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# 1 Is the judgment bias test a good tool to assess the quality of horse management?

2 Giovanna Marliani\*<sup>a</sup>, Alessia Balboni<sup>a</sup>, Carlotta Tiberi<sup>a</sup>, Rachele Malavasi<sup>b</sup>, Aldo Gardini<sup>c</sup>,
3 Pier Attilio Accorsi<sup>a</sup>

<sup>a</sup>Department of Veterinary Medical Sciences, University of Bologna, Ozzano dell'Emilia
(BO), Italy

<sup>6</sup> <sup>b</sup>School of Ethical Equitation, Moncigoli di Fivizzano, +3498551997, Italy

<sup>7</sup> <sup>c</sup>Department of Statistical Sciences P. Fortunati, University of Bologna, Bologna, Italy

#### 8 Abstract

9 Animal welfare should include the possibility of animals experiencing positive emotions. Emotions influence the cognitive process, and judgment bias tests (JBTs) are employed in 10 different species, to assess the optimistic or pessimistic expectation of an individual and its 11 affective state. Only recently the JBTs have been applied to horses. This research aims to 12 investigate the relationship between a spatial JBT and hypothalamic-pituitary-adrenal axis 13 (HPA) chronic and acute activation in forty-one animals hosted in different kinds of 14 15 environments: traditional stables (TS), natural boarding (NB), and ethological stable (ES). Fecal (FC) and horsehair (HC) cortisol concentrations were quantified for each subject 16 17 through Radio-Immuno-Assay (RIA). Body condition score (BCS), as an indirect index of animal motivation towards food, and personality traits were measured to explore their 18 19 possible influence on JBT results. Horses had to distinguish a positive position (P), where a bucket full of food was positioned, from a negative one (N), with an empty bucket. Then, 3 20 21 intermediate positions (Near Negative-NN, Near positive-NP, and Medium-M) with an empty bucket were presented to the subject one at a time. Only 20 subjects out of 41 completed the 22 23 JBT and were included in the statistical analysis, and both BCS and P position, whether at the right or the left of the subject, seems to have influenced the inclusion rate. Only the ES group 24 registered a significantly lower score in NN, suggesting a more optimistic affective state, 25 whereas NB and TS did not significantly differ in their responses. Despite this, horses from 26 NB recorded higher FC concentration than TS subjects during all the phases of the test, but 27 lower HC levels, which could suggest a generally lower level of chronic stress but its 28 interpretation presents several confounding factors. These results put into question whether 29

JBT is indeed a good test to monitor the quality of the management, as it does not seem to reflect the chronic physiological state of the animals and could be influenced by a state of acute stress, caused by the test procedure. Due to these confounding factors, this procedure should be accompanied by other indicators. Finally, to include more animals and exclude possible biases, the structure of the JBT and the employment of food as a reward should be evaluated considering the peculiarities of the species and individual motivations.

36 Keywords Cognitive test; Equine; Emotions; Welfare; Cortisol; Stress

# 37 Introduction

The consideration of emotional aspects in animal welfare science has increasingly gained importance (Broom, 2003). According to the 5 Domains Model, animal management should reduce situations that negatively affect animals and promote the experience of positive emotions (Mellor and Beausoleil, 2015; Mellor et al., 2020). However, the investigation of welfare is often focused on external circumstances, while affective states are difficult to explore (Duncan, 2006; Mellor et al., 2020).

Cortisol is commonly employed in animal welfare studies as a physiological indicator of 44 stress. Nevertheless, the increase of this glucocorticoid can occur as a normal non-stress-45 physiological response or can be caused by both negative and positive situations, such as 46 pain, anxiety, and fear as well as excitement or pleasure (Koolhaas et al., 2011; Ralph and 47 48 Tillbrook, 2016). Therefore, it is always important to contextualize and try to understand what potentially has caused the activation of the hypothalamic-pituitary-adrenal axis (HPA), 49 which leads to the increase of cortisol (Ralph and Tillbrook, 2016). Feces and horsehair are 50 51 used as non-invasive methods for the determination of cortisol concentrations (Palme, 2012; Duran et al., 2017). While feces evidence the increase of plasmatic cortisol according to the 52 53 animals' metabolism (in horses after about 24 hours) (Mostl et al., 1999), horsehair reflects the individual history, reflecting chronic stress conditions (Duran et al., 2017). 54

However, stress and, consequently, cortisol levels can be influenced by both emotional and physical stressors. Therefore, to investigate emotions in non-vocal animals, research relies on indirect indications of how the animal perceives the environment, considering that emotional states can influence memory, attention, and judgment (Duncan, 2006; Mendl et al., 2009). In particular, Judgment bias tests (JBTs), modulated by human sciences (Bethell, 2015), have been employed in animal research to investigate how the environment or routine procedures affect animals' affective states (Burman et al., 2009, Neave et al., 2013; Daros et al., 2014).
In animal research, judgment bias represents the animal's response to a neutral stimulus and,
evaluating if the individual expects from this stimulus either a positive or a negative outcome,
it defines the subject as optimistic or pessimistic (Mendl et al., 2009).

