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RESEARCH ARTICLE

Serum Cortisol Concentration is a Reliable Tool to Assess Stress Level among Stereotypic and Non-Stereotypic Thoroughbred Horses

Abdul Saleem Alias Baboo, Muhammad Naeem*, Atique Ahmed Behan and Nasir Rajput

Faculty of Animal Husbandry and Veterinary Sciences, Sindh Agriculture University, Tandojam, Pakistan
*Corresponding author: mnrajput@sau.edu.pk

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The study was conducted to investigate the impact of different housing systems on the development of equine behavior through variations in serum cortisol concentrations. N=120 x TBP (Thoroughbred Pakistan) of three different groups (A=40 horses 2years not gelded, B= 40 age 3 years and C= 40 four years gelded) and four distinct types of housing systems. Single Housing System No Interaction (SHSNI), the horses didn't have any interaction among them, Single Housing System Limited Interaction (SHSLI), Double Housing System (DHS), a pair of horses were kept in house and Group Housing System (GHS), where (n=40) horses were having complete interaction among them, were considered in the study. The study was conducted on Remount Depot Mona (known as Home of Horses) which is in Mandi Bahuddin, Punjab, Pakistan. The duration of study was 120 days and conducted in four phases (Phase-I SHSNI, Phase-II SHSNI, Phase- III DHS and Phase- IV GHS were used) and each phase was comprised of 30 days duration. Before shifting to any housing system, the blood was collected to measure cortisol concentration at zero day and then after every 30th day again blood was collected prior to shifting to next housing system. Blood was collected from each horse for serum cortisol analysis through Cortisol kit. Competitive Enzyme immunoassay (CEIA) was used for quantification of cortisol in samples by using commercially available cortisol ELISA kits. Analysis of variance by considering randomized complete block design was performed for statistical analysis. The cortisol concentration exhibited no significant fluctuations within the groups, but a noteworthy disparity was observed between the housing systems ($P<0.05$). The cortisol concentration measured at 0th day was 1.53 µg/dL which was decreased 0.58 µg/dL in SHSNI and increased 0.59 µg/dL in SHSLI reached slightly above the average value at 0th day, then it decreased (0.57 µg /dL) in DHS and again increased in GHS (0.58 µg /dL) and reached to 1.55 µg /dL. The study concludes that SHSLI is a more suitable housing system than all others, needed to be adopted for the better welfare of the horses than other housing systems. This house has maximum social interaction, less stress and stereotypes with limited injuries as compared to alternative housing systems. Further investigation can be performed to standardize that type of housing which maintains the minimum stress level of Thoroughbred horses.

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INTRODUCTION

Stress represents a complex coping mechanism that arises in response to any alteration in the environment or management leading to a stressful situation. Numerous studies have been conducted to investigate how stress affects immune cells, considering their functions and quantities (Davis *et al*., 2008). Lower counts of blood lymphocytes and higher numbers of neutrophils, particularly among T cells, serve as typical indicators of stress responses (Popescu and Diugan, 2017; Olvera-Maneu *et al*., 2023). Stress hormones play a functional role in activating the Hypothalamic Pituitary Adrenal axis (HPA-axis), prompting the secretion of carefully coordinated stress-related hormones. These hormones counteract the adverse effects of stressors that jeopardize

the homeostasis of horses (Pawluski *et al*., 2017). Stress can be categorized into acute and chronic types based on its duration. Acute stress responses bring about changes in leukocyte numbers, potentially bolstering immunity and mobilizing resources as needed. Conversely, chronic stress, with its prolonged duration, may disrupt immune functions, ultimately impacting the overall health of animals (Dhabhar, 2009). An axiom aptly reminds us that "one cannot be cured in the same environment in which they got sick." When horses are kept in isolation, devoid of social interaction, they often experience boredom, frustration, and psychological discomfort, which can lead to the development of stress and coping mechanisms such as stereotypes.

