ARTICLE INFORMATION	Fill in information in each box below
Article Type	Research article
Article Title (within 20 words without abbreviations)	Behavioral and cardiac responses in mature horses exposed to a novel object
Running Title (within 10 words)	Temperament comparison with behavioral and cardiac parameters in mature horses
Author	Kyung Eun Lee1, Joon Gyu Kim2, Hang Lee1, Byung Sun Kim2
Affiliation	1 College of Veterinary Medicine, Seoul National University, Seoul 08826, Republic of Korea 2 Department of Equine Science, Cheju Halla University, Jeju 63092, Republic of Korea
ORCID (for more information, please visit https://orcid.org)	Kyung Eun Lee (https://orcid.org/0000-0002-6938-6621) Joon Gyu Kim (https://orcid.org/0000-0002-6246-2097) Hang Lee (https://orcid.org/0000-0003-0264-6289) Byung Sun Kim (https://orcid.org/0000-0003-1008-2305)
Competing interests	No potential conflict of interest relevant to this article was reported.
Funding sources State funding sources (grants, funding sources, equipment, and supplies). Include name and number of grant if available.	This study was partially supported by the Research Institute for Veterinary Science, Seoul National University.
Acknowledgements	We are grateful to Myung Sun Chun and Janne Winther Christensen for the valuable comments and advices to this manuscript. We also thank Gemma Pearson for support which was critical for HRV data correction.
Availability of data and material	Upon reasonable request, the datasets of this study can be available from the corresponding author.
Authors' contributions Please specify the authors' role using this form.	Conceptualization: Kim BS. Data curation: Lee KE, Kim BS, Kim JG. Formal analysis: Lee KE. Funding acquisition: Lee H. Investigation: Lee KE, Kim BS, Kim JG. Methodology: Kim BS, Kim JG. Project administration: Lee H. Resources: Kim BS, Kim JG. Software: Lee KE, Kim JG. Supervision: Kim BS. Visualization: Lee KE, Lee H. Writing - original draft: Lee KE. Writing - review & editing: Lee H, Kim BS, Kim JG.
Ethics approval and consent to participate	All procedures were approved by the Institutional Animal Care and Use Committee at Seoul National University (license: SNU-190121-3-1).

CORRESPONDING AUTHOR CONTACT INFORMATION

For the corresponding author (responsible for correspondence, proofreading, and reprints)	Fill in information in each box below
First name, middle initial, last name	Byung Sun, Kim
Email address – this is where your proofs will be sent	bskim0929@chu.ac.kr

Secondary Email address	bskim0929@naver.com
Address	Cheju Halla University, 38, Halladaehak-ro, Jeju-si, Jeju 63092, Republic of Korea
Cell phone number	+82-10-3314-3176
Office phone number	+82-64-741-7663
Fax number	+82-64-741-6585

6

For the corresponding author (responsible for correspondence, proofreading, and reprints)	Fill in information in each box below
First name, middle initial, last name	Hang, Lee
Email address – this is where your proofs will be sent	hanglee@snu.ac.kr
Secondary Email address	
Address	Bldg. # 85-812 Seoul National University College of Veterinary Medicine, 1 Gwanak-ro, Gwanak-gu, Seoul 08826, Republic of Korea
Cell phone number	+82-10-8900-2754
Office phone number	+82-2-880-1274
Fax number	+82-2-888-2754

7
8

9   **Abstract**

10   This study aimed to investigate whether breed, sex, and age affected temperament differently (more or less

11   neophobic) in mature horses during a novel object test. The study included Jeju crossbred (n = 12, age = 9.42 ± 4.57

12   y), Thoroughbred (n = 15, age = 10.73 ± 3.09 y), and Warmblood horses (n = 12, age = 13.08 ± 3.55 y) with the

13   females (n = 22, age = 11.36 ± 4.24 y) and geldings (n = 17, age = 10.65 ± 3.66 y). Jeju crossbreds (Jeju horse ×

14   Thoroughbred) are valuable considering their popular usage in South Korea, but limited studies have explored

15   temperament of Jeju crossbred horses. A trained experimenter touched the left side of the neck with a white plastic

16   bag (novel object). The test ended when the horse stopped escape response and heart rate dropped to baseline.

