SC variations took place in both ride categories, showing much more modest, or even negative variations, in the subsequent VGs. Our study is in agreement with previous studies performed during endurance competitions, that also registered the highest SC increases in the first half of the rides [22–24]. This was also reported previously in human athletes, whose cortisol levels increased after short-term and decreased after prolonged, i.e. lasting several hours, exercise [50]. This drop is believed to result from the negative feedback system generated by the high cortisol levels induced by exercise. Two mechanisms were proposed for athletes to bypass the negative feedback. First, interleukin-6 released from working muscles induced by low glycogen contents seems to act as a hormone, stimulating, similarly to cortisol, the maintenance of glucose homeostasis during exercise and mediating exercise-induced lipolysis [51]. The second mechanism could be the inherent ability of the individual to override the serotonergic mechanisms (that inhibit CRH release and therefore the HPA axis) involved in central fatigue, which are not necessarily related with training level [52]. This drop could also be connected to a decrease in a first moment from a decrease in the emotional stress content experienced by horses. It was also proposed that the initial higher levels could be associated with excitement and not with body demand [53]. Therefore, the variations reported in other studies at similar magnitudes but in much lighter exercises could reflect the emotional stress [27].

One of the proposed added values of the use of $IRT^{OT}$ is its potential independency from the effort effect and thereby providing a valid mean of evaluating the emotive reaction to effort stressors in exercised horses [20]. We could only find very few associations between SC and $IRT^{OT}$. This is line with other studies that investigated SC and $IRT^{OT}$ simultaneously during exercise [15, 36, 38, 39, 54]. One study could establish an association between the two biomarkers in exercise, but only after an ACTH stimulating challenge test [55] and another, during clipping, a non-exercise activity [48]. In this study, the highest $IRT^{OT}$ rise in subsequent vet gates was fromVG1 to VG2 (+ 3.1%), that also corresponded to the highest SC drop (-20%). Yet, between VG2 and VG3 both variations were negative. This could indicate that a decrease in cortisol production during effort response (the physiological response) might not represent the emotional reactivity to prolonged effort.

# SC levels and $IRT^{OT}$ association with competition outcome

Elevations of basal cortisol concentrations in response to emotional stress are believed to be detrimental, but not necessarily to sport performance [56]. Indeed, in the more inexperienced horses of the 40K ride, the higher levels of SC before and during the ride were associated with a better performance, reflecting most likely the extra necessary physiological response to effort (Table 1 and Supplementary Fig. 1). In the 80K category, cortisol behaved differently. It was not the pre-exercise SC level that influenced the results *per se*, but the magnitude of increase from PI to VG1@30 Km that was associated with a higher placement group (Table 1). Moreover, the group finishing in the Top5 showed a significantly lower SC than the slower G2 in the second-to-last vet gate or VG2. This may mean an extra effort of less well prepared horses of G2. If, on one hand, cortisol was shown to increase with level of effort intensity, it was also shown to be higher in untrained horses when subjected to the same amount of exercise than trained ones [57].

$IRT^{OT}$ was proposed as an alternative biomarker capable of quantifying emotional reactivity to effort, as opposed as a straight measure of effort like cortisol [35, 37, 38]. A lower and higher $IRT^{OT}$ before and after exercise, respectively, i.e., a higher variation after exercise, was reported to be associated with better performances by analysing 130 Spanish Standardbred horses in harness races [38]. The same authors concluded that a variation of -0.97% represented the break-point under which physiological stress developed. In this study, the 80K category horses with a lower $IRT^{OT}$ at the PI and a greater rise into the final VG3 were better placed in the final classification (Table 1). Furthermore, this rise was associated with a shorter recovery time in VG3, but not in VG1, which might be attributed to the initial excitement. The 40K ride horses showed very few associations with $IRT^{OT}$. A reason for that could be that they started with an already higher $IRT^{OT}$ at the PI.Negro *et al*. (2018) estimated that an ocular temperature of 37.61ºC before the race with a variation of + 7.57% were the optimal values for the best performance. Our lower number of horses precluded these calculations. Yet, horses classified in the Top5 when compared with G3 had an average $IRT^{OT}$ of 33.8ºC and 35.33ºC with a variation of + 10.65% and + 1.78%, respectively.

