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Infrared thermography as a tool in welfare assessment of equines handled in paddock and stall

[*Termografia infravermelha como ferramenta na avaliação do bem-estar de equinos manejados soltos ou estabulados*]

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ABSTRACT

This study aims to describe the effects of stabling on thermographic characteristics of adult equine limbs, to compare the level of animal welfare between horses kept loose and those under continuous stabling and to investigate whether the Infrared Thermography (IRT) can be an effective tool to evaluate the welfare of equines. 24 gelding horses of Brazilian Sport Horse breed, aged between 5 and 9 years and weighing 500 ± 50kg were used in a 6-week trial. They were divided into 2 groups: 1) Paddock - equines kept in paddocks and ridden exclusively on urban patrol; 2) Stall - animals that ridden exclusively on urban patrol, but were kept in continuous stabling, housed in 12m² with concrete and bedless stables. Every animal was evaluated once a week over six weeks. Animal behavior and temperature of the eyes and distal extremities of the limbs as well as blood samples were collected. The confined horses showed stereotyped behavior and higher serum cortisol, indicating lower animal welfare when compared to those managed in a pasture. Limb IRT showed predictive potential for identifying chronic stress as the discriminating analysis showed 74.5% hits while eye temperature was not efficient for this purpose.

Keywords: physiological parameters, lairage, thermography, behavior

RESUMO

O presente estudo teve por objetivo descrever os efeitos da estabulação sobre características termográficas dos membros de equinos adultos, para comparar o nível de bem-estar entre cavalos mantidos soltos e aqueles sob estabulação contínua e para investigar se a Termografia Infravermelha (TIV) pode ser uma ferramenta efetiva na avaliação do bem-estar em equinos. 24 cavalos machos castrados da raça Brasileiro de Hipismo, com idade entre 5 e 9 anos e peso de 500 ± 50kg foram utilizados em um experimento de 6 semanas de duração. Os animais foram divididos em 2 grupos: 1) Pasto - animais mantidos em piquetes e utilizados exclusivamente em patrulhas urbanas; 2) Baia – animais utilizados exclusivamente em patrulhas urbanas, mas mantidos em estabulação contínua, em cocheiras de 12m² de concreto sem cama. Todos os animais foram avaliados uma vez por semana durante 6 semanas. O comportamento animal, a temperatura dos olhos e extremidades distais bem como amostras de sangue, foram colhidos. Os cavalos estabulados apresentaram comportamento estereotipado e maior concentração sérica de cortisol, indicando menor grau de bem-estar quando comparados aos animais manejados soltos em piquetes. A termografia dos membros apresentou potencial para identificar estresse crônico em cavalos uma vez que a análise discriminante mostrou 74,5% de acerto preditivo enquanto a temperatura máxima do olho não foi eficiente para esse propósito.

Palavras-chave: parâmetros fisiológicos, estabulação, termografia, comportamento

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INTRODUCTION

Animal welfare can be defined as an individual's level of physical, psychological, and behavioral balance in relation to their attempts to the environmental adaptation (Broom, 1986; Broom and Johnson, 1993; Broom and Molento, 2004). Not meeting the needs often impairs welfare and leads to stressful conditions that make it impossible to express properly the animal performance (McGreevy *et al*., 2018) or alter the individual's health condition (Broom and Molento, 2004). Of interest, choosing management practices that favor the application of the animal welfare concepts may represent an improvement in the living conditions of the animal and mitigation of spending on inputs, veterinary procedures and handling time.

There are several reasons why stables are widely accepted as a handling tool for horses, including the reduction of the area required for breeding, higher population density and individualized nutritional control (Brown, *et al*., 2003; Connysson, *et al*., 2019). However, housing may lead to the suppression of the natural behavior of horses, as it implies restriction to grazing, walking and social interaction (Yarnell *et al*., 2015), as well as it imposes to the horse a regimen of physical activity of greater intensity and less frequency, and, in general, long lasting periods of fasting. Such conditions may harm the life quality of the horse (McGreevy, 2012). Prolonged stabling may present even worse effects, especially regarding to the locomotor apparatus of equines: the influence of nutritional handling of stabled horses on the microcirculation of the limbs is well known (Martins Filho *et al*., 2008) but the effects due movement restriction are not clear.

In turn, the diagnosis or measurement of stress degree, as well as the evaluation of the health of locomotor system require restraint and animal manipulation. Such factors undoubtedly disturb and generate stress to animals whose relationship with humans is rare or poorly stablished (Broom and Molento, 2004) as, unfortunately, is common to see in Brazilian horse farms and facilities. Besides, there are evidence that Infrared Thermography (IRT) can be a valuable tool for the recognition of physiological and pathological events (Bouzida *et al*., 2009). In this sense, the possibility of use a precise and non-invasive method that could be performed remotely aiming to assess the degree of welfare would have great implications for animal management. Thus, this technique presents great potential since it allows to obtain images at a distance, which in turn represents the surface temperature of the animals (Souza *et al*., 2008).

Therefore, the present study aimed to describe the implications of stabling on the thermographic characteristics of adult equine limbs, to compare the level of animal welfare of the research groups and to investigate whether the IRT can be an effective tool to evaluate the welfare of equines.

MATERIAL AND METHODS

The present study was approved by the Ethics Committee on Animal Use and Care of the University of Brasília (CEUA/UnB) under protocol No. 20/2018.

The experiment was developed in the 1st Regiment of Mounted Police of the Federal District, and in the 2nd Regiment of Mounted Police of the Federal District, in Brasília/DF. The climate of the region is type AW according to Köppen's classification, with a mean annual temperature of 21.8 °C, with 13 °C and 31 °C as an absolute minimum and maximum, respectively.