17   Behavioral score and escape duration were measured as behavioral variables. Multiple variables related to heart rate

18   (HR) and heart rate variability (HRV) were measured to reflect emotional state. These included basal HR (BHR),

19   maximum HR (MHR), delay to reach maximum heart rate (Time to MHR), standard deviation of beat-to-beat

20   intervals (SDNN), root mean square of successive differences (RMSSD), and ratio of low to high frequency

21   components of a continuous series of heartbeats (LF/HF). Statistics revealed that Thoroughbreds had significantly

22   higher behavioral scores, and lower RMSSD than Jeju crossbreds ($p < 0.05$), suggesting greater excitement and fear

23   to the novel object in Thoroughbreds. None of the behavioral or cardiac parameters exhibited sex differences ($p <$

24   0.05). Age was negatively correlated with SDNN and RMSSD ($p < 0.05$), indicating that older horses felt more

25   anxiety to the novelty than younger horses. Thoroughbreds and females had distinct correlations between behavioral

26   and HRV variables in comparison with other groups ($p < 0.05$), implying that escape duration might be a good

27   indicator of stress, especially in these two groups. These results are expected to improve equine welfare, safety and

28   utility, by providing insights into the temperament of particular horse groups, to better match reactivity levels with

29   specific functions.

30

31   **Keywords**: equine, temperament, novelty, behavior, heart rate, heart rate variability

32

33

34

35   **Introduction**

3

36   As prey animals, horses have evolved a strong susceptibility to fear-eliciting stimuli, increasing survival

37   among individuals that react quickly to any hint of potential danger [1]. This characteristic contributes to

38   determining horse temperament and is heritable. Thus, domestic horses share this neophobic response with their

39   wild ancestors. Temperament has a strong genetic component in horses [2], and this explains why, even after

40   habituation, innately fearful horses will still exhibit flight responses to novel objects [3]. Given that horse

41   characteristics are very important to riders and breeders for improving safety and horse welfare [4], there is

42   considerable interest in understanding equine temperament.

43   In multiple animal species, behavioral, heart rate (HR), and heart rate variability (HRV) responses are the

44   most commonly used variables in predicting temperament traits [5]. Many studies have evaluated the temperament

45   and emotional state of horses through observing behavioral and cardiac changes after novel object tests [6-8]. The

46   consensus from novel object experiments is that behavioral and cardiac activity are significantly correlated and they

47   are complementary measures of temperament each other, which should improve reliability. However, limited studies

48   have investigated behavioral and cardiac parameters from a temporal perspective in temperament tests. Thus,

49   temporal variables were included in this study (escape duration for behavior, and time to maximum heart rate

50   (MHR) for HR), to explore both the degree of the surprise response to the novel object, and the duration of that

51   response. These parameters may help further elucidate the temperament traits of horses. The current study

52   investigated breed, sex, and age differences in temperament using behavioral, HR and HRV responses to an

53   unfamiliar, complex, stimulating object in mature horses.

54   In Jeju (a native Korean pony) crossbred horse (Jeju horse × Thoroughbred), which is one of the subject

55   horse breeds, several reports explored the genetic basis of temperament, with the estimation of genetic parameters,

56   and genetic correlations between temperament traits [9, 10]. However, relationships between behavior, physiological

57   responses, and temperament, have not been studied in this breed. These horses are especially common in youth

58   horseback riding in South Korea [11], because they are similar in size to ponies. It is widely known among people

59   that Jeju crossbred horses are bolder and less prone to startle, compared with other breeds in the same environment,

60   and their advantages include strong hooves, a high feed efficiency, and endurance [12]. Horseback riding is an

61   increasingly popular recreational activity in South Korea, and given this economic significance, further studies on

62   Jeju crossbreds are warranted.

63   This study aimed to explore breed, sex, and age differences in the temperament using a novel object test in

64   mature horses, ages of which were in range of being ridden as horseback riding. Our subjects included Jeju

4

65 crossbreds, Thoroughbreds, and Warmbloods, the three most popular breeds for horseback riding in South Korea.

66 We hypothesized that breed, sex, and age would differentially affect horse temperament. Our results will benefit the

67 selection of horses with a temperament appropriate for a specific purpose, thus increasing their utility and welfare.

68 Horses with an optimal temperament will also improve human safety when horseback riding, potentially increasing

69 the activity's popularity and economic benefits.