Stressors can be classified into three primary categories: physical stressors (resulting in physical discomfort), psychological stressors (leading to mental discomfort), and social stressors (associated with social factors). Behavioral responses to these stressors are often of great significance and are typically evaluated by analyzing the levels of stress-related hormones in the body (Proudfoot and Having, 2015; Schmucker *et al*., 2022; Delank *et al*., 2023). Social stressors are frequently induced by management practices, with housing systems playing a pivotal role in this context. Confining housing systems, such as individual stabling, tends to promote social isolation and stress among horses. This is because horses naturally thrive in free-roaming environments, engage in pasture grazing, and participate in intricate social interactions (Krueger *et al*., 2021). Several studies have reported associations between single housing systems and changes in heart rate, as well as fluctuations in glucocorticoid levels (Vickers, 2017). Additionally, individual horses may encounter challenges in adapting to these housing conditions and may exhibit limited responsiveness to environmental stimuli (Ruet *et al*., 2019).

In recent times, the world is increasingly adopting group housing systems. However, it's important to note that in group housing systems, there is a heightened level of social contact among individuals, which can lead to agonistic interactions and the development of social stress. The nature of agonistic interactions among horses is primarily influenced by individual personalities, prior experiences, the composition of groups based on age and sex, as well as the stability of the housing system. Aggressive interactions often arise due to changes in social structure or alterations in group compositions (Christensen *et al*., 2011; Hartmann *et al*., 2012). Both housing systems, whether individual or group, contribute to the development of social stress and can impair immunocompetence. Cortisol, a glucocorticoid steroid hormone, is considered a major stress hormone. The measurement of cortisol concentration at various levels has proven to be an effective and highly reliable method for assessing the stress status of horses (Schmucker *et al*., 2022). Salivary, fecal, and blood cortisol concentrations serve as well-established indicators of stress in horses (Bachmann *et al*., 2003; Ayala *et al*., 2012; Dembek *et al*., 2023).

Variation in cortisol concentration triggers or induces the "fight or flight" response, which consists of two primary reactions. The first response involves the production of epinephrine (adrenaline) and norepinephrine hormones. When a horse encounters a stressful situation, the amygdala evaluates the stressor and sends signals to the hypothalamus. The hypothalamus, in turn, activates the sympathetic nervous system, leading to the production of epinephrine and norepinephrine. Additionally, adrenaline hormones are secreted from the adrenal medulla of the adrenal gland. Functionally, these hormones regulate the initial stage of the stress response, preparing the body for action. The second response is mediated by the production of cortisol, a stress hormone. The amygdala signals the hypothalamus, which activates the Hypothalamic Pituitary Adrenal (HPA) axis. This activation leads to the release of corticotrophin-releasing hormone (CRH or CRF). CRH acts on the anterior pituitary gland, stimulating the production of Adrenocorticotropic Hormone (ACTH). ACTH, in turn, activates the zona fasciculata layer of the adrenal cortex in the adrenal gland, causing the secretion of cortisol as a stress hormone (Pawluski *et al.,* 2017). Functionally, cortisol increases the level of glucose in the bloodstream through processes like gluconeogenesis (production of glucose from sources other than carbohydrates), proteolysis (breakdown of proteins), and lipolysis (breakdown of fats). Simultaneously, it reduces tissue sensitivity to insulin, causing less glucose to be consumed by tissues and more to remain in the blood. This elevated blood glucose level provides the horse with the necessary energy for the skeletal muscles to engage in the "fight or flight" response.

Currently, well-designed housing systems for horses, including single, double, pair, and group configurations, offer various levels of tactile or visual social interaction and freedom of movement. However, there is a prevailing shift towards individual boxes in modern equine husbandry (Hoffmann *et al*., 2012; Fureix *et al*., 2012). In these individual box systems, horses are often kept alone, leading to a sense of confinement and isolation. In single boxes, horses experience complete isolation and a lack of social interaction. These conditions are considered predisposing factors that can result in frustration, boredom, psychological anxiety, and stress (Yarnell *et al*., 2015). While many studies have examined the effects of transport, competition, and exercise on horses, there has been relatively limited reporting on immune parameters within the context of different housing systems. Building on previous research (Bachmann *et al*., 2003; Ayala *et al*., 2012; Schmucker *et al*., 2022; Dembek *et al*., 2023), our study aimed to measure cortisol serum concentrations to assess the impact of different housing systems on stress development in Thoroughbred horses in Pakistan.