Twenty-four gelding horses of the Brazilian Sport Horse breed aged between 5 and 9 years and with a mean weight of 500 ± 50kg were randomly selected and divided into 2 experimental groups: 1) Paddock group (PG) - with 12 horses kept in paddocks and that exclusively patrolled about 10 hours, remaining at rest approximately for 36 hours until the next patrol; 2) Stall group (SG) - with 12 animals kept in a continuous stall regime, housed in 12m² (3m x 4m) stalls, concrete floor, without bedding, also exclusively exercising urban patrol of 10 hours of work interspersed by 36 hours of rest. It is important to emphasize that, except in cases of police occurrence, patrols represent a long lasting low intensity exercise as they are fulfilled, in general, at walk (Ferreira II, 2018). All animals were fed with grass and alfalfa hay (*Cynodon spp.* and *Medicago sativa*) in addition to 5kg of pelletized concentrate/animal/day, and in both experimental groups, the concentrate feed was divided into three meals (earlier, noon and evening). Water was offered *ad libitum* to both groups, but the hay to SG was also divided into three meals, while PG had it available continuously in the paddocks. All horses included in this experiment were submitted to a clinical examen by an experimented veterinarian to ensure there was no limb injury or disease.

The animals were evaluated once a week for six weeks with the purpose of collecting blood samples, acquiring thermal images using IRT camera and collecting behavioral and climatic (environmental) data. Sampling was always carried out at the same time, starting at 08:30 am.

During the experimental period, air temperature (T_{air}) and relative humidity (RH) data were obtained using a portable digital weather station (Instrutemp®, ITWH-1080), installed 2 meters far from the horse containment pen. The animal containment procedure occurred after sampling behavioral data (described below). Blood samples were collected after thermographic image acquisition. Thus, the stress of handling did not interfere with the behavioral and thermographic parameters evaluated.

Initially, a digital camcorder (Sony®, HDR CX-130) recorded each animal for 5 minutes without human presence in the places where they are commonly housed (i.e., SG in the stalls and the PG in the field) according to methodology used by McBride and Cuddeford (2001), Smulders *et al*. (2006) and Lesimple *et al*. (2020). After that, the horses were taken to a shaded area, protected from drafts and the distal region of the limbs were brushed to remove any dust or mud that might be on the target surfaces of the thermography. After this procedure, 10 minutes were timed without any intervention on the animal to stabilize the transient heat generated by brushing. Thermal images were then obtained following the protocol described by Soroko and Howell (2018), considering 2m between the camera and the limbs (for the acquisition of infrared images of the limbs) and 1m between the camera and the horse eyes (for the acquisition of infrared images of the eyes). All thermal images were acquired at a perpendicular angle to the target region.

Thermographic images were acquired always at the same time in the morning and focused on distal extremities of the limbs (dorsal surface of the hoof, metacarpal-metatarsophalangeal joint, metacarpal-metatarsal joint and radio-carpal-metacarpal / tibia-tarso-metatarsal joint) and the eyeballs of each animal. For this purpose, a thermograph camera model T420 (FLIR®, Wilsonville, OR, USA) was used. Emissivity ($\mathcal{E}$) was set at 0.98 based on the recommendation of the camera manufacturer for biological tissues.

Images were analyzed using the software Flir Tools® (v5.13, FLIR Systems) through the ellipse, circle and box tools (Figure 1). The maximum and average temperatures of each region were measured. These two variables (maximum and average) were chosen as study variables as minimum temperatures in an image is more prone to mistakes on image analysis: the “analysis window” could mistakenly include a part of the floor or background (that are invariably colder than animal) or even a foreign object could be misplaced on image and can artificially push down the minimum temperature. Also, maximum temperatures are well described for inflammatory process. The difference in temperature between members of the same animal regarding the influence of T_{air} was analyzed by regression analysis.

Blood samples were collected by jugular puncture once at each visit. Vacuum tubes (Vacuplast®) containing clot-activating factor were used for collection. Immediately after collection, the blood was stored in an isothermal container with ice and transported to the laboratory for further analyses. Blood was centrifuged at 1420g for 5 minutes and serum was frozen at -20 °C until their analysis. Serum was used for quantification of lactate (Lac), Magnesium (Mg^{+2}), alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatine kinase (CK) and lactate dehydrogenase (LDH) using commercial kits for automatic biochemical analyzer (COBAS® C111, Roche). The measurements of serum cortisol (CORT) were performed by ELISA technique using commercial kits of competitive immunoenzymatic assay (AccuBind® ELISA) according to the supplier.

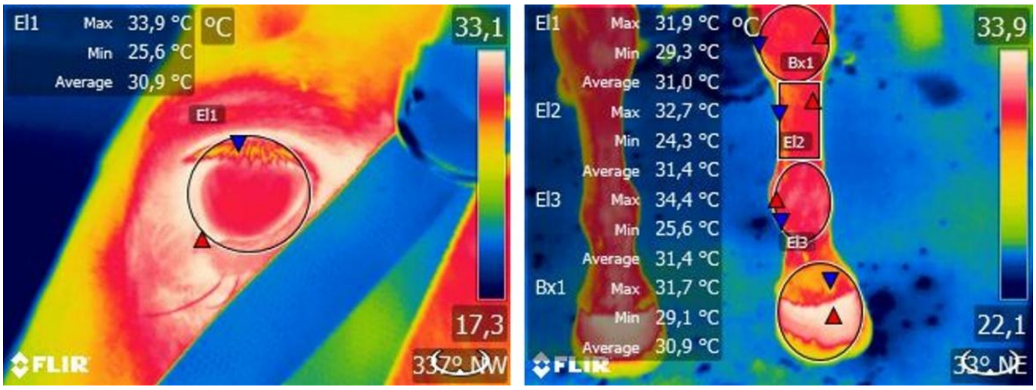

Figure 1. Example of acquisition and analysis of thermal images from forelimb and left eye in gelding Brazilian Sport Horse kept in paddocks or in continuous stabling.