70

Materials and Methods

72 *Animals and management*

73 The study included 12 Jeju crossbred horses (eight mares and four geldings), 15 Thoroughbred horses

74 (eight mares and seven geldings), and 12 Warmblood horses (six mares and six geldings). Jeju crossbreds ages were

75 9.42 ± 4.57 years; Thoroughbreds 10.73 ± 3.09 years; and Warmbloods 13.08 ± 3.55 years. Age distributions did not

76 significantly differ within both breed and sex groups ($p > 0.05$). Horses were part of the horseback riding classes at

77 the Cheju Halla University (Jeju island, Republic of Korea), and were not ridden more than once a day. On non-

78 riding days, they were exercised for an hour on a horse-walker. All subjects were housed under comparable

79 conditions in individual box stalls. Water was provided *ad libitum* in automatic drinkers, while feed (hay and

80 concentrates) were provided three times a day. All animals were checked to ensure that they were in good condition,

81 and had no obvious cardiac abnormalities that could affect HRV. All the horses in the University facility were

82 subject to the same management principles regardless of the breed, sex or age.

83

84 *Experimental design*

85 Test environment

86 The experiments took place in a 30 m × 70 m indoor riding arena, located in a building adjacent to the

87 stables. All horses had been exercised in the site at least 5 days a week for over a year.

88

89 Test objects

90 The stimulus in the novel object test was a white plastic bag (25 x 30 cm). The plastic bag was tied to the

91 end of a slender, flexible stick about 1 m long. Plastic bags are a complex stimulus combining visual, auditory, and

92 tactile elements, making them a common choice for such studies [13-15].

Test procedures

The test was conducted between 09:00 and 11:00 . A halter and with a 5 m lead rope was used to lead the horses. A handler already well known to the subjects, who was skilled in training horses and very experienced with this test, did not change during the whole experiments. The horse was affixed with HR equipment upon entry to the arena, and was left for 10 minutes (min) to adapt to the situation. During this period, the experimenter stood quietly next to the horse with hiding the stimuli behind his back, but without touching the subject. An assistant familiar with the horses then measured baseline HR. Subsequently, the experimenter gently exposed the stimuli and touched the area between the horse's left shoulder and neck at intervals of about one second. If the horse exhibited flight responses, the tester loosened the lead rope but held onto the end. The test ended when evasion responses ceased and HR back at baseline. Throughout the experiment, the second experimenter checked HR, and informed the first experimenter when he should stop the touching action.

Behavioral observations

Two variables for behavior were used; behavioral score and escape duration (Table 1). Behavioral score was assessed using the following scale by an assistant: 1 (very fearless, no reaction) to 5 (very fearful, showing flight response) (Table 2). Behavioral scoring system was adapted from previous studies [16, 17]. An assistant recorded videos of the novel object test at approximately 4 m from the subject horse. Videos were later assessed for behavioral score and scored to calculate escape duration, beginning from stimulus initiation to the cessation of responses.

Monitoring and videotaping of heart rate (HR) and heart rate variability (HRV)

Heart rate was recorded continuously from 2 min before stimulus introduction to the end of the experiment, using a Polar V800 Equine heart rate sensor (Polar Electro OY, Kempele, Finland). Normally 5 min intervals are recommended for HRV analysis, but previous findings identified that there were no significant differences in some HRV features between 5 min and 2 min intervals when detecting mental stress [18]. All horses were familiar with the sensor because it is used for periodic health examinations. The device consisted of an electrode belt with a built-in transmitter and a wristwatch receiver. Water and conductive gel were used to improve electrode-to-skin contact. Data were uploaded to Polar Flow software for storage, and analyzed using Kubios HRV

software (Kubios standard 3.2.0, Biomedical Signal Analysis Group, Department of Applied Physics, University of

Kuopio, Finland). Measurements of HR and HRV occurred after visual inspection to correct any artifacts by setting

the custom filter to 0.3, according to the previous study [19].