MATERIALS AND METHODS

The purpose of this study was to assess how various designs of housing systems impact the development of stereotypies among horses. The research was carried out at the Remount Depot Mona, which was established in 1902 and is situated in Mandi Bahuddin, Punjab, Pakistan as shown in Fig. 1. A total of 120 horses were divided into three distinct groups based on their gelding status. In Group A, the animals were 2 years old not gelded, while in Groups B and C, the animals were 3 and 4 years old, respectively consisted of gelded horses. These 120 horses

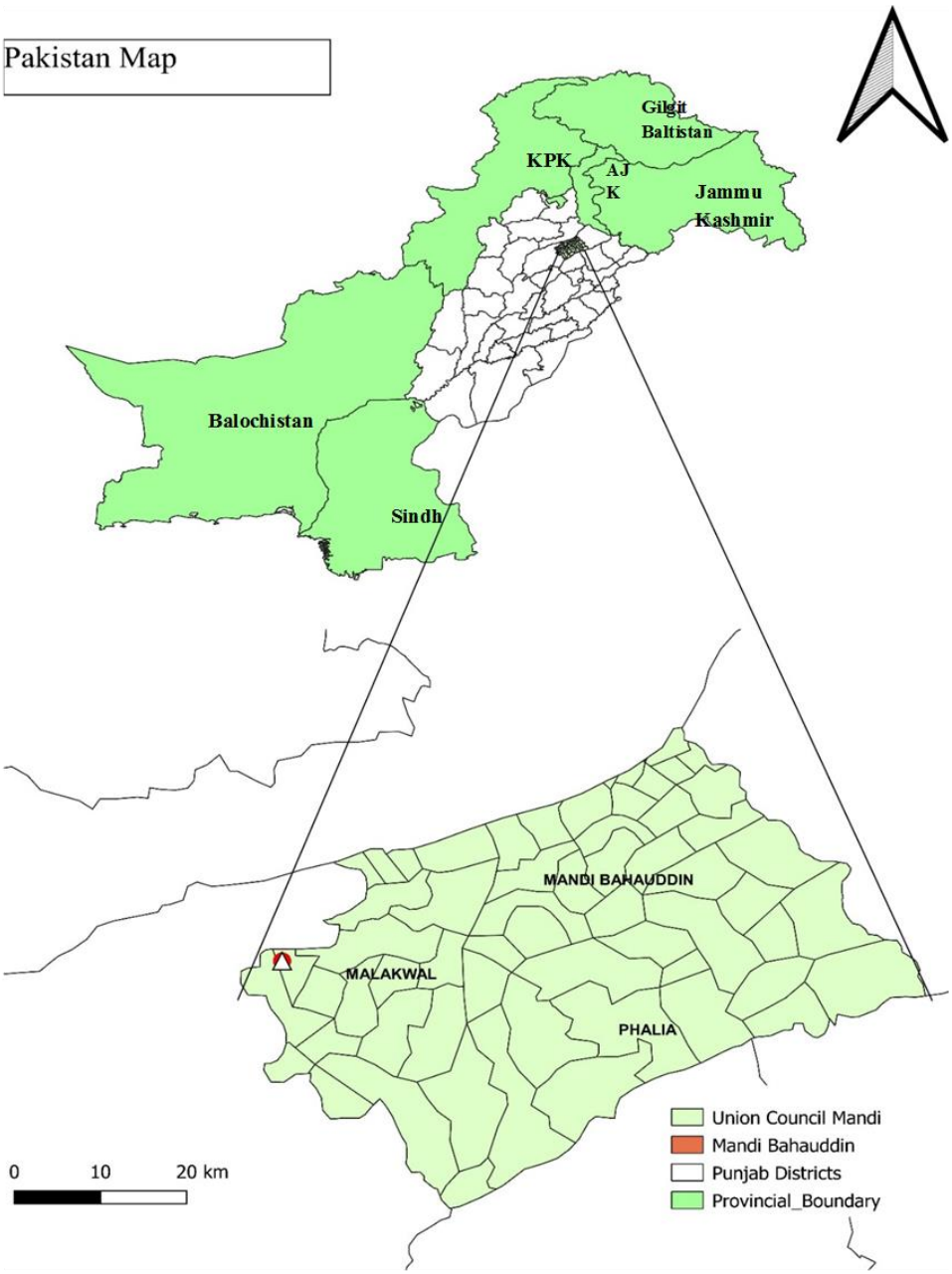

Fig. 1: Map of study site.