Aiming to do behavioral evaluation (5 minutes per animal), the direct focal observation method proposed by Bosholn and Anciães (2017) was used, with annotations on a spreadsheet always performed by the same observer (Smulders *et al*., 2006; Lesimple *et al*., 2019; Minero *et al*., 2015), totaling at the end of the experiment, 30 minutes of observation for each horse of both groups. Only pathological behaviors were recorded and analyzed according to frequency and lasting, according to methodology used in other domestic species by Haverbeke *et al*. (2009).

The experimental design was entirely randomized, with two experimental groups (PG and SG). Statistical analysis was performed using SAS® (v9.4, Cary, North Carolina), considering 5% of significance level. For multivariate analysis (discriminant, correlation and principal components) R program (Team, 2021) was used as auxiliary tool in order to confirm SAS® results and help in making figures. Repeated measures analysis was performed over time, with the experimental group and the experimental day as fixed factors and T_{air} and RH as covariates. The covariance matrix used was the CS (compound symmetry) type. Then, means were submitted to Tukey's test.

Discriminant analysis using thermograph images, climatic components and blood variables was performed aiming to predict which group each animal belongs to. PROC STEPDISC was used to select a subset of variables used to distinguish between the two treatments by PROC DISCRIM.

Correlation analysis was performed using PROC CORR considering all blood parameters studied, the environmental variables (T_{air} and RH) and temperature obtained by IRT. The behavioral data were submitted to descriptive statistics and correlation analysis, considering, for each recorded behavior, the duration and frequency of presentation. For heatmap correlation figures, data was plotted using CORRPLOT R package (Wei and Simko, 2021).

Principal Component analysis (PCA) was performed using all variables measured (climatic, thermographic and serum biochemistry) for both experimental groups using PROC PRINCOMP and R program. Then, data was plotted using factoextra (Kassambara and Mundt, 2020) and factoMiner (Lê *et al*., 2008) R packages.

RESULTS

Repeated measures analysis did not show significant difference between the two treatments (PG and SG) in any experimental day. Thus, only general results of thermographic images and weather during the trial are shown in Table 1. However, the analysis of temperature from IRT images on the same animal showed significant regression according to T_{air} (Table 2). Serum biochemical variables also did not show significant differences between treatments (Table 3).

Table 1. Means and standard deviation of air temperature (ºC), relative humidity (%), mean and maximum temperatures (ºC) of thoracic member and pelvic limb regions for both experimental groups during the trial

Variable	PG		SG	
	Mean	SD	Mean	SD
T_{air}	22.8	1.99	22.9	2.40
RH	69.7	9.70	58.5	10.80
$T_{maxhoof}$	33.3	2.97	30.1	4.03
T_{mhoof}	30.2	2.81	27.1	3.26
T_{maxeye}	36.5	0.56	36.1	0.65
T_{meye}	34.5	0.76	34.0	0.83
T_{maxA}	31.1	2.01	28.6	2.89
T_{mA}	29.7	2.06	27.2	2.77
T_{maxP}	31.9	1.77	29.9	2.69
T_{mP}	30.2	1.96	28.4	2.73
$T_{maxfetlock}$	31.4	2.22	28.8	3.01
$T_{mfetlock}$	29.7	2.27	27.2	2.85

PG – Paddock group; SG – Stall group; SD – standard deviation; T_{air} – Air temperature; RH – Relative humidity; $T_{maxhoof}$ – maximum hooves temperature; T_{mhoof} – mean temperature of hooves; T_{maxeye} – maximum temperature of eyes; T_{meye} – mean temperature of eyes; T_{maxA} – maximum temperature of forelimbs; T_{mA} – mean temperature of forelimbs; T_{maxP} – maximum temperature of pelvic limbs; T_{mP} – mean temperature of pelvic limbs; $T_{maxfetlock}$ – maximum temperature of fetlocks; $T_{mfetlock}$ – mean temperature of fetlocks

Table 2. Temperature differences between limbs according to air temperature (T_{air})

Region	Comp	T_{air} (°C)				SE	Regression	
		18	21	24	26		Linear	Square
Tarsus	R/L	1.3	< 1	< 1	< 1	0.741	0.0014	ns
Metacarpus	R/L	1.2	1	< 1	< 1	0.001	ns	0.0337
Metatarsus	R/L	1.3	1	< 1	< 1	0.370	0.0037	ns
Fet_F	R/L	1	< 1	< 1	< 1	0.001	ns	0.0393
Hoof_H	R/L	1.7	1.3	< 1	< 1	0.002	ns	0.0238
Carpus/Tarsus	A/P	2.7	2	1.2	< 1	0.001	ns	0.0001
Fetlock	A/P	2.8	1.2	< 1	1.1	0.021	0.0003	0.0021
Hoof	A/P	1.3	1	< 1	< 1	0.061	0.0353	ns

Comp = comparison; SE = standard error; R/L = right and left; A/P = anterior and posterior; Fet_F = Fetlock forelimb; Hoof_H = Hooves hindlimb; ns = not significant.

The animals in the PG did not show stereotypic behaviors. On the other hand, animals from SG did it (Table 4). There was significative correlation between stereotypic behaviors and CORT as well as between themselves (Figure 2). Only those behaviors that achieve minimum values to be assessable were plotted.