During recent times, the JBT has been applied in horses (Freymond et al., 2014; Löckener et 65 al., 2016). Domestic horses' welfare and mental health depend on both their relationship with 66 human beings and their management (Hausberger et al., 2009; Freymond et al., 2014). 67 68 Methods of horses' management are varied and differ in terms of freedom of movement, the possibility of interaction with conspecifics, and the kind of feeding and its routine. In some 69 70 countries, domestic horses are traditionally housed in individual stalls, where they spend most of their time (Yarnell et al., 2015). This kind of management is considered practical for 71 72 humans because, for example, it is easier to detect any diseases, control the consumption of water and food, and manage the horse for various routine operations (McGreevy, 2012; Ruet 73 74 et al., 2019). The natural boarding system of the horse tries to reproduce the living conditions of feral equines. Horses usually live in a group in paddocks designed to stimulate movement, 75 76 exploration, relationship with conspecifics, and body care (McIlwraith and Rollin, 2011; Saba et al., 2013). Finally, in Italy, there is a new kind of facility, called Ethological Stable, that 77 has intermediate characteristics of the traditional stable and natural boarding system, and it's 78 79 characterized by bigger stalls than traditional ones and separated by grates with windows, which allow the horses to see and touch each other (Marliani et al., 2021). Whichever 80 management method is considered, it has a profound effect on the welfare of the individual 81 (Yarnell et al., 2015; McGreevy, 2012; Ruet et al., 2019), and JBT could be helpful for the 82 assessment of the welfare impacts of different housing systems, but its application in horses 83 requires further investigation. 84

This research evaluated horses under 3 different management systems: traditional stable, natural boarding, and ethological stable. Each subject underwent a JBT, fecal and horsehair sampling, and an evaluation of personality traits and Body Condition Score (BCS). We tested if the type of management can influence horses' judgment bias. We also investigated the influence of other independent variables (personality, age, and BCS) on judgment bias, and we examined if JBT results correlated to cortisol levels found in feces and horsehair.

#### 91 Material and Methods

#### 92 Stables

#### 93 Natural Boarding System (NB)

This system hosted 21 horses, living 24/7 together in a large paddock of about 6 ha. The 94 paddock was composed of heterogeneous spaces with large areas and corridors, huts and 95 muddy and sandy soil areas (according to season). Trunks and objects, where animals can 96 97 rub, were scattered within the paddock, to stimulate the movement of animals, and the entire 98 area was arranged in the shape of a labyrinth. Hay and water were available ad libitum and 99 horses were occasionally allowed to access grassy lawns. This system was frequented daily by at least 1 person, from a few hours to the entire day. Most of the horses were used for 100 101 occasional horse trekking and horse-riding activities in the sandy field (maximum 2 hr/day) by riders of varying ages and experience. Only 2 of the horses included in the statistical 102 analysis were not involved in these activities. Horses were barefoot and ridden without a bit. 103

#### 104 Traditional Stable (TS)

This facility housed 55 horses during the study. The stable was organized in 2 parallel 105 corridors that had separate rows of individual stalls (3.00 m x 3.50 m). Each stall had wood 106 chip bedding, a sliding door and a window that allowed the horse to look out into the 107 corridor, facilitating visual contact with the other horses in the stable. The facility had 108 individual paddocks (10.00 m x 10.00 m; or 20.00 m x 20.00 m) where horses were pastured 109 110 for a different lag of time that varied depending on weather conditions and the owner's decisions, not every day and only during the daytime. Hay was provided at 7.00 am and 4.00 111 pm and a supply of mixed grains were provided at 8.00 am and 8.00 pm. Most of the horses 112 113 housed in this stable were involved in sporting activities, in particular jumping, dressage, and vaulting with beginners and competitors. The stable was frequented every day and had a 114 115 carousel, a round pen, and some sand courts.

116 Ethological Stable (ES)

The system was the same as described by Marliani et al., 2021. During the study, the stable hosted 11 horses. The stable included 12 stalls, called Big Box© (8.00 m x 5.00 m), arranged in rows of 6. Each stall was divided into 2 sections of 20m2: the front part consisted of highdensity impact rubber mats that provided the horse with rigid support; the rear part consisted of a soft and semi-permanent element of river sand. This system of double litter allowed the

horse freedom of movement and choice of the ground on which to rest. The walls of the Big 122 Box<sup>©</sup> were of metal gratings, with windows between stalls, so that subjects were able to see 123 and touch each other. There was a paddock of 1.50ha organized with large areas and 124 corridors, and 6 paddocks of 2.00ha each. Horses, individually or in a small group, stayed in 125 paddocks all day and were brought into the stall for the evening, or in case of bad weather. 126 127 Hay was available throughout the day and cereals (barley, oats, and corn), apples, and carrots were provided at 7.00 a.m. and 7.00 p.m., according to a personalized food plan. There were 128 also a round sand pen and a covered sand rectangular field. Horses were engaged principally 129 130 in flatwork, country riding, and walks, and they were not used for competitions.