A recent study proposed $IRT^{OT}$ as an indicator of physical fitness in ranch horses [40], as opposed to a purely psychological reaction to effort The rise was attributed to an increase of blood flow in muscles and peripheral heat dissipation and correlation was found with creatine kinase (CK), indicating a possible association with muscle damage. More studies are warranted to investigate the meaning and usefulness of $IRT^{OT}$.

# Pain and failure to qualify

In this research, most likely due to the small sample, we could not find a difference or association between eliminated or classified horses and SC levels or $IRT^{OT}$. However, with due reservations due to small sample, and considering the ROC analysis results, we propose a value higher than 0.23 ng/ml SC for a 62% prediction of elimination, with 80% sensibility and 82% specificity.

Responses of cortisol and $IRT^{OT}$ to various aversive procedures involving fear [48] and perceived or real pain [58] have been reported. To our knowledge, the proportion in which musculoskeletal pain contributes to variations of these biomarkers during exercise was not studied before. As noted earlier, the overlap of responses to different stressors by the HPA axis in exercise precludes the analysis of the impact of each factor separately and the setting of thresholds. This is especially true in a competition context, where standardisation of exercise is not possible.

# SC Circadian Rhythm

The control study to assess the circadian rhythm was performed only during the day time, corresponding to the competition schedule, so a full 24 h period was not studied. However, the SC registered peak at 10:00 in SC in (Supplementary Fig. 3) agrees with the results reported by Bohak et al. (2013). The same horses in the competition showed a peak at 2:00 PM supporting a disruption of the circadian rhythm as shown before in endurance competitions [22].

## Limitations Of The Study

# Volume and Food Contamination in SC Determination

In this study, we used the saliva collection protocol described by Peeters et al. (2001). However, in further studies, due to the progressive natural dehydration of the horses, which likely justifies the diminished saliva volume observed as the competitions progressed, an increase in the time of contact of the Salivette® with the oral cavity should be considered with the progression of the ride. How the level of salivary free cortisol is affected by reduced saliva warrants investigation [21]. Yet, high and low flow rates in normal adult humans did not show a difference in concentration in SC [59]. In this study, even if the sample was smaller, the highest increases of SC concentration still occurred in VG1, when horses were supposedly less dehydrated, and not in VG3.

It was also noticed that many saliva samples after extraction were contaminated with food. In order to investigate a possible interference with the results a small trial was performed in five horses after a mouth wash to compare clean saliva and saliva posteriorly contaminated and incubated with different types of food (hay, granulated and grass). No significant differences ($p > 0.05$) were found between the different samples (MM *et al.* 2019, unpublished data). A recent study also showed that food contamination did not alter SC levels significantly [60].

# Non Controlled Interferences with IRT$^{OT}$

The same operator recorded IRT$^{OT}$ measurements during the research study and distance from the operator to the eye was measured at all times. However, we recognise that our values might have been affected by the interference of the environmental conditions throughout the day. Ambient conditions, surface moisture, brightness, sun reflection and wind breeze are some of the variables that have been reported to interfere with IRT shooting [20]. A controlled environment is challenging to achieve in endurance competitions, without interfering with the pace of the competition and time management of the competitors.

# Other parameters not quantified

How circadian rhythm, even if disrupted, could have influenced the variations from PI into VG1 was not taken into account. Also, the impact of different transportation time and characteristics, even if short-distance could have an impact in basal SC and IRT$^{OT}$ was not quantified. Trainers were not questioned about previous training of their horses. Prior competition history, including completions/eliminations rates and previous speed/recovery times/position records were not consulted.

In our study measurement of body temperature was not included due to its perceived invasiveness and practicality in young horses and less experienced as in qualifier rides. Soroko et al. (2016) did not find a post-exercise correlation between rectal temperature and maximum eye temperature in 19 racehorses.

## Future Directions

Currently, it is still challenging to untangle emotional distress and experienced pain from the effort stressor in the exercising horse. As the scientific community has recognised these limitations, there has been a shift in the last years to investigate behavioural indicators of distress due to pain, such as the grimace score and conflict behaviours. An interesting, innovative approach is the use of artificial intelligence through video analysis of facial pain expression to assess animal welfare through physical manifestations [61]. However, to exhaust the topic usefulness of biomarkers in identifying horses at risk during endurance competitions, more extensive studies are needed at high-level competitions, to collect statistically significant samples of horses that failed to qualify.