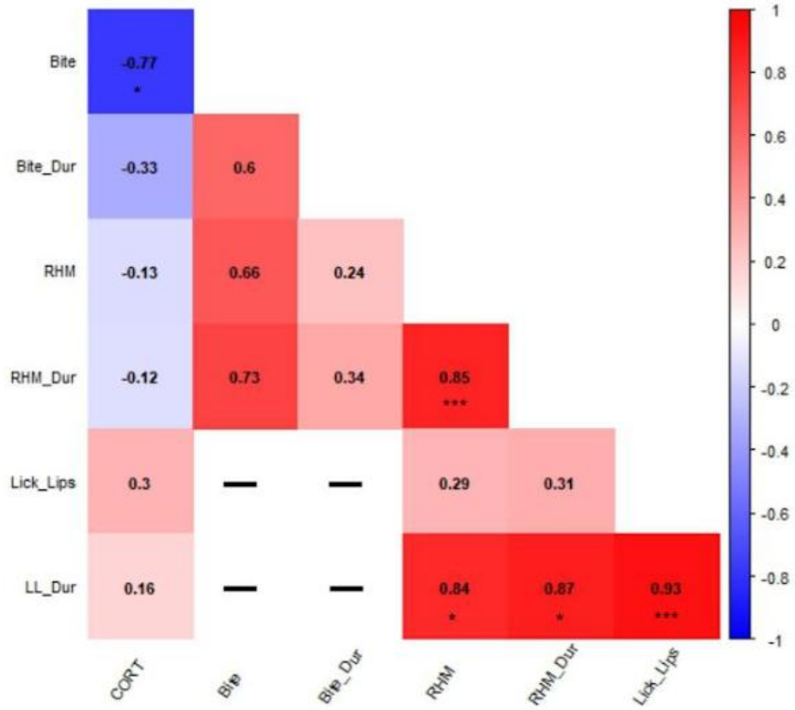

Figure 2. Correlation analysis of Stereotypic behaviors for gelding Brazilian Sport Horse kept in continuous stabling (SG group). CORT – cortisol; Bite_Dur – lasting of bite behavior; RHM – random head movements; RHM_Dur – lasting of random head movements behavior; Lick_Lips – Lips licking behavior; LL_Dur – duration of lips licking behavior.

Table 3. Mean and standard deviation of serum biochemistry variables measured during the trial by experimental group and experimental day

Analyte	Group	Experimental Day												Reference Value (mean ± SD)[1]
		D1		D2		D3		D4		D5		D6		
		M	SD	M	SD	M	SD	M	SD	M	SD	M	SD	
ALT (U/L)	PG	7.1	1.7	8.8	2.4	8.2	2.0	8.6	1.9	6.7	2.5	8.6	1.7	11 - 40
	SG	10.5	2.2	11.3	2.6	10.8	5.0	9.9	1.8	9.7	1.2	9.1	2.6	(27 ± 14)
AST (U/L)	PG	283.2	36.7	281.7	46.5	295.7	85.7	291.8	64.9	279.5	43.1	310.9	41.9	226 - 366
	SG	288.9	50.8	286.6	49.9	343.1	126.4	280.1	50.8	286.3	55.6	268.5	76.1	(296 ± 70)
CK (U/L)	PG	278.3	212.0	226.6	70.7	258.6	126.5	274.1	69.8	310.2	165.6	281.7	100.5	2.4 - 23.4
	SG	237.4	117.0	278.0	90.3	292.7	125.7	212.1	46.4	222.3	58.8	265.5	124.8	(12.9±5.2)
Lac (mmol/L)	PG	1.5	0.2	1.2	0.2	1.6	0.2	1.5	0.3	1.7	0.5	1.9	0.3	1.11-1.78
	SG	0.9	0.3	1.3	0.2	1.5	0.2	1.4	0.2	1.7	0.2	1.5	0.2	(ni)
LDH (U/L)	PG	301.4	74.3	295.1	57.4	264.7	74.2	280.5	60.7	289.8	65.5	314.5	65.7	162 - 412
	SG	277.4	82.7	298.2	71.8	297.4	89.2	291.6	80.8	300.4	90.7	308.0	93.6	(252 ± 63)
Mg (mg/100mL)	PG	1.8	0.2	2.0	0.1	2.1	0.1	2.0	0.2	2.0	0.2	2.1	0.2	2.2-2.8
	SG	1.7	0.1	2.0	0.2	2.0	0.1	2.2	0.2	2.2	0.2	2.1	0.2	(2.5±0.25)
CORT (µg/100mL)	PG	13.2	6.6	12.7	6.7	13.7	7.0	13.5	6.4	16.1	7.4	13.9	4.9	1.30 - 2.93
	SG	10.9	2.3	10.6	2.3	13.5	3.2	10.2	3.3	11.8	4.2	15.0	6.4	(ni)

ALT – alanine aminotransferase; AST – aspartate aminotransferase; CK – creatine kinase; Lac – lactate; LDH – lactate dehydrogenase; Mg – magnesium; CORT – cortisol; M – mean; SD – standard deviation; D1-D6 – experimental days; PG – paddock group; SG – stall group; ni – not informed;
[1]According to (Kaneco *et al.*, 2008)

Table 4. Morbidity, frequency and duration of stereotypic behaviors observed in Stall Group (SG)

Behavior	Morbidity (%)	Frequency		Duration (s)	
		Mean	SD	Mean	SD
Bite	9.72	9.71	6.97	16.14	13.11
Lick	5.56	2.75	0.96	3.50	1.73
Random Head Moviment	26.39	8.00	9.48	11.26	20.53
Aerophagy	4.17	2.00	1.00	3.67	2.08
Lick Lips	16.67	30.67	50.60	28.58	37.59
Walking in Bay	11.11	2.62	2.07	17.37	10.34
Dig	2.78	24.50	27.58	18.00	21.21
Weaving	1.39	1.00	-	1.00	-

SD – standard deviation;

The variables selected by PROC STEPDISC procedure that influenced the distinction between SG and PG were T_{maxLFH} (maximum temperature of left fetlock hindlimb), T_{air}, CORT, ALT, Lac, T_{mP} (mean temperature of pelvic limbs), $T_{maxcarpAR}$ (maximum temperature of right forelimb carpus), T_{maxRFH} (maximum temperature of right fetlock hindlimb) and CK. The discriminant analysis considering these variables was able to correctly classify the animals in 88.3% of the cases (Table 5). Furthermore, canonical analysis using these variables could distinguish experimental groups with 77.8% of canonical correlation (Figure 3). On the other hand, the analysis considering only thermographic parameters was correct in 74.5% (Table 5) and canonical correlation alone was able to produce one canonical variable with 56.8% of correlation.