131 Subjects

Forty-one adult horses were involved in the study: 16 subjects from NB, 14 horses from TS,
and 11 subjects from ES (Table 1). All horses had been resident in each stable for at least the
last 3 months. The history of each subject and the characteristics of the stables were collected
using 2 questionnaires.

## 136 Body Condition Score (BCS) and Personality Evaluation

An evaluation of BCS was made for every horse. This is a nutrition assessment system, in 137 which a score is assigned based on the visual (rib and bone prominence) and manual 138 evaluation of the panniculus adipose in 6 main regions: neck, shoulder, withers, thorax, 139 140 lumbar region, and tail attachment. First of all, the amount of fat present is visually evaluated, assessing the visibility or not of bone and rib prominences. The score is from 1 to 9, where 1 141 corresponds to excessive emaciation and 9 is a condition of excessive nutrition (Henneke et 142 143 al., 1983). The BCS was collected and considered in the statistical analysis for all the horses, except for 3 (2 NB horses who suffered from Cushing and 1 ES individual). 144

145 To assess the personality of the subjects involved in the study, a translated version of the

146 HPQ (Horse Personality Questionnaire) (Lloyd et al., 2007) was used. The HPQ was

147 completed by the horse's owner or caregiver. For each of the 25 items, they were asked to

assign a score on a Likert scale from 1 to 7, where 1 indicates that the trait was not at all

149 manifested, while 7 corresponds to the extreme expression of the character trait. The data

150 were analyzed according to Lloyd et al. (2007), obtaining a score for each of the 6 personality

151 components (Dominance, Anxiousness, Excitability, Protection, Sociability, and

152 Inquisitiveness).

#### 153 Judgment Bias Test

The protocol employed in the study was a modification of those proposed by Freymond et al. (2014) and Henry et al. (2017). It was a Go/No-Go JBT with spatial stimuli. Black buckets were employed for the test, to prevent the horses seeing the contents. In each stable, the test was conducted in a fenced arena with a sandy substrate, familiar to the subjects and sufficiently far from disturbances.

159 In the arena, a start-line and a semicircular stop-line were drawn on the ground using the 160 wood-chip, to allow the experimenter to see better when the horses passed the line to stop the stopwatch. The test was carried out by 2 trained experimenters. Experimenter 1 (E1) was in 161 charge of preparing the bucket and placing it behind the finish line, 9 meters from the start 162 line (Figure 1). Experimenter 2 (E2) had the task of leading the horse into the test arena and 163 placing it behind the start line. Only when E1 had placed the bucket, the E2 release the horse 164 and take a step to the side. Once the horse finished the trial, E2 retrieved the horse and 165 brought it back to the start line. The trial was completed when the horse passed the finish line 166 with one of the forelegs, or after 60 seconds, which was the maximum latency considered. E2 167 measured the time taken by the horse to reach and pass the stop line. While the horse 168 completed the trial, the experimenters remained stationary and directed their gaze towards the 169 170 ground to avoid influencing the behavior of the horse. The experimenter who led the horse was always the same for the same horse to get the horse used to the same person and 171 172 minimize biases due to the possible stress caused by different experimenters. The JBT was 173 divided into 4 consecutive days: habituation, training, and 2 sessions of the test.

#### 174 *Habituation*

The first day was employed for the phase of habituation, during which horses got used to the setting. During this phase, 1 horse at a time was led into the arena by an experimenter, then the second experimenter positioned the bucket with a reward, which consisted of a small amount of oats, at the positive position (P). Oats was chosen as a reward because usually horses are highly motivated by them. The habituation was completed when horses, released at the start line, independently approached the bucket 3 consecutive times. The horses were randomly allocated to either the left or right position of the test apparatus.

# 182 Training

For the training phase the negative position (N), corresponding to an empty bucket, was set at 183 the opposite in respect of the positive (P), 7.20 meters apart. The 2 positions were presented 1 184 at a time, and the same position was presented no more than twice in a row. The training 185 always finished with P. E2 recorded the latency of each subject to reach and pass the finish 186 line. If the horse didn't move towards the bucket in 60 seconds, E1 went towards the horse, 187 took it, and led it again to the start position and the trial was considered completed. The 188 training ended when the latencies to reach the last 3 positive buckets were lower than that to 189 reach the last 3 negative ones (Mendl et al., 2010). Following the protocol suggested by 190 191 Henry et al. (2017), each training session was composed of 3 positive positions and 2 negative locations, and finished always with the positive one. The minimum number of 192 sessions employed was two. If the horse failed to distinguish the 2 positions after 5 training 193 sessions, it was excluded from the JBT. 194

195 *Testing* 

The day after the training, the testing phase was performed in 2 consecutive days. During the test, 3 intermediate positions, 1 at a time, between N and P were presented: NN (Near-Negative), M (Middle), and NP (Near-Positive). These were placed 1.80 meter from each other and the bucket was always empty. Before the beginning of the test, N and P were presented as a reminder of the training, a maximum of 6 times. Each session followed the scheme proposed by Henry et al. (2017), where ambiguous locations were preceded alternately by positive and negative locations:

• first session (first day testing phase) P-N-NP-P-N-M-P-N-NN-P

• second session (second day of testing phase) N-P-M-N-P-NN-N-P-NP

The second session always ended with an N position where the bucket was full of food, to exclude horses employed their sense of smell during the test (Mendl et al., 2010).