## Conclusion

The rise of SC was abrupt only in the first part (VG1) of the ride and then either decreased or rose at a very modest level. The pre-exercise higher cortisol or IRT$^{OT}$ levels in less experienced horses (40K ride) seem to indicate an increased susceptibility to stress but did not seem detrimental to performance. However, a value higher than 0,23 ng/ml SC for a 62% prediction of elimination, with 80% sensibility and 82% specificity, was proposed, with due reservations due to the small sample, based on ROC analysis results. In more experienced. horses (80K ride) a lower pre-exercise $IRT^{OT}$ at the PI with a higher variation into VG3 were associated with a better performance

SC and $IRT^{OT}$ can potentially be used in association to characterise physical effort and emotional stress in endurance competitions. Still, its impact in performance should be put into context with the competing horses' level.

## Declarations

# Ethics approval and consent to participate

Written owner's consent was obtained for all horses participating in this study. The department of animal welfare of the Portuguese Directorate-General for Food and Veterinary Affairs with the number 0421/000/000/2016 has approved this project.

# Competing interests

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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# Author's Contributions

(1) Conception and design of the study, or acquisition of data, or analysis and interpretation of data

(2) Drafting the article or revising it critically for important intellectual content

(3) Final approval of the version to be submitted

# Supporting Data

Supporting data can be accessed by request to the corresponding author.

# About the author:

This research is part of the PhD thesis under the title “ Usefulness of non-invasive and objective methods in the assessment of the welfare of horses in endurance competitions” developed by the first author.