were allocated to four distinct housing systems and phases. SHSNI (Phase-I), SHSLI (Phase-II), DHS (Phase-III), and GHS (Phase-IV) houses were used. Each horse received one hour of morning exercise daily, from 6:30 AM to 7:30 AM, followed by a reasonable period to alleviate stress and physiological discomfort through physical activity to assess the impact of housing systems on cortisol concentration, a randomized complete block design was employed in the study, with housing systems treated as blocks. Statistical analysis was conducted using analysis of variance, considering the randomized complete block design. The cortisol concentrations displayed non-significant variations among the groups, but a significant difference was observed among the various housing systems ($P<0.05$). To facilitate adjustment and adaptability of the horses to immediate changes in their environment and to mitigate the physiological impact of acute stress, a generous period of 30 days was provided within each housing system. This allowed the horses to normalize and develop resilience. Furthermore, the study also observed stereotypic and non-stereotypic behaviors in horses aiming to establish a correlation with serum cortisol concentrations. A 5 mL blood sample was collected from the jugular vein using blood collecting tubes containing anticoagulant heparin. These samples were carefully stored in a cool ice box to prevent any deterioration and were subsequently dispatched to the laboratory for serum cortisol analysis. On day zero, the initial reading was taken before the horses were transferred to the SHSNI housing systems. Subsequently, on every 30th day, blood samples were collected from each housing system, and the results were meticulously recorded on data collection sheets. The comprehensive dataset is presented in Table 1. Cortisol concentration was determined following the method described by Abdollahpour *et al*. (2020). A CEIA (Competitive Enzyme Immunoassay) was employed to quantify cortisol in the samples, utilizing a commercially available cortisol ELISA kit provided by AccuBind (Monobind Inc., Lake Forest, CA, USA; product code: 3625-300A, with a

Table 1: Impact of housing systems in the increase/decrease of cortisol concentrations

GROUP	SHSNI		SHSLI	DHS	GHS
	0th Day	30th day	60th day	90th day	120th day
A	1.54	0.96 (-0.58)	1.52 (+0.56)	0.97 (-0.55)	1.53 (+0.56)
B	1.51	0.94 (-0.57)	1.56 (+0.62)	0.96 (-0.60)	1.56 (+0.60)
C	1.56	0.97 (-0.59)	1.54 (+0.57)	0.98 (-0.56)	1.57 (+0.59)
Mean±SEM	1.53	0.95^{a}	1.54^{bd}	0.97^{ac}	1.55^{d}

Values followed by different superscript letter differ significantly ($p < 0.05$)

sensitivity of 0.25 µg dL−1). The necessary reagents for the CEIA included antibodies, antigens, and enzyme-antigen conjugate. The reaction between these reagents and the sample is detailed below:

$$AgAbBtn + EnzAgAbBtn\ AgAbBtn + Streptavidincw \Rightarrow \text{immobilized complex}$$