Correlation analysis for both groups is generally, similar (Figure 4). However, some correlations are present in SG while are absent in PG and vice versa, as CK*AST (58% in SG and not significant in PG) and LDH*AST (60% in SG and not significant in PG) or AST*CORT (57% in PG and not significant in SG) and T_{maxA}, T_{maxP} and $T_{maxfetlock}$ (maximum temperature of fetlocks) that presented positive and significative correlation with RH for PG and were not significant in SG.

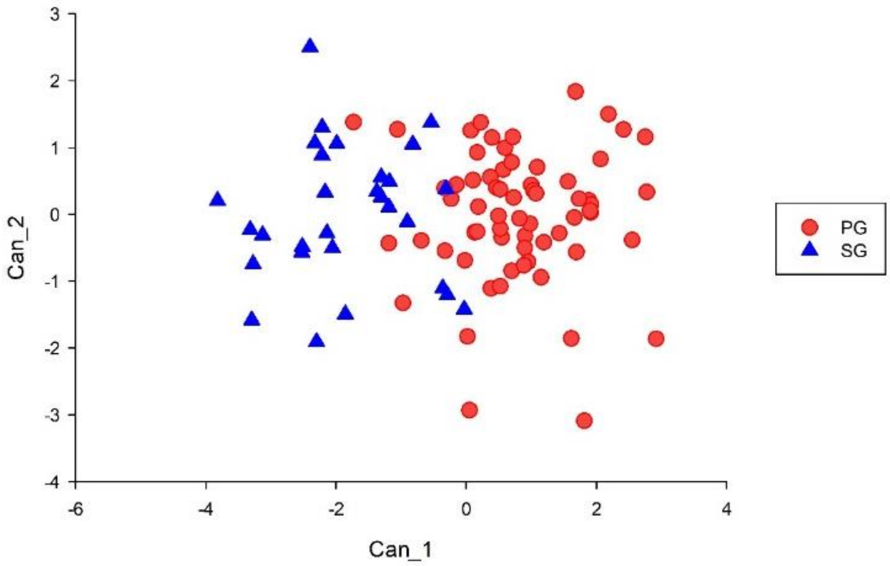

Figure 3.First two canonical variables discriminating the two experimental groups (gelding Brazilian Sport Horse kept in paddocks or in continuous stabling).

Table 5. Discriminant analysis considering STEPDISC chosen variables and Thermographic variables for SG and PG

Experimental Group		STEPDISC variables			Thermographic variables		
		SG	PG	Total	SG	PG	Total
SG	(n)	23	6	29	24	17	41
	(%)	79.31	20.69	100	58.54	41.46	100
PG	(n)	5	60	65	10	55	65
	(%)	7.69	92.31	100	15.38	84.62	100
Total	(n)	28	66	94	34	72	106
	(%)	29.79	70.21	100	32.08	67.92	100
Error Rate		0.2069	0.0769	0.117	0.4146	0.1538	0.255
Priors Probability		0.3085	0.6915	-	0.3868	0.6132	-

PG – paddock group; SG – stall group;

Principal component analysis (PCA) could explain 56.72% of variability in the first two eigenvectors. A major part of thermographic variables was the eigenvector 1 while the serum variables were mainly on eigenvector 2 (Figure 5A). The two experimental groups presented slight differences in the PCA distribution (Figure 5B).

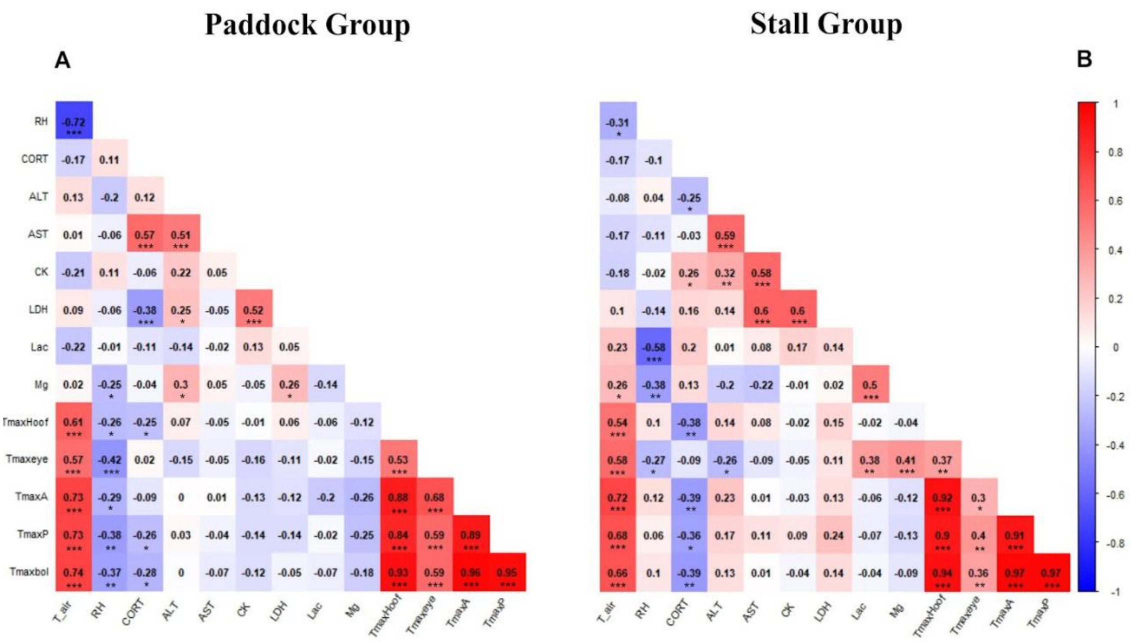

Figure 4. Correlation analysis of climatic, serum and thermographic variables in gelding Brazilian Sport Horse kept in paddocks (Paddock Group – A) or in continuous stabling (Stall group -B). T_{air} – Air temperature; RH – Relative humidity; CORT – cortisol; ALT – alanine aminotransferase; AST – aspartate aminotransferase; CK – creatine kinase; LDH – lactate dehydrogenase; Lac – lactate; Mg – magnesium; $T_{maxhoof}$ – maximum hooves temperature; T_{maxeye} – maximum temperature of eyes; T_{maxA} – maximum temperature of forelimbs; T_{maxP} – maximum temperature of pelvic limbs; $T_{maxfetlock}$ – maximum temperature of fetlocks.