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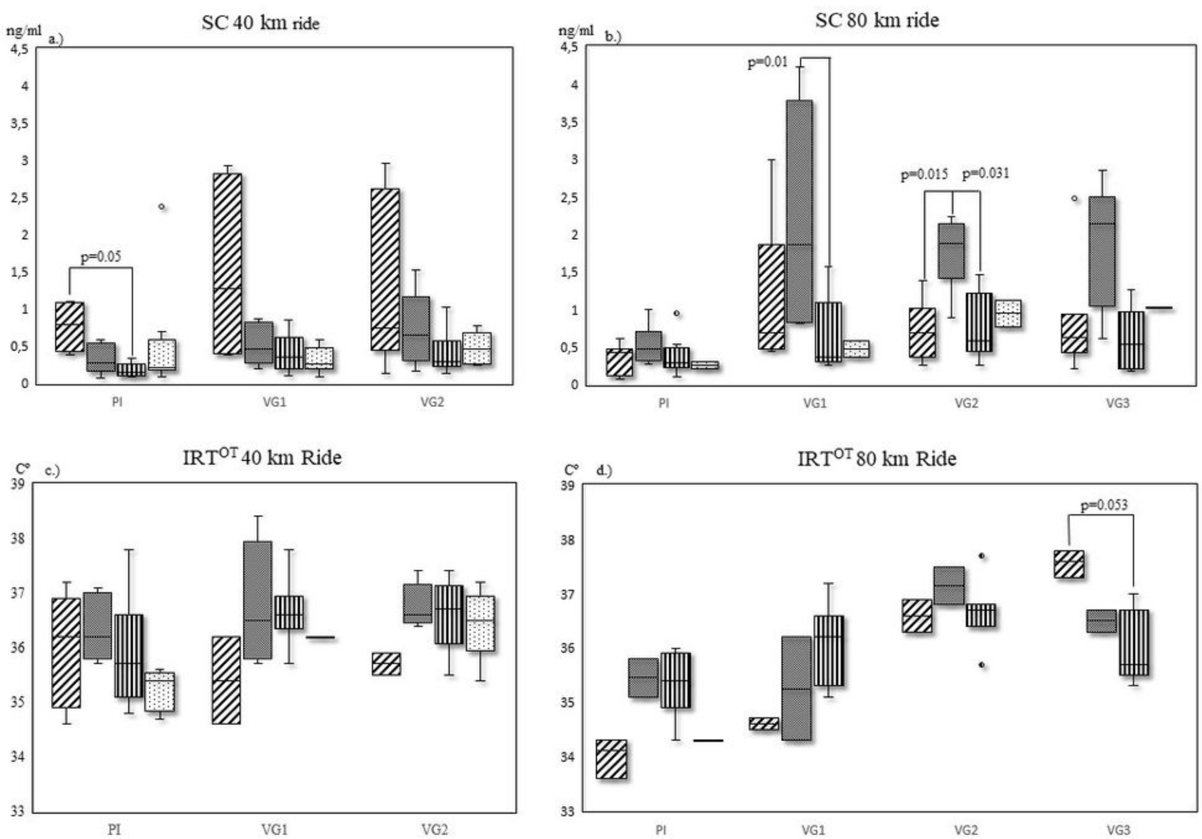
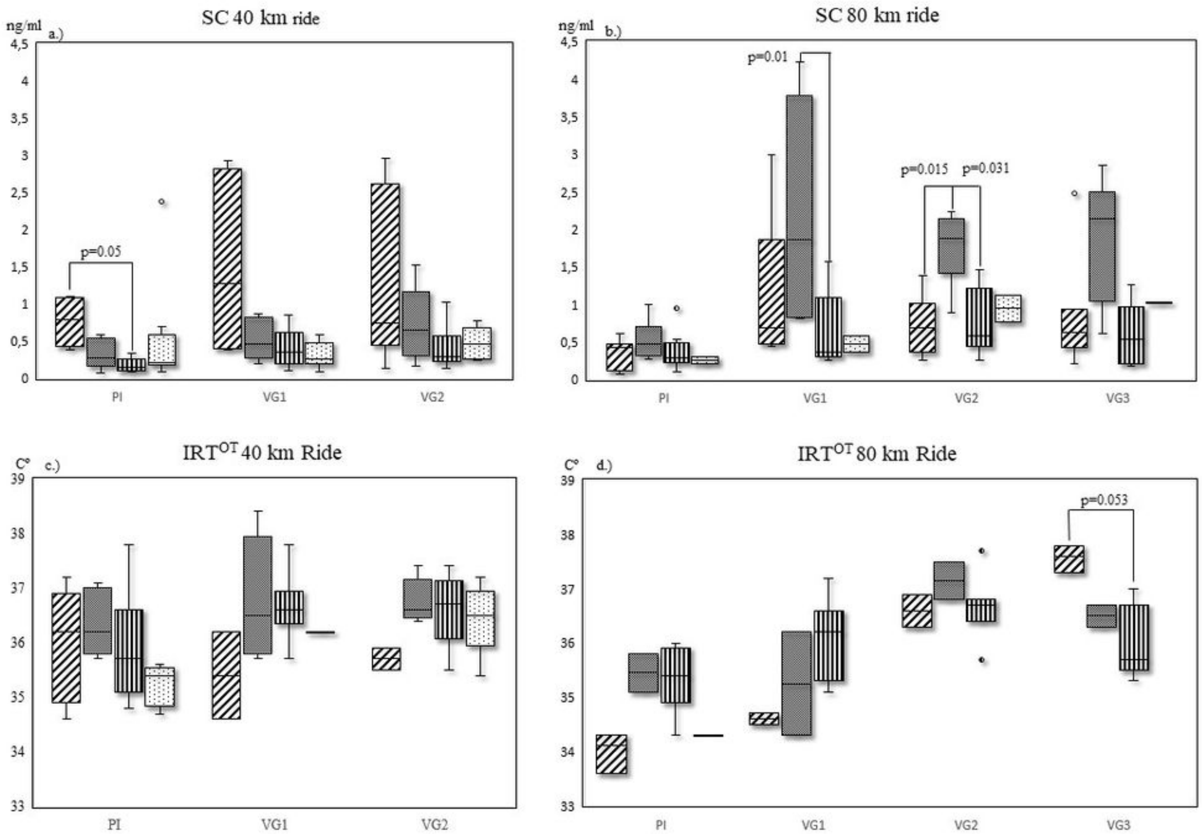
# Figures




### Figure 1

Clustered boxplots of SC (Salivary Cortisol) and IRTOT (Ocular Temperature measured by Infrared Thermography) by group position a.) Salivary Cortisol (SC) in 40 and b.) 80 km rides, and c.) Infra-Red Thermography Ocular Temperature (IRTOT)in 40 and b.) 80 km rides with horses grouped in Top5 (1th-5th), G2 (6-10th), >11th and FTQ (Failed to Qualify), aligned from left to right, at Preinspection (PI) and Vet Gates (VG).. Horizontal X-Axis: Vet Gates (VG); Vertical Y-Axis: SC in ng/dl and IRTOT in centigrade C.

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# Supplementary Files

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