207 The test sessions were videotaped using a Sony HDR-CX240E camera placed on a tripod

208 outside the arena. All videos were analyzed using the software Solomon Coder, to record

209 precisely the time that horses employed to reach buckets.

- 210 To avoid biases caused by differences in baseline running speeds, due to size and/or age of
- 211 individuals, raw latencies recorded to reach the intermediate positions were transformed into
- scores, according to the formula proposed by Mendl et al. (2010).
- Adjusted latency = (mean latency to ambiguous location -mean latency to P) / (mean latency
- to N –mean latency to P)
- 215 This formula returns 0 for the P and 100 for N.
- 216 Collection of Fecal and Horsehair Samples
- Fecal samples were collected immediately after defecation, for a total of 4 samples for eachhorse that completed the test. The collection was made during:
- 1. the habituation (P1), which was representative of the baseline cortisol concentration beforethe beginning of the whole procedure;
- 221 2. the training (P2), which was representative of cortisol level during habituation;
- 3. the first session of the test (P3), which was representative of training;
- 4. the second session of the test (P4), representative of the first day of the test.
- A total of 86 fecal samples were collected. Some samples were missing for the NB group.
- 225 One horse was excluded because he suffered from Cushing disease, which alters cortisol
- concentration, and some could not be collected for other reasons (1 sample during the
- habituation; 4 samples during the training, and 1 sample for the first session of the test). Each
- fecal sample was placed in a non-sterile container identified with date, time, subject name,
- refrigerated to  $+4^{\circ}$ C, transported to the laboratory and frozen to  $-20^{\circ}$ C.
- 230 Horsehair samples were collected from the base of the mane of each horse who completed the
- JBT and stored at room temperature in identified non-sterile containers, for a total of 20
- samples. The sample of the horse who suffered from Cushing was excluded from the
- 233 statistical analysis.

## 234 Cortisol Assay

- 235 Cortisol concentrations were determined by Radio Immuno-assays (RIAs). Cortisol was
- extracted from fecal specimens (500 mg, wet weight) with methanol-water solution (v/v 4:1)

and ethyl ether, whereas for horsehairs 60 mg of trimmed horsehairs (1-3 mm) of a subject

- were put in a glass vial with 5 ml methanol (Accorsi et al., 2008). All samples were dried
- under an air-stream suction hood at 37°C and the dry-residue was dissolved into phosphate-
- 240 buffered saline (PBS) 0.05 M, pH 7.5. Cortisol metabolites assay in both feces and horsehair
- 241 were carried out according to Tamanini et al. (1983). The cortisol RIA was performed using
- an antiserum to cortisol-21-hemisuccinate-BSA (anti-rabbit), at a working dilution of 1:20
- 243 000 and 3H-cortisol (30 pg/tube vial) as a tracer. Validation parameters of analysis were:
- sensitivity 0.19 pg/mg, intra-assay variability 5.9%, inter-assay variability 8.7%.
- 245 Radioactivity was determined using a liquid scintillation  $\beta$  counter and a linear standard
- curve, ad hoc designed by a software program (Motta and Degli Esposti, 1981).

#### 247 Statistical Analysis

To analyze the collected data, non-parametric statistical methods were employed, given that 248 the small size of the sample did not guarantee the reliability of the normality assumption. In 249 particular, to assess the presence of differences among the management, the Kruskal-Wallis 250 test with Dunn's adjustment for multiple comparisons was adopted. To test for the presence of 251 correlation between numerical variables, Spearman's correlation coefficient was considered. 252 To assess the influence of the position of nominal and numerical variables on the inclusion or 253 the exclusion of the horses from the test, we employed a Chi-squared test and a Mann-254 Whitney test. To compare the fecal cortisol concentration during the different phases of the 255 256 test we employed a Friedman test, with a Wilcoxon pairwise as post-hoc test. Statistical 257 significance was set at P < 0.05. The whole statistical analysis was carried out in the R environment (R Core Team, 2021). 258

# 259 **Results**

#### 260 Task Acquisition

Of the 41 horses considered, only 20 horses completed the JBT and were considered for the statistical analysis (NB n = 10; ES n = 4; TS n = 6). Eleven horses did not pass the habituation phase (NB n = 6; ES n = 1; TS n = 4) and 4 subjects the training (ES n = 4). Moreover, 6 did not maintain a significantly different latency between P and N during the reminders before the test phase (ES n = 2, TS n = 4). The rate of excluded animals was similar for the 3 types of management.