Where: AbBtn = Biotinylated Antibody, Ag = Native Antigen, EnzAg = Enzyme-Antigen Conjugate, AgAbBtn = Antigen-Antibody Complex, EnzAgAbBtn = Enzyme-Antigen Conjugate-Antibody Complex, Ka = Rate Constant of Association, K-a = Constant Rate of Disassociation K = ka/k-a = Equilibrium Constant, Streptavidincw: Streptavidin Immobilized on wells, Immobilized Complex: sandwich complex bound to solid surface. Due to instantaneous reaction between biotin attached with antibody, streptavidin immobilization on microwell occurred. This effects separation of antibody bound fraction after decantation or aspiration. The enzyme activity is inversely proportional to native antigen concentration. The concentration of cortisol was calculated by standard curve. 6 ampules (0, 1, 4, 10, 20, and 50 µg/dl) 1 ml each, 1 ml Cortisol Enzyme Reagent, 7 ml Steroid Conjugate Buffer (red dye buffer), 7 ml Cortisol Biotin Reagent (conjugated anti-cortisol biotinylated mlgG, green dye), Microplate Coated with 1 µg/ml Streptavidin, 20 ml Wash Solution Concentrate, 7 ml Substrate A (tretramethyl benzidine (TMB) in buffer), 7 ml Substrate B (hydrogen peroxide (H2O2) in buffer) 8ml Stop Solution (1N HCl) were used for preparation of reaction mixture. Working Enzyme Reagent was prepared by adding 0.7 ml of Cortisol Enzyme Reagent in 7 ml of Steroid Conjugate Buffer. Dilution of concentrate wash buffer was done in ratio of 1:50 with distilled or deionized water. Working Substrate Solution was formed by mixing 7 ml of substrate B with 7 ml of substrate A completely. Before starting the procedure, all reagents, standards, and samples were acclimatized with room temperature. Twenty-five (25) µl sample was pipetted into each well. After that, 50µl working cortisol enzyme reagent was added to wells. The wells were moderately swirled for almost 30 seconds. Next, 50 µl of cortisol biotin reagent was added to each well. The microplates were swirled moderately once again for at least 30 seconds to mix all material in wells. Afterwards, the microplates were enclosed and were incubated for 1 hour at 25°C. After incubation, the material of all wells was thrown away by flipping the microplates. Each microplate washed 3 times with wash buffer. Wash buffer with quantity of 350 µl/well was filled and flipped sharply to empty the material. After final washing to microplate and tapped on blotting paper to get rid of buffer. Followed by washing, 100 µl working substrate solutions was added to wells and wells were incubated at 25°C for 15 minutes. After incubation, the reaction was stopped by adding 50µl stop solution in each well and moderately mixed. Absorbance readings of all samples and standards were obtained within 15-20 minutes after reaction stopped by using an ELISA Strip Reader Stat FAX 303 Plus (Awareness Technologies), with 450 nm wavelengths and had 620nm reference wavelength. Standard curve of cortisol was created by using six standards of 0, 1, 4, 10, 20, and 50 µg/dl concentration. The curve was stored and used to calculate concentration in µg/dl of all samples by using lin-log method. In this study, a statistical model based on a two-factor factorial design under a randomized complete block design was employed. The age of the animals and the housing systems were considered as blocking factors for each other. To analyze the statistical significance of serum cortisol concentration, a two-way analysis of variance (ANOVA) was applied. Subsequently, a modified Tukey's test was conducted for comparing means. All statistical analyses were carried out using SPSS Software (Schork and Remington, 2000.)

RESULTS

The study results indicated that the concentration of cortisol hormone did not exhibit significant changes among the groups ($P>0.05$). However, there was a significant association observed between the cortisol hormone concentration and housing systems ($P<0.05$). Analysis using Tukey's test on the cortisol concentration levels revealed a significant effect of housing systems over time, from day 0 to the 120th day. Specifically, in the SHSNI housing system, cortisol concentration decreased across all age groups. Conversely, in the SHSLI system, there was an increase in cortisol levels. In the DHS system, cortisol concentration decreased, while in the GHS system, cortisol levels increased. Overall, the findings underscored a notable and statistically significant relationship between the housing system and cortisol concentration in Thoroughbred horses. At day zero, before transitioning to the SHSNI housing system, the average serum cortisol level for all 120 horses was measured 1.53 µg/dL. In the SHSNI housing system, the serum cortisol value recorded on the 30th day was 0.95 µg/dL. The averages for group A, B, and C were 0.96 µg/dL, 0.94 µg/dL, and 0.97 µg/dL, respectively. Moving on to the SHSLI housing system, the serum cortisol value recorded on the 60th day was 1.54 µg/dL. The average cortisol levels for group A, B, and C were 1.52 µg/dL, 1.56 µg/dL, and 1.54 µg/dL, respectively. During Phase II in SHSLI, normal average serum cortisol values were observed, and 26 horses exhibited improvements in their behavior, with a reduction in the prevalence of stereotypies. In the DHS housing system, the serum cortisol value recorded on the 90th day was 0.97 µg/dL. The averages for group A, B, and C were 0.97 µg/dL, 0.96 µg/dL, and 0.98 µg/dL, respectively. The second-lowest serum cortisol levels were observed in DHS, where horses were housed in pairs, as opposed to the individual housing systems used in both SHSNI and SHSLI. In the GHS housing system, which facilitated intense social interaction both visually and through tactile contact, the serum cortisol value recorded on the 120th day was 1.55 µg/dL. The averages for group A, B, and C were recorded at 1.53 µg/dL, 1.56