Six HR and HRV parameters were determined per horse (Table 3). Basal HR (BHR) 5 s before the

introduction of the plastic bag, and maximum HR (MHR) during the test were measured. Delay to reach MHR

(Time to MHR) from the beginning of the test was measured in seconds. Standard deviation of the beat-to-beat

intervals (SDNN), the root mean square of the successive differences (in milliseconds) (RMSSD), and the low

frequency/high frequency ratio (LF/HF) were analyzed as HRV parameters (Table 3). SDNN measures the state of

balance between sympathetic and parasympathetic activities of the heart. RMSSD is normally used to estimate high

frequency beat-to-beat variations that represent vagal regulatory activity [5]. Lower SDNN and RMSSD values

indicate a shift towards sympathetic dominance, while higher values of these parameters reflect a shift towards

parasympathetic dominance. The LF/HF ratio indicates sympathetic activity under physical or psychological

stressors. An increase in LF/HF means a regulatory shift toward sympathetic dominance [8].

Statistical analysis

Data normality was determined with QQ-plots and the Shapiro-Wilk's test. Non-normal data (escape

duration, Time to MHR, SDNN, RMSSD, and LF/HF) were log-transformed. Significance was set at $p < 0.05$.

Between-breed differences in behavior and HR parameters were analyzed with a one-way analysis of variance

(ANOVA), followed by a post-hoc Tukey's HSD test. Independent sample t-tests were conducted to look for sex

differences in response variables. A breed $\times$ sex interaction was tested using a two-way ANOVA and Tukey's HSD

test. Age effects were analyzed with Pearson correlations. Pearson correlation coefficients between behavioral and

cardiac parameters were calculated within breed and sex.

Results

Two-way ANOVA revealed that the breed $\times$ sex interaction was not significant in each dependent parameter ($p >$

0.05).

Breed

150   Breed significantly affected behavioral score, but not escape duration (Table 4). Thoroughbreds had the

151   highest behavioral scores (3.73 ± 0.93), followed by Warmblood (3.58 ± 0.95) and Jeju crossbred horses (2.75 ±

152   1.01). Importantly, Thoroughbreds showed a significantly higher reactivity than Jeju crossbreds ($F_{2,36}$ = 3.556, p =

153   0.039). However, Warmbloods were intermediate in their responses, and did not show striking differences compared

154   with the others. Escape duration did not differ among breeds (p > 0.05). Jeju crossbreds had a significantly higher

155   BHR and RMSSD (BHR: 44.42 ± 5.79; RMSSD: 68.50 ± 21.18) than Thoroughbreds (BHR: 40.27 ± 4.19; RMSSD:

156   49.12 ± 16.81) (p < 0.05), with Thoroughbreds and Warmblood horses similar in both parameters (Table 4).

157   Furthermore, MHR, Time to MHR, SDNN, and LF/HF did not vary among breeds (p > 0.05).

158

159 *Sex & Age*

160   Females and geldings did not differ significantly in behavioral or cardiac parameters (Table 4, p > 0.05).

161   Age was not correlated with any behavioral reactions (Table 5, p > 0.05). Age did not affect any HR

162   variables or LF/HF, but SDNN and RMSSD decreased significantly with increasing horse age (SDNN: r = -0.488, p

163   = 0.002; RMSSD: r = -0.416, p = 0.008).

164

165 *Correlations between behavioral and cardiac parameters in the novel object test*

166   Behavioral score was correlated with MHR in all breeds (Table 6), with the highest positive correlation in

167   Jeju crossbreds (r = 0.807, p = 0.002). However, other significant correlations between behavioral and cardiac

168   indices were only detected in Thoroughbreds, and not in the others. Specifically, among Thoroughbreds, behavioral

169   score was negatively correlated with SDNN (r = -0.644, p = 0.010), while escape duration was negatively correlated

170   with both SDNN (r = -0.699, p = 0.004) and RMSSD (r = -0.614, p = 0.015).

171 For the most part, behavioral score was positively correlated with MHR in both sexes (Table 6), but not correlated

172 with any other HR or HRV parameters. Escape duration was not significantly correlated with HR variables, but was

173 negatively correlated with both SDNN (r = -0.451, p = 0.035) and RMSSD in females (r = -0.516, p = 0.014).

174

175 # Discussion

176   A survey by Sackman and Houpt regarding horse personality which was assessed by horse owners,

177   revealed that Thoroughbreds were the most nervous breed, whereas ponies had a less nervous personality [20].

Warmbloods had an intermediate level of nervousness. Other previous studies concur with this. A study exploring horse temperament reported that Thoroughbreds were ranked highly in breeds showing high excitability and anxiousness [21]. Another study demonstrated that ponies had lower anxiety scores than sport horses [22]. The results of the current study concur with these studies in that Thoroughbreds had significantly higher behavioral scores than other horse breeds. Jeju crossbred horseshad the lowest behavioral score in our study.