- All the horses included employed the same time to reach N when there was both the emptyand the full bucket (last position of the second test phase).
- 269 Twenty-two horses had the positive bucket on their left side, 19 on their right. Considering
- this variable and the entire group in a chi-squared test, there was a significantly higher
- 271 probability that the horses who had the P location on their left side was excluded from the test
- 272  $(\chi 2 = 5.47, df = 1, P-value = 0.02)$ . Indeed, considering the subjects included in the test, 13
- had P on their right side and 7 on their left.
- The BCS of the excluded horses was significantly (W = 104; r = -0.359; 95% C.I. [-0.614, -
- 275 0.109]; P-value = 0.02) lower (Median  $\pm$ IQR: 4  $\pm$  1.5, n = 19) than that of the included
- 276 subjects  $(5 \pm 2.0, n = 19)$  (Figure 2).
- 277 JBT Scores
- 278 Comparing the 3 different stables, scores recorded in NP ( $\chi 2= 4.2957$ , df = 2, P-value = 0.12)
- and M ( $\chi 2 = 1$ , df = 2, P-value = 0.61) did not significantly differ between the groups, but
- there was a significant difference for NN position ( $\chi 2= 8.3082$ , df = 2, P-value = 0.02).
- Indeed, the horses of ES had an NN score significantly lower  $(6.46 \pm 5.94)$  than subjects of
- 282 NB (100.00  $\pm$  46.50; P-value = 0.01) and than those of TS (72.34  $\pm$  35.75; P-value = 0.04)
- 283 (Figure 3).
- 284 Considering the entire group of horses, the scores recorded in NP (Median  $\pm$  IQR: 10.24  $\pm$
- 44.63), M (2.88  $\pm$  17.34), and NN locations (71.09  $\pm$  67.00) were significantly different,
- according to the Kruskal-Wallis test with Dunn's post-hoc test ( $\chi 2= 18.7236$ , df = 2, P-value <
- 287 0.001). In particular, NN was significantly different from NP (P-value < 0.01) and M (P-
- value < 0.001), but NP and M did not differ (P-value = 0.34). No correlation was found
- between the scores recorded for the intermediate positions and personality traits and age, but
- there was a significant negative correlation between the score in NP and BCS (S = 1907.1, P
- 291 < 0.01, rho = -0.67). Body condition score differs between groups ( $\chi 2$ = 9.0149, df = 2, P-
- value = 0.01) and it was significantly higher in NB ( $6.22 \pm 1.72$ ) horses than in TS ones (3.83)
- $\pm 0.75$ , P-value = 0.01), while ES subjects recorded an intermediate value (5  $\pm$  0), and did not
- differ neither from TS (P-value = 0.14) and NB (P-value = 0.64).
- 295 Cortisol Results

- Fecal cortisol (FC) baseline level (P1) and its concentration during the habituation phase
- 297 (P2), training (P3) and the first session of the test (P4) were determined from the fecal
- samples collected during the 4 days of the JBT. Comparing the FC concentration in P1, P2,
- P3 and P4, it was not significantly different in both TS groups ( $\chi 2=2$ , df = 3, P-value = 0.57)
- 300 and ES ( $\chi 2= 8.1$ , df = 3, P-value = 0.051).
- 301 The FC baseline concentration was not significantly different between the 3 groups of horses
- 302 ( $\chi 2= 0.56$ , df = 2, P-value = 0.75). However, during habituation ( $\chi 2= 6.67$ , df = 2, P-
- value = 0.04), training ( $\chi 2$ = 11.59, df = 2, P-value < 0.001) and the first day of the test ( $\chi 2$ =
- 11.97, df = 2, P-value < 0.001), subjects from NB registered a significantly higher
- 305 concentration of FC than the TS group. The ES group showed a significantly higher
- 306 concentration than TS horses in P3 (P-value = 0.03) and, with a significant tendency, in P4
- 307 (P-value = 0.07). (Table 2).
- 308 Considering horsehair cortisol (HC), its concentration resulted significantly different in the 3
- groups (chi-squared = 13.93, df = 2, P-value < 0.001). It was higher in TS horses than NB
- 310 individuals  $(1.03 \pm 0.16 \text{ pg/mg}; 0.21 \pm 0.19 \text{ pg/mg}; P < 0.001)$ , while the ES group, which
- had intermediate values ( $0.38 \pm 0.14 \text{ pg/mg}$ ), did not differ significantly from either of the
- others (ES vs. NB, P-value = 0.20; ES vs. TS, P-value = 0.14). In addition, as suggested by
- Heimbürge et al., 2019, we analyzed the influence of age and sex. We found a significant
- difference for sex (W = 9, r = -0.54, P-value = 0.01), and female (0.25  $\pm$  0.12) had HC levels
- than geldings ( $0.52 \pm 0.66$ ). In addition, HC concentration was significantly positively
- correlated with age (S = 615.39, P-value = 0.047; rho = 0.46), but the groups did not differ
- significantly for age ( $\chi 2=5.1301$ , df = 2, P-value = 0.08). No significant results were found
- considering the influence of sex (W = 27, P-value = 0.96) and age (S = 1025.2, P-value = 0.82,
- s19 rho = -0.05) on the fecal cortisol baseline (P1).