Table 2: Cortisol release (µg/dL) in different housing systems

Days	Cortisol variation in different housing system		
	Minimum	Maximum	Mean±SEM
0th Day	1.58	1.98	1.79±0.07[a]
30th Day SHSNI	1.15	1.27	1.20±0.02[b]
60th Day SHSLI	1.57	1.97	1.78±0.05[a]
90th Day DHS	1.20	1.33	1.27±0.02[b]
120th Day GHS	1.58	1.98	1.79±0.06[a]

Values followed by different superscript letters differ significantly (p<0.05). SEM= standard error of mean

Table 3: Effect of stereotypies on serum cortisol level (µg/dL) in different housing systems

Stereotypies	SHSNI		SHSLI	DHS	GHS
	0th Day	30th day	60th day	90th day	120th day
Kicking	1.70±0.08	1.18±0.04	1.69±0.08	1.25±0.05	1.69±0.08
Biting	1.80±0.02	1.22±0.01	1.79±0.02	1.29±0.02	1.79±0.02
Weaving	1.68±0.04	1.20±0.01	1.67±0.04	1.26±0.02	1.67±0.04
Crib-biting	1.06±0.15	0.88±0.07	1.05±0.15	0.89±0.08	1.05±0.16
Mean±SEM	1.56±0.11[a]	1.12±0.03[bc]	1.55±0.11[ad]	1.17±0.04[bc]	1.55±0.07[ad]

Values followed by different superscript letters differ significantly (p<0.05). SEM= standard error of mean

Table 4: Comparison of cortisol release in different stereotypies

Stereotypies	Stereotypies effect on cortisol concentration		
	Minimum	Maximum	mean±SEM
Kicking	1.18	1.69	1.50±0.11[a]
Biting	1.12	1.64	1.44±0.12[b]
Weaving	1.59	2.24	1.99±0.14[c]
Crib-biting	0.51	0.61	0.57±0.02[d]

Values followed by different superscript letters differ significantly (p<0.05). SEM= standard error of mean

Table 5: Stereotypic vs non stereotypic cortisol concentration (µg/dL) in different housing systems

Days	A1	A2	B1	B2	C1	C2
0th day	1.66	1.90	1.69	1.98	1.58	1.95
30th day	1.19	1.23	1.15	1.23	1.15	1.27
60th day	1.65	1.88	1.69	1.96	1.57	1.97
90th day	1.23	1.29	1.23	1.33	1.21	1.33
120th day	1.65	1.89	1.69	1.97	1.58	1.98
Mean±SEM	1.48±0.06[a]	1.64±0.12[ab]	1.49±0.08[ac]	1.692±0.14[ad]	1.42±0.05[d]	1.70±0.14[ad]

Values followed by different superscript letters differ significantly (p<0.05). SEM= standard error of mean

Table 6: Stereotypic vs non-stereotypic horses impact cortisol concentration (µg/dL) in different housing systems.

Groups	SHSNI		SHSLI	DHS	GHS
	0th day	30th day	60th day	90th day	120th day
A1	1.66	1.19	1.65	1.23	1.65
A2	1.90	1.23	1.87	1.29	1.89
B1	1.69	1.15	1.69	1.23	1.69
B2	1.98	1.23	1.96	1.33	1.97
C1	1.58	1.15	1.57	1.21	1.58
C2	1.95	1.27	1.97	1.33	1.98
Mean±SEM	1.79±0.03[a]	1.20±0.002[bc]	1.78±0.03[ad]	1.26±0.002[b]	1.79±0.03[ad]

Values followed by different superscript letters differ significantly (p<0.05). SEM= standard error of mean

Table 7: Stereotypic vs non-stereotypic on cortisol concentration (µg/dL)

Variable	Stereotypic vs Non- stereotypic on cortisol concentration		
	Minimum	Maximum	mean±SEM
A1	1.19	1.66	1.48±0.11[a]
A2	1.23	1.90	1.63±0.15[ab]
B1	1.15	1.69	1.49±0.12[ac]
B2	1.23	1.98	1.69±0.17[abd]
C1	1.15	1.58	1.41±0.09[bcde]
C2	1.26	1.98	1.71±0.16[ad]