The neophobic response in horses is largely genetic [23]. A previous study exploring horse temperament has revealed the close association in temperament between Thoroughbreds and Arabians, which was inferred because of the origins of Thoroughbreds in Arabians, Barbs, and Turkomen breeding stock [20]. Jeju crossbred horses originated from crosses between Thoroughbred and Jeju horses. Thoroughbreds are renowned as being of nervous disposition [20], but Jeju horses are demonstrated to have a moderate and bolder temperament [12]. Therefore, the Jeju crossbreds may have attenuated the vigilant traits in Thoroughbreds, which was identified in the present study. Kim revealed that the gentleness, patience, and aggressiveness, patience, and aggressiveness had moderate heritabilities, and that sensitivity had a high heritability in the temperament of Jeju crossbred horses [10]. So, selected breeding for appropriate temperament would be effective for optimal utility in this breed.

Significantly higher BHR in Jeju crossbreds compared to Warmbloods was attributed to their different body size. It was identified that body size and metabolic rate have significant relationship, so resting heart rate is faster in small equine breeds compared to larger ones [24]. In the current study, the results of cardiac activity after exposed to the novelty suggested that Thoroughbreds were more sensitive than Jeju crossbreds. There was no meaningful difference among breeds in MHR and Time to MHR, but RMSSD of HRV differed between Jeju crossbreds and Thoroughbreds. Individuals exposed to stressful situations have a lower vagal tone which means a regulatory shift toward sympathetic dominance, as seen in the lower RMSSD of Thoroughbreds compared with Jeju crossbreds, indicating increased vulnerability to stress. A change in HR may result from a combination of simultaneous transitions in activity, within both the vagal and sympathetic branches, so it has a limit in being used for accurately assessing sympathovagal balance as an index of psychological stress. On the other hand, HRV provides more accurate information on ANS (autonomic nervous system) regulation traits, and that is the reason why HRV is often coupled with HR parameters to obtain a clearer measure of stress in many animals [5]. Because changes in the sympathetic nervous system indicate the arousal dimension of the effect [25], our findings seem to suggest that Jeju crossbreds are less astonished by the novel object compared with the others.

206   It was stressed out the importance of gonadotropin action for the maturation of male, and the increase in

207   sex related genes expression with increasing age [26]. A previous study revealed significant temperament

208   differences between stallions and mares, as well as between stallions and geldings, but not between mares and

209   geldings [27]. This outcome is likely because geldings are castrated and produce considerably less testosterone than

210   stallions. The resultant effects on secondary sex traits could explain the lack of clear temperament differences

211   between mares and geldings. Our result concurs with this hypothesis. The current study did not reveal any obvious

212   sex differences in behavioral parameters.

213   Differences in HR and HRV responses between females and geldings were also not detected. A previous

214   evaluation of temperament using behavioral and cardiac responses did not find clear sex differences between mares

215   and geldings [28]. Another study examining sex differences in novel-object response among young horses (9–22

216   months old) revealed that sex did not influence HR variation [8]. Our results in this study are in line with the

217   previous findings, given that geldings have similar testosterone production as juvenile male horses.

218   The effect of age on temperament is debatable. A previous study reported that older horses had a

219   significantly higher behavioral reactivity to novel stimuli than younger horses [27]. In contrast, another study on

220   horses < 22 months old found that behavioral response frequency to a novel object decreased with increasing age

221   [29]. Yet other study suggested that age had no influence on horse temperament [30], as did the current study,

222   because no correlations between age and behavioral parameters was identified in this study. With regard to cardiac

223   variables, age was not significantly correlated with HR parameters in this study, but was negatively correlated with

224   some HRV variables. A low SDNN and RMSSD typically signals sympathetic activation associated with stressful

225   circumstances. Previous research showed that older horses were less behaviorally responsive to stressful stimuli than

226   younger horses, but exhibited a shift toward control by the sympathetic nervous system, which was indicated by a

227   lower standard deviation of the beat-to-beat intervals (SDRR, in ms) and RMSSD [31]. SDRR is regarded as the

228   same as SDNN, and representation of the data is identical [32]. These partially matched with the results of this

229   study. In this study, even though behavioral responses and age had positive correlation coefficients, the correlations

230   were not significant. However, it was revealed that older horses showed significantly decreased SDNN and RMSSD

231   values than younger horses, implying a higher degree of stress.