#### 320 Discussion

- 321 The concept of animal welfare includes the possibility of animals experiencing positive
- 322 emotions, whereas negative emotional and experiential states, such as fear, are sufficient
- 323 elements to compromise it (Dawkins, 2006). The primary function of emotions is to guide
- individual decisions and they are strictly related to the cognitive process (Paul et al., 2005;
- Roelofs et al., 2016; Mendl et al., 2009). In particular, the judgment bias indicates how an
- individual judges a certain situation in dependence of their emotional state (Paul et al., 2005).

The evaluation of judgment bias is considered useful for comparing the emotional state of individuals of the same species living in different environmental situations (Bethell, 2015). This research aimed to understand the usefulness of JBT in the evaluation of welfare in horses in different housing and management systems by comparing results between JBT and chronic HPA activation. We also considered other independent variables, such as BCS, age and personality traits, to determine their influence on the outcome of the test.

Only 20 on 41 horses completed all the phases of JBT, which poses serious doubts about the 333 334 possibility to generalize the results (Roelofs et al., 2016). However, as the study of JBT in horses is relatively recent and the difficulty of this species in learning and memorizing the 335 336 spatial discrimination between P and N has already been reported (Hintze et al., 2018), we consider this study to be a useful addition to the current literature. The learning processes of 337 the subjects across the trial was not influenced by the type of management, similar to what is 338 reported by Henry et al. (2017). However, we identified 2 other factors that could have 339 influenced the exclusion rate: the position of the positive bucket and the BCS. As for the 340 341 position of the bucket, we cannot exclude a phenomenon of lateralization. Lateralization in 342 vertebrates is the different specialization of the 2 cerebral hemispheres and it can be observed both in motor and sensorial (olfaction and sight) activities (Vallortigara and Rogers, 2005). 343 Horses have eyes in a lateral position, with decussation of 80-90% of optic fibres (Brooks et 344 345 al., 1999). Similar to other species, they usually use the right eye, so the left hemisphere, to investigate novel objects, and the left eye to evaluate objects with an emotional negative 346 valence (De Boyer Des Roches et al., 2008). The positioning of the positive bucket on their 347 left side could have negatively influenced the value that the subjects have assigned to the 348 349 object. Moreover, the positive position was the first and only position presented to the horse 350 during the entire habituation phase. Another factor that seems to affect the inclusion/exclusion rate is the BCS of the horses. Indeed, the excluded subjects have a BCS 351 significantly lower than the included ones. BCS is an indirect measure of the adequate 352 nutritional intake and health state of the individuals, and a potential index for the presence of 353 pathologies. A healthy equid should have a BCS between 4 and 6 (Henneke et al., 1983; 354 355 Yngvesson et al., 2019) and in this range fall the median values registered in all the 3 stables. However, if we consider the BCS as a potential indicator of motivation towards food (Henry 356 357 et al., 2017), horses with a lower BCS could be less motivated and this can negatively influence the learning of a discrimination task based on food rewards (McCall, 1989). In 358 359 addition, we should consider the different management and the different activities the horses

are used for. Indeed, in the 3 management we noticed that NB horses had a significant higher
BCS than TS. Not only feeding routine, health status and environment can influence BCS,
but also the type of exercise (Christie et al., 2006; Zoller et al., 2019). Therefore, the
differences that we found in the 3 systems can be also due to the differences of equestrian

activities in which horses were involved.

The 20 horses included in the test obtained different scores for each intermediate position, demonstrating that they can differentiate NP, M, and NN. All individuals included showed the same latency to reach N also in the last trial when the bucket was filled, demonstrating that they did not use their sense of smell to deal with the JBT (Mendl et al., 2010).

The first of our predictions was that independent variables, such as body condition score 369 (BCS), age and personality traits, do not influence the JBT results. No correlation was found 370 between JBT scores and both age and personality traits. Regarding personality traits, this 371 result agrees with Lalot et al. (2017) that did not evidence any correlation between 372 personality traits and an optimistic bias in canaries. On the contrary, in another study by 373 Barnard et al. (2018), it was found that dogs with higher sociability and excitability 374 approached faster the ambiguous probes of a spatial JBT than those characterized by high 375 separation-related anxiety and dog-directed fear. Therefore, the relationship between 376 personality and JBT seems to be complex as its outcomes are influenced not only by 377 personality traits but also by transient affective states (Whittaker and Barker, 2020), which 378 379 are difficult, if not impossible, to describe in a non-vocal species. Moreover, the HPQ was 380 validated in English and we employed a translated version of the HPQ, so we cannot exclude the presence of possible bias due to the use of a different language. Instead, a negative 381 382 correlation was found between BCS and the score in NP, so the latency to reach the nearpositive position was lower for horses with a higher BCS. This result is in agreement with the 383 384 previous result about the inclusion rate and confirms the fact that a different motivation among individuals can influence the JBT result. 385