A1, B1 and C1 horses shown stereotypies while A2, B2 and C2 are non-stereotypic horse. Values followed by different superscript letters differ significantly (p<0.05). SEM= standard error of mean

µg/dL, and 1.57 µg/dL, respectively. During Phase IV in GHS, a second normal serum cortisol value was observed. Across all housing systems, four different stereotypic behaviors were observed, including kicking, biting, weaving, and crib-biting. Thirteen horses that initially exhibited stereotypic behaviors were able to correct their abnormal behavior, resulting in a reduction in the prevalence of stereotypes. The study found a significant effect of stereotypies on serum cortisol levels (µg/dL) in different housing systems, as detailed in Tables 3 and 4. Moreover, Tables 5, 6, and 7 as well as in Fig. 3, 4 and 5 provide a comprehensive breakdown of cortisol concentrations in stereotypic vs. non-stereotypic horses across different housing systems and age groups. Notably, a significant association was observed between the cortisol concentration of stereotypic horses and non-stereotypic ones.

DISCUSSION

According to Luescher *et al*. (1998), social isolation stands out as the primary stressor for stabled horses, with reduced social contact leading to a higher incidence of stress-related behaviors in Thoroughbred stabled horses. These findings align with a similar study conducted by Yarnell *et al*. (2015), which reported abnormal fecal cortisol concentrations in horses housed in single units with no contact (SHNC). In single housing, horses experience a sense of isolation, which contradicts their natural social behavior. While horses can somewhat adapt to a separation environment, complete isolation and a lack of interaction can induce stress, as evidenced by variations in serum cortisol concentration. Aurich *et al*. (2015) also noted abnormal cortisol salivary concentrations in horses housed individually, in contrast to other housing systems. Loneliness appears to be a direct factor linked to the presence of stress hormones. The physiological behavior of the organism plays a crucial role in inducing stress. Pawluski *et al*. (2017) similarly reported findings where single-housed horses exhibited low cortisol hormone levels. In another study conducted by Hovey *et al*. (2021), cortisol levels as low as 1.48 µg/dL were observed, serving as an indicator of chronic stress. A significant contributing factor to the reduction in serum cortisol concentration could be attributed to the architectural design of the housing system, which did not allow individually housed horses to engage in visual or tactile social interactions. Horses, being highly social animals, thrive in group settings. When they experience complete isolation, confinement, and a lack of social interaction due to high walls without windows or barriers, they tend to develop feelings of frustration, boredom, and psychological anxiety. Therefore, these various factors can collectively contribute to the development of stress.

In the paired housing system, where horses were kept together in pairs, stereotypies still emerged. One of the primary contributing factors to this phenomenon was that these horses had not previously experienced close contact with each other, especially from an early phase. As a result, they had not developed the behavior of sharing personal space, food, freedom, and establishing a hierarchy. This lack of prior shared experience led to non-cooperative behavior when they were first introduced to pair housing. These findings align with the research by Christensen *et al*. (2002), which reported that

Fig. 2: Different Housing Systems **A)** SHSNI**; B)** SHSLI**; C)** DHS**; D)** GHS.

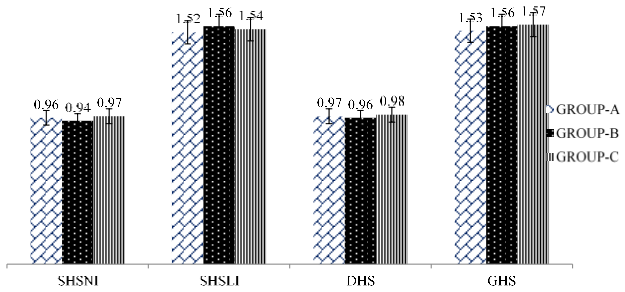

Fig. 3: Cortisol averages values (µg/dL) of different age groups in different housing systems.

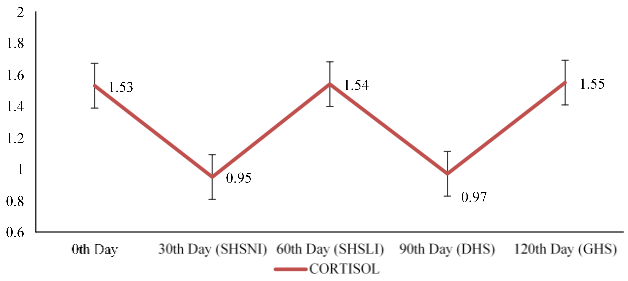

Fig. 4: Cortisol level fluctuations in different housing system.