232   This phenomenon might be due to training obscuring the natural responses of horses to unexpected stimuli,

233   as demonstrated by a previous study [33]. Mature 'schooled' horses trained to stay in place do not always exhibit

234   typical behavioral responses, whereas younger horses with less training tend to run away when surprised. This

235 influence of training suggests that behavioral and HR reactivity to the novel object test may not be fully reflective of

236 temperament. However, with additional HRV data, we might better predict stress levels, increasing the accuracy of

237 temperament assessments. Therefore, even if the older horses in this study did not reveal or show lower behavioral

238 or HR responses due to training when compared to younger horses, they were able to be more nervous to the

239 unfamiliar objects than young horses.

240   Many studies on horses have demonstrated significant correlations between behavioral and cardiac

241 variables during novel object tests [6, 34, 35], which concluded that both of these methods should be used to draw

242 comprehensive assessment of stress reactions. Our study corroborated previous researches showing that behavioral

243 score was highly correlated with MHR in all horse groups. Increased HR is accepted as a proxy for fear in novel

244 object tests in horses [5, 8], therefore, the current study suggests that the stronger the behavioral score, the more fear

245 the horses felt normally.

246   In each breed and sex group, the correlation results between behavioral and cardiac parameters highlighted

247 the characteristics of each group. A previous study with Hanoverian horses identified there were strong correlations

248 between both reactivity and the time to calm down, and both HR and RMSSD, after a horse was exposed to a

249 suddenly moving stimulus [28]. In the present study, Thoroughbreds and females exhibited the highest correlations

250 between behavioral and cardiac parameters compared with other breed or sex groups. Lower HRV indices (SDNN

251 and RMSSD) indicates a shift in physiological control to the sympathetic nervous system after a lack of sufficient

252 parasympathetic counteraction [5]. Horses with a low parasympathetic response are more stressed [8, 36]. It was

253 reported that evaluations of emotional state in horses with a behavior reaction are easier to perform in

254 Thoroughbreds than other breeds, and the frequency of escape attempts is the most appropriate behavioral reactivity

255 for the assessment of the emotional state of that breed [37]. Our results show that there are significant correlations

256 between behavioral reactions and emotional state, especially in Thoroughbreds and females. These two groups

257 specifically showed high correlation frequency between escape duration and both SDNN and RMSSD compared to

258 other breed and sex groups. The results suggest that escape duration might be useful to detect stress levels,

259 especially in certain horse groups for which behavioral differences are not detected. Thus, the potential value of

260 escape duration indices in predicting the stress levels of animals, and complementing other HR and HRV parameters

261 with more horse breeds need to be further explored.

262   Like many traits, temperament has both environmental and genetic components. According to the previous

263 study [38], the effect of habituation is applied to very specific object, so horses could show surprising responses to

11

264 the novel thing even after habituation to the similar object. For example, the fear reaction to the tarp remained after

265 one week despite the habituation to the umbrella. In this study, environmental factors were minimized as much as

266 possible through testing horses that were born on a single farm, or had resided there for at least 1 year by the time of

267 the experiment. However, differences in recent living background and previous experience which was unknown to

268 researcher among some of subjects before the experiment could lead to environmental effects overshadowing

269 hereditary effects in the temperament test. Therefore, studies focusing on environmental factors are necessary for a

270 better understanding of horse temperament in the future.

271

272 # Conclusion

273   In conclusion, our study identified clear differences between breeds in behavioral and cardiac parameters

274 when responding to a novel stimulus. The temperament of Jeju crossbreds, derived from a Korean native pony, is

275 rarely studied, despite their popular usage in South Korea for horseback riding. In particular, Thoroughbreds

276 exhibited greater responses, and hence more stress than Jeju crossbreds. Sex differences in temperament were not

277 detected. However, age was negatively correlated with SDNN and RMSSD, possibly due to increased experience

278 with training among older horses. Thoroughbred and female groups had stronger correlations between escape

279 duration and certain HRV indices, implying that escape duration might be used as stress indicator. The findings of

280 this study will improve the prediction of sensitivity of responses of horses to novel objects based on breed, sex, or

281 age. Horses can be matched on the basis of level of experience of the rider (less nervous horses more suitable for

282 beginners) with the results, thus lowering the risk of horseback riding. These findings will contribute to efforts

283 aimed at improving horse welfare and safety, while also providing means to increase the potential economic

284 benefits.