Secondly, we predicted that different management styles would have affected JBT results.
Only the ES group showed a significantly lower latency to reach NN, suggesting that those
horses had greater anticipation of a positive outcome at that location, and thus experienced a
general more positive affective state. NN is usually associated to negative outcome and a
slower latency to reach this probe location is usually interpreted as index of fear and anxiety,
so of a pessimistic affective state (Barnard et al., 2018). Indeed, our results are similar to

those of Karagiannis and colleagues (2015) that found that dogs suffered from separation 392 related problems approach significantly slower NN probes but not the other locations. 393 However, this result should be considered with caution, because only 4 of 11 horses 394 completed the JBT in the ethological stable. Contrary to our predictions, NB and TS did not 395 score differently to reach intermediate positions, although these 2 management systems are 396 397 extremely different. It cannot be excluded that the manipulation by strangers of NB horses and, overall, the separation from their herd caused a mild state of distress, which could have 398 altered the results of JBT. This hypothesis can be partially confirmed by the analysis of fecal 399 400 cortisol, which evidenced an increasing level of glucocorticoids in the feces (representative of daily levels of stress) and a significantly higher FC concentration in NB in respect to TS 401 subjects during the JBT procedure. According to Roelofs et al. (2016), involving social 402 animals individually in JBT could increase stress perception. Indeed, it is well known that 403 social isolation induces stress in most gregarious species, manifested through behavioral and 404 405 physiological changes such as increasing vocalization, locomotion, heart rate, and plasma cortisol levels (Mal et al., 1991; Wolff, Hausberger and Le Scolan, 1997; Lansade and 406 407 Bouissou, 2008), and the horse is a social animal that forms preferential bonds within the herd to guarantee social stability (Beaver, 2019). Therefore, the procedure itself could have 408 409 been stressful for NB horses, which are also usually less used to being manipulated by strangers, and this can have determined that they did not differ from the TS group in their 410 411 latency to reach intermediate positions. All these elements suggest that JBT may not be reliable to evaluate the quality of the management style for these individuals because it is 412 413 affected by contingent confounding factors.

Finally, we predicted that acute and chronic activation of the HPA axis would be different 414 according to the management system and this would be reflected in the JBT results. As for 415 the acute activation, the fecal cortisol results are partly in accordance with the JBT results, as 416 explained in the previous paragraph. In any case, we recommend collecting fecal samples at 417 least 2 days after the end of the JBT procedure, to check the level of cortisol also during the 418 second day of testing and at the return to the baseline. Fecal cortisol concentrations help us to 419 420 measure not only cortisol base line, but also animal's stress response, avoiding the invasiveness of blood collection. It reflects an average of the plasma cortisol secreted and 421 422 metabolized by the animal over the course of a species-specific period (in horses about 24 423 hours) (Mostl et al., 1999; Sheriff et al., 2011). Therefore, the daily collection of fecal

424 cortisol allowed us to monitor the stress of animals possibly caused by the entire procedure of
425 JBT, considering also that it could be a stressful disruption of their daily routine.

We obtained surprising results when we considered horsehair cortisol concentration, whose 426 results, if matching with those of JBT, would have reinforced the role of JBT as a useful 427 method to assess welfare in managed horses. Indeed, HC concentration indicates the chronic 428 activation of the HPA axis and it is commonly employed to assess the chronic stress 429 conditions in animal welfare studies because the concentration of cortisol in the hair reflects 430 431 the history of the subject (Prandi et al., 2010). We found that NB horses had significantly lower horsehair cortisol level than TS horses, suggesting a lower activation of the HPA axis 432 433 in the long term, whereas ES horses recorded intermediate values of HC and did not differ significantly from the others, which is in line with the management style that we could 434 435 consider "intermediate" between TS and NB. These results are in accordance with Placci et al. (2020), who found a most favorable endocrine framework in horses kept in natural 436 boarding, suggesting that this kind of management style matched most with the ethological 437 and physiological needs of the species. From these results, we would have expected that NB 438 439 horses also had the best judgment bias, and vice versa for the TS group, whereas NB and TS did not score differently to reach intermediate positions. Therefore, in our study horsehair 440 cortisol did not correlate with JBT results, which is quite surprising given that JBT is 441 442 considered a helpful method in the assessment of good management. Cortisol level should be discussed considering several limitations. In particular sex, breed and seasons (Aurich et al., 443 2015; Heimbürge et al., 2019) are the principal influencing factors that was not possible to 444 control in this study, both for the availability of the structures and for the exclusion of most of 445 the horses from the JBT. Analyzing also our statistical results, female and younger animal 446 seems to have a generally lower HC level. This could have influenced the lower HC found in 447 NB group, considering in TS group there were only geldings. Also, the season could be 448 another potential confounding factor. Indeed, NB was tested in spring, while TS and ES in 449 autumn and summer. Mazzola and collegues (2021) found higher cortisol concentration in the 450 hair of horses during summer and autumn, in accordance with Aurich et al. (2015), which 451 452 found that salivary cortisol level was lower in March and April. In the feces baseline, we did not find any difference between sexes and no correlation with age. Also, the season seemed 453 454 to not have any influence, because baseline cortisol level did not show any difference among 455 the 3 groups.