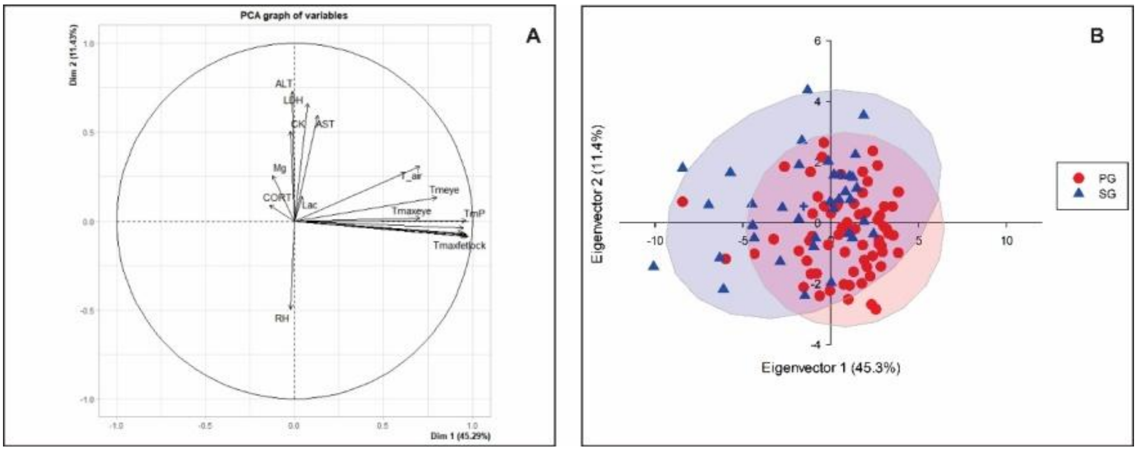

Figure 5. Principal component analysis showing the first two eigenvectors of climatic, serum biochemistry and thermographic variables (A) of gelding Brazilian Sport Horse kept in paddocks (PG) or in continuous stabling (SG) – (B) T_{air} – Air temperature; RH – Relative humidity; CORT – cortisol; ALT – alanine aminotransferase; AST – aspartate aminotransferase; CK – creatine kinase; LDH – lactate dehydrogenase; Lac – lactate; Mg – magnesium; T_{maxeye} – maximum temperature of eyes; T_{meye} – mean temperature of eyes; T_{mP} – Mean temperature of pelvic limbs; $T_{maxfetlock}$ – maximum temperature of fetlocks. *Other termographic variables as $T_{maxhoof}$ – maximum hooves temperature; T_{maxA} – maximum temperature of forelimbs; T_{maxP} – maximum temperature of pelvic limbs is not tagged in the figure due overlap position.

DISCUSSION

This research was performed aiming to elucidate the differences on animal welfare and physiologic variables (especially in terms of thermographic characteristics of limbs) according to housing management. We hypothesized that animals reared in paddocks are emotional and physiologically different from animals reared in stalls and theses differences can be expressed by changes in peripheral blood stream accessed by IRT technology. Besides that, this trial was also done in order to investigate whether the IRT could be an effective tool or not to evaluate these differences and to predict or diagnose the welfare of equines. According to Redaelli *et al*. (2014), physiological and emotional state of the animal can cause variation in skin temperature due peripheral vasoconstriction. Thus, we selected different thermographic targets that could be easy accessed by IRT camera (eyes and limbs) and can (at least in theory) be modulate by physiological and emotional state of animal.

It was expected that limb temperatures, at least in the anterior members, were lower in SG: since these animals had their movement restricted in any way and considering that the frog, the V-shaped pad of soft horn between the bars on the sole of the equine hoof, works like a secondary blood pump (Back and Pille, 2013), as lower movement, as greater blood stasis and, thus, lower the temperature. However, this was not observed here. Perhaps, movement restriction caused by stabling is not enough to substantially alter blood circulation in the limbs. Of interest, Hoffmann *et al*. (2001) observed lower blood flow on forelimbs in horses when animals weight bearing. But unfortunately, they did not measure how long sampled animals stayed completely parked before ultrasound examen.

The differences in temperature between analogous regions (hooves, fetlocks, carpals, tarsus, metacarpals, and metatarsals) tended to follow environment temperature (T_{air}), coinciding with the findings of Mogg and Pollitt (1992) and Palmer (1983) that observed an increase in the gradient of temperature between limbs as the environment temperature decreased. In this sense, the findings of the present research support the proposal of the aforementioned scientists, since with T_{air} at 18 °C, almost all of the comparisons showed a difference greater than 1 °C and, with T_{air} at 26 °C, only one of the gradients was greater than 1 ºC. These thermal differences for analogous regions may be explained by the large concentration of arterio-venous anastomoses at the distal end of equine limbs (Mogg and Pollitt, 1992). Thus, vasoconstrictions can generate irregular and asymmetric thermal patterns when the environment temperature is reduced (Soroko and Morel, 2016).

Nonetheless, the comparisons whose gradients reached the highest values were between the ipsilateral limbs (A/P, Table 2). This larger difference is believed to be due to the proportion of weight borne by the forelimbs compared to the hindlimbs. While the first support about 60% of the animal's weight, the hindlimbs account for only 40% of the weight (McGreevy *et al*., 2018). Therefore, Baxter and Morrison (2008) pointed out that when a horse is forced to remain with most of its body weight supported continuously on one limb, the normal biomechanics of the hoof impairs blood flow, resulting in greater heat loss at the distal end and favoring, in extreme cases, the appearance of tissue fatigue, injury, and necrosis. In the same study, it was also pointed out that healthy parked horses still perform constant movements, changing, contralaterally, the pair of limbs that supports most of the body weight, in the movement known as “quasi-static”, and thus, enabling the continuity of blood flow in their distal extremities.