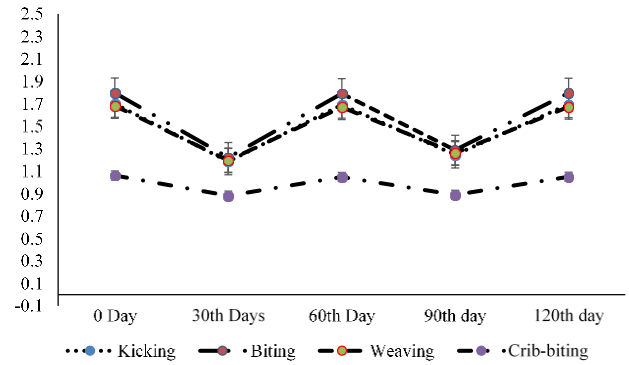

Fig. 5: Stereotypies induced cortisol level fluctuations in different housing system.

horses previously housed individually tended to exhibit more aggression when placed in group settings, possibly due to a lack of shared behavior and experiences. Research by Gmel *et al*. (2022) also supported these observations, as it found that individual box designs that allowed for visual contact between horses were associated with lower levels of stereotypic behavior in stabled horses. Cooper *et al*. (2000) similarly reported, through empirical studies, that allowing close visual and tactile contact with neighboring horses significantly reduced the occurrence of stereotypies. Rushen and Mason (2006) concluded that stereotypies serve as indicators of chronic stress, and they observed lower cortisol concentrations in horses displaying stereotypic behaviors compared to non-stereotypic horses. Numerous authors have conducted studies and reported that social housing can effectively reduce stress (Schwarz and Gazzarin 2013; Ruet *et al*., 2019; Gmel *et al*., 2022). Additionally, the decrease in the prevalence of stereotypes was influenced by the presence of sharing behavior among horses. Throughout the history of domestication, primary attention and priority have been directed toward the physical welfare of horses, encompassing aspects like comfort, shelter, protection from severe weather, predator avoidance, and access to food, and prevention of injuries. However, there has been a lack of adequate consideration given to the psychological stress and anxiety that horses may experience due to confinement and a deficiency in social interaction.

Conclusions: Horses, originally highly social herd animals accustomed to living in close-knit groups with intense social interaction, exhibit the development of stereotypies and a reduction in cortisol concentrations, indicative of stress, when subjected to the minimal social interaction found in single housing systems. Different housing systems, with varying levels of isolation, significantly influence the development of stereotypic behaviors. The assessment of stress through the analysis of serum cortisol concentrations is a dependable and scientifically grounded method. In conclusion, the study suggests that the SHSLI housing system, with its enriched environment, is more suitable, resulting in reduced psychological discomfort, fewer injuries, and fewer health hazards for horses compared to other housing systems. Every effort should be dedicated to creating a naturalistic environment that addresses and minimizes contributing factors. Horses that do not respond to these improvements, particularly those unable to adapt to modern stable conditions through adopting or social learning behavior, may need to be excluded from the breeding population. It is advisable to update the single housing system by incorporating provisions for social contact, either visual or vocal, to prevent horses from experiencing loneliness and to reduce the development of stereotypic behaviors. Further investigations could delve into the epigenetic profiles of horses and mares with the goal of standardizing housing designs that closely mimic a naturalistic environment while ensuring uncompromised social and psychological well-being. These studies may aim to explore the impact of epigenetic factors on stress levels or investigate the role of genes and breeds in the development of stereotypies, psychological discomfort, and stress by assessing stress hormone levels. A comprehensive study could also be devised to assess the

impact of various training methods and physical exercises on stress levels. This assessment could involve the evaluation of stress-related hormones such as ACTH, LRH, and cortisol concentrations to establish and understand the relationship between stress and physical activity more thoroughly.

Authors contribution: MN, AAB and NR conceived and designed the study. ASAB executed the experiment, ASAB and MN analyzed the data. All authors designed the manuscript and approved for the final version.

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