#### 456 Conclusion

This is the first research using JBT in horses which also considers fecal and horsehair cortisol 457 analysis. Considering the endocrinological results, with all their limitation, we cannot exclude 458 that the procedure of the JBT itself can induce acute stress to some subjects and this is a 459 potential confounding factor for the evaluation of the living environment on the immediate 460 affective state of these animals. Moreover, the horsehair cortisol, which reflects the 461 accumulated stress in the long term, did not match JBT results, thus posing even more doubts 462 on the reliability of this test per se as applied to the evaluation of management. However, 463 horsehair cortisol evaluation presents several limitations and potential confounding factors. 464 465 According to our results, the use of the JBT test on horses seems much affected by individual motivation for food, as emerged by the high exclusion rate and by the fact that horses highly 466 motivated for food (and present both in the natural boarding and the traditional stable 467 management) had the best results for NP score, thus reducing the possibility to impartially 468 evaluate the effect of management on the individuals. 469

In conclusion, we strongly suggest further research about the application of JBT in this
species, both to ensure a higher rate of inclusion by reducing confounding factors, and to
avoid the evaluation of management on its sole basis. We also recommend the selection of a
multidisciplinary approach in the welfare assessment of horses.

#### 474 Ethical Considerations

- 475 The study was not invasive or the animals and was evaluated and approved by the scientific
- 476 Ethics Committee for Animal Welfare of DIMEVET (Department of Veterinary Medical
- 477 Sciences) of the University of Bologna. The trial was monitored for Animal Welfare.

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# 481 **Conflict of Interest**

482 The authors declare no conflict of interest.

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# 625 Tables

626**Table 1.** The number of total mares, geldings, and stallions for each stable and mean age  $\pm$ 627standard deviation of each group considered for the study. In brackets the number of mares,628gelding and stallions and mean age  $\pm$  standard deviation of horses that completed the test.

629

	Natural Boarding	Traditional Stable	Ethological Stable
Mares	7 (4)	4 (0)	5 (1)
Geldings	9 (6)	9 (6)	6 (3)
Stallions	-	1 (0)	-
Age (years)	$14 \pm 6 (12 \pm 6)$	$18 \pm 5 \; (17 \pm 4)$	$18 \pm 6 (19 \pm 3)$

#### 630

631 Table 2. Results of the Kruskal-Wallis test with Dunn's adjustments for multiple comparisons

632 of fecal cortisol concentration (pg/mg) recorded in each phase of the test, considering a time-

lag of 24 hours from their collection. The median  $\pm$  IQR were reported for each kind of

634 management, and the letters indicate the different groups according to Dunn's test.

Phase	Ethological Stable	Natural Boarding	Traditional Stable
P1 (baseline)	$3.04 \pm 1.57^a$	1.71±4.40 <sup>a</sup>	2.33±0.47 <sup>a</sup>
P2 (habituation)	3.06±0.42 <sup>b</sup>	6.80±1.86 <sup>a</sup>	3.26±3.39 <sup>b</sup>
P3 (training)	5.92±2.63 <sup>a</sup>	7.52±5.21 <sup>a</sup>	3.49±1.34 <sup>b</sup>
P4 (test1)	7.08±4.22 <sup>a,b</sup>	9.71±3.12 <sup>a</sup>	$2.45 \pm 1.74$ <sup>b</sup>
a h = n < 0.05			

635 a,b = p < 0.05

636

# 637 Figures

**Figure 1.** Representation of the test arena. P = Positive location; NP = Near Positive

639 intermediate location; M = Medium intermediate location; NN = Near Negative intermediate 640 location; N = Negative location. P and N were randomly on the left side or the right side of

641 the horse.



642 643

Figure 2. Boxplot of the Body Condition Score (BCS) of excluded and included horses. The
box plots show the median and 25th and 75th percentiles; the whiskers indicate the values
within 1.5 times the inter-quartile range, IQR. The outlier is indicated by the black circle; *P*-

647 value < 0.05 is indicated by \*.



Figure 3. Boxplot of JBT results of each group (ES = ethological stable; NB = natural
boarding system; TS = traditional stable). The box plots show the median and 25th and 75th
percentiles; the whiskers indicate the values within 1.5 times the inter-quartile range, IQR.
The outlier is indicated by the black circle. P-value = 0.01is indicated by \*\*, and P-value <</li>
0.05 is indicated by \*.



659