Nonetheless, as infrared thermography is sensitive enough to detect disease in early stages (Schaefer *et al*., 2007; Turner, 2001), Soroko and Howell (2018) recommended not to rule out the possibility that an asymmetry of thermal patterns of analogous regions happens due to subclinical inflammation, suggesting the need for further investigations in order to ensure the health condition of the animals. Of interest, previous exams and the management used for the horses in this experiment can ensure the absence of diseases or debilitating conditions of the limbs. Therefore, taken together, these results corroborate with the thesis of stabling does not seem to pose a great challenge in terms of body movement, although it represents a different condition from that experienced by horses raised in open fields. In other words, we can hypothesize that just confine animals to a loose-box could offer them the minimum movement to overlook any difference in terms of limb blood flow in relation to PG.

Blood biochemistry also showed no difference between experimental groups (Table 3). With exception of CK and CORT, all other variables measured showed to be within the reference values or close to that. Considering the theoretical lower movement in SG animals, our attention was turned to possible muscle damage. Nevertheless, there were no signs of muscle damage: although the CK values observed in this study exceeded the normal range for the species, which is between 2.4 IU/L and 23.4 IU/L (Kaneco *et al*., 2008), they are below from common values reported during muscle injury (above 5000 IU/L) (Sharkey, 2017). The AST concentrations observed reinforce the absence of muscle damage since they are within the expected range for healthy animals, between 226 IU/L and 366 IU/L, according to Kaneco *et al*. (2008). Thus, increase of CK concentration reported here may be reflect of the increased permeability of the sarcolemma due to intensity and duration of urban patrol activities, which is in agreement with Kingston (2008). Corroborating this hypothesis, Valberg (2018) highlighted that small elevations in serum CK concentration may be a consequence of training, transport, or strenuous exercise. Therefore, the short interval (less than 24 hours) between the patrol activities and the sampling agree with Teixeira-Neto *et al*. (2008), Ferraz *et al*. (2010) and Padilha *et al*. (2017), whose affirmed that the increase in serum CK concentration is a consequence of muscle effort and physical adaptation to exercise which the animals are subjected to. Therefore, in agreement to Ferreira II (2018), with regard to musculoskeletal capacity, it is possible to infer that urban patrols satisfy the minimum exercise requirements to maintain animal musculoskeletal health.

In contrast, the values observed for CORT draw attention in both groups, as they are above from normal values for the species, which would be between 1.3µg/100mL and 2.93µg/100mL (Kaneco *et al*., 2008). Since it is known that the serum concentration of this substance presents a daily variation rhythm in horses (Beech, 2013), humans (Silva *et al*., 2017), pigs (Ekkel *et al*., 1996) and cattle (Thun *et al*., 1981), we could hypothesize that the high concentration of the hormone was a bias due to the time of day (morning) for sampling: there is a well-established knowledge that the highest concentrations of CORT in the bloodstream occur in the morning (Bohák *et al*., 2013; Pawluski *et al*., 2017), and thus, it is evident that the period in which the collections were performed in the present experiment favored the observation of the high values of CORT, coinciding with results achieved by Peeters *et al*. (2011). However, Bohák *et al*. (2013) and Giannetto *et al*. (2015) sought to trace the circadian rhythm of CORT in horses and observed that the maximum concentration of serum cortisol did not exceed 5µg/100mL, which differs from the data obtained in the present study, in which there were records of average above 14µg/100mL for both, PG and SG. Interestingly, the means of CORT in PG were higher on almost all days of the experiment, probably reflecting an acute stress due their capture and containment for sampling and an unfriendly relationship with humans, which was ratified by the reaction of the animals to flee in the presence of the keepers and researchers. This behavior was also observed by Minero *et al*. (2015).

However, the absence of stereotypic behaviors in PG points to stress arising from the normal breeding environment and interrelationships with individuals of the same or other animal species (field challenges), similar to that observed by Beaver (2019). On the other hand, elevated CORT concentration (above 15µg/100mL) was also observed in SG and, moreover, it happened in association with the presence of stereotypies, indicating that these animals were experiencing a condition of distress and sub-optimal welfare. Although the clear physiologic relationship of increased CORT, by activation of the hypothalamic-pituitary-adrenal axis, in face of an acute stressor, which does not necessarily represent a negative event (Morberg and Mench, 2000), there are controversies about the response of this hormone in chronic stress situations (Pawluski *et al*., 2017).

Ladewig (2000) proposed that a chronic stressor is a sequence of exposures to one or several acute stressors and face these repeated stressful stimuli cause an initially exacerbated response that, over time, culminates to a modulation of such response towards an alternance between sensitization and desensitization if a real adaptation does not occur. Alternatively, as expressed by Grandin and Johnson (2009), an animal that is in an adverse environment and presents stereotypies, by self, may be intend to calming or stimulating itself. Perhaps, these behaviors reflect moments of greater or lesser adaptation, within a range of time and in an undoubtedly challenging environment in terms of welfare. Therefore, the presence of stereotypies reinforces the above hypothesis, as these behavioral changes are believed to be an attempt to adapt as a result of the unsuitable environment and negative emotional experiences, such as frustration, boredom, or anxiety (Henderson, 2007; McGreevy, 2012). Besides, cohering with this thesis is the negative correlation of CORT and the habit of biting environmental structures (Table 1). Thus, Freymond *et al*. (2015) reported that biting horses showed variable salivary cortisol concentration (higher, lower and equal) compared to those who did not have this habit. However, when they were prevented from performing stereotypic behavior, these animals reached very high peaks in salivary concentration of the hormone.

With this, the observation of oral stereotypies, such as biting and licking structures in the environment, random movements with the lip and aerophagia indicate that the animals from SG could not develop a natural feeding behavior, for which they were highly motivated by their biology: the ingestion of several portions of forage plants, with small volumes and long fibers, for at least 16 of the 24 hours of the day (Henderson, 2007; Wickens and Heleski, 2010). McGreevy (2012) and Rochais *et al*. (2018), in turn, reported that equines submitted to long periods of fasting are more susceptible to start presenting oral stereotypies, even if their nutritional requirements are met and their body condition score come to be satisfactory. Such conditions seems to happen in the present trial since the stabled horses' feed was provided only three times a day.

Nevertheless, the presence of locomotor stereotypes in SG indicates that the environment also prevents the horses performing behaviors inherent to the nature of the species: moving throughout the day, in groups, in search of the best feeding places, feeding and resting, having the possibility of fleeing from an eventual predator (Beaver, 2019; Lesimple *et al*., 2020). In this sense, the findings of Hampson *et al*. (2010) reinforce our results, as they observed that while equines kept in 4-hectare paddocks traveled about 6 km per day, those kept in solaria 6m wide by 6 m long traveled only 1km approximately. It is clear, therefore, that the restriction on movement imposed by stall housing is detrimental to the welfare of these animals.

The use of thermal images from eyes aiming to predict or evaluate the degree of stress was showen to be inaccurate and, thus, unable to reach a minimally satisfactory assessment. Our results (Figure 1) agree with those observed by Soroko *et al*. (2016) and Gómez *et al*. (2018), who reported no correlation between CORT and T_{maxeye} (maximum temperature of eyes). Conversely, Valera *et al*. (2012), Dai *et al*. (2015) and Fenner *et al*. (2016) obtained results suggesting the opposite: T_{maxeye} showed to be a good parameter for predicting the degree of stress since there was a correlation with salivary cortisol concentration (Valera *et al*., 2012) and with heart rate elevation (Dai *et al*., 2015; Fenner *et al*., 2016). De Santis *et al*. (2017) tried to explain these divergent results by proposing that T_{maxeye} may reflect both physiological and emotional events, chronic or acute, making it difficult to interpret the data. Trindade *et al*. (2019) support the hypothesis of De Santis *et al*. (2017), as they observed that T_{maxeye} showed a positive correlation not only with serum CK values but also with cortisol concentration in farm horses. Thus, further studies are recommended to clarify how and if T_{maxeye} can be used as a predictive parameter of stress level.

The discriminanting ability of the surveyed information was high (88.3%). Thus, in 88.3% of the cases, using variables choosen by STEPDISC procedure (T_{maxLFH}, T_{air}, CORT, ALT, Lac, T_{mP}, $T_{maxcarpAR}$, T_{maxRFH} and CK), it was possible to correctly distinguish in which experimental group the animals belonged to (Table 5 and Figure 3). Although the high hit capability to distinguish experimental group (and therefore, to distinguish which animal was stabled and which was not), use these parameters implies sophisticated mathematical skills and some data processing capacity as well as it is time consuming. Therefore, doing this in the field is unfeasible and then, alternatively, it was used only thermographic variables in order to distinguish experimental groups. This approach, in turn, achieved 74.53% hits (Table 5). Thus, if in one hand high accuracy requires mathematical treatments, computing, and time, on the other hand, with a relative low cost on accuracy (13.77% of difference between methods) it can make its use feasible daily, if computer technology is developed to automatically reach the variables used.

Correlation analysis showed some differences between treatments. For example, there was a positive correlation between CK and CORT for SG that did not happen for PG. Similarly, there were correlations between CK and AST, and LDH and AST that were not reported for PG. These correlations *per se* seems to be in line with a muscular wearing caused by urban patrols as discussed above, but once it has seen only on SG, it could be hypothesized that continuous stabling causes slower decrease of those enzyme levels, maybe, due less movement when compared with PG. On the other hand, there was a correlation between AST and CORT for PG. Flament *et al*. (2012) used ducks to investigate the rule of corticosterone, cortisol, AST and other serum enzymes on animal welfare. They suggested that the increase in AST levels could be linked with acute stress caused by handling and transportation. This agrees with our observation of an acute stress (also described above) caused by the procedure of capture and containment of animals for sampling.

Of interest, the correlations between RH and thermographic variables showed different patterns between PG and SG. There was no correlation between these variables for SG, whereas there were negative correlations for them on PG. Thus, it can be suggested that RH is no longer important as the animal is stabled and, so far, protected in terms of climatic changes, while animals reared in paddocks did not account with this shelter. Therefore, the general effect of humidity in stabilizing the temperature could be noticed on PG as the correlations were negative.

The principal component analysis (PCA) presents two well divided axis: eingenvector 1 (horizontal) showing thermographic and environment variables and eigenvector 2 (vertical) with serum biochemistry variables (Figure 5A). When PCA consider separately the experimental groups (Figure 5B), it is noteworthy some differences on variance between PG and SG as the ellipsis of SG are dislocated for the left on eingenvector 2 and slight rotate anticlockwise while ellipsis for PG seems to a circle close to the figure center. Thus, it seems that PG animals present a relation between thermal and serum biochemistry more equilibrated (less variable) than SG animals.

CONCLUSION

Opposite to what was expected, the continuous stabling does not interfere in the thermoregulation of the equine limbs. However, keeping the horse stabled continuously represents a biological challenge large enough to impair the animal welfare. Of interest, the interaction with humans may also represent a source of negative emotional states as evidenced by serum cortisol values observed here.

Further studies are still needed to ensure the reliability of interpretation of maximum eye temperature as a stress detector method in horses. Nevertheless, simultaneous use of physiological and thermographic indicators managed to distinguish, which animal belonged to stabling or paddock treatments. Additionally, with little loss of accuracy, it was possible to correctly classify from which group each animal belong to using only thermographic predictors. Thus, thermal variables of limbs, obtained from infrared thermography, seem to have predictive potential in the diagnosis of chronic stress.

The continued development and validation of novel, quantifiable methods for stress assessment using behavior and physiology are essential to increase our knowledge and understanding of stress identification in horses.

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