285

286 # References (Vancouver or NLM style)

287 1.   Shapira I, Sultan H, Shanas U. Agricultural farming alters predator-prey interactions in nearby natural

288 habitats. Animal Conservation. 2008;11(1):1-8.

289 2.   Monika B, Jarosław K, Beata M, Leszek S. Relationships between thoroughbreds' contribution in the

290 pedigree and the level of fearfulness and performance in warmblood stallions. Acta Veterinaria. 2018;68(3):288-

291 300.

292   3.   Leiner L, Fendt M. Behavioural fear and heart rate responses of horses after exposure to novel objects:

293   Effects of habituation. Applied Animal Behaviour Science. 2011;131(3-4):104-9.

294   4.   Graf P, von Borstel UK, Gauly M. Importance of personality traits in horses to breeders and riders. Journal

295   of Veterinary Behavior: Clinical Applications and Research. 2013;8(5):316-25.

296   5.   Von Borell E, Langbein J, Després G, Hansen S, Leterrier C, Marchant-Forde J, et al. Heart rate variability

297   as a measure of autonomic regulation of cardiac activity for assessing stress and welfare in farm animals—A review.

298   Physiology & behavior. 2007;92(3):293-316.

299   6.   Christensen JW, Keeling LJ, Nielsen BL. Responses of horses to novel visual, olfactory and auditory

300   stimuli. Applied Animal Behaviour Science. 2005;93(1-2):53-65.

301   7.   Lansade L, Bouissou M-F, Erhard HW. Fearfulness in horses: A temperament trait stable across time and

302   situations. Applied Animal Behaviour Science. 2008;115(3-4):182-200.

303   8.   Visser E, Van Reenen C, Van der Werf J, Schilder M, Knaap J, Barneveld A, et al. Heart rate and heart rate

304   variability during a novel object test and a handling test in young horses. Physiology & Behavior. 2002;76(2):289-

305   96.

306   9.   Kim NY, Seong HS, Kim DC, Park NG, Yang BC, Son JK, et al. Genome-wide analyses of the Jeju,

307   Thoroughbred, and Jeju crossbred horse populations using the high density SNP array. Genes and Genomics.

308   2018;40(11):1249-58.

309   10.   Kim NY, Son JK, Cho IC, Shin SM, Park SH, Seong PN, et al. Estimation of genetic parameters for

310   temperament in Jeju crossbred horses. Asian-Australasian Journal of Animal Sciences. 2018;31(8):1098-102.

311   11.   Choi SC, Yang JH, Ryu EA. A study on the diversification strategy of under-utilized horses. 2019.

312   12.   Yoon MJ, Kim BS, Do KT, Jung HJ, Kim JY, Choi YJ, et al. Research for developing a Korean riding

313   horse breed (HALLA HORSE). 2019.

314   13.   Hintze S, Murphy E, Bachmann I, Wemelsfelder F, Würbel H. Qualitative behaviour assessment of horses

315   exposed to short-term emotional treatments. Applied Animal Behaviour Science. 2017;196:44-51.

316   14.   Kim BS, Kim JG, Chung SH. Assessment of heart rate and behavioral response for the prediction of

317   effectiveness of desensitization training in horses. Annals of Animal Resource Sciences. 2018;29(1):33-41.

318   15.   Søndergaard E, Jago J. The effect of early handling of foals on their reaction to handling, humans and

319   novelty, and the foal–mare relationship. Applied animal behaviour science. 2010;123(3-4):93-100.

320   16.   Brunberg E, Gille S, Mikko S, Lindgren G, Keeling LJ. Icelandic horses with the Silver coat colour show

321   altered behaviour in a fear reaction test. Applied Animal Behaviour Science. 2013;146(1-4):72-8.

322   17.   Christensen JW, Malmkvist J, Nielsen BL, Keeling L. Effects of a calm companion on fear reactions in

323   naive test horses. Equine Veterinary Journal. 2008;40(1):46-50.

324   18.   Castaldo R, Montesinos L, Melillo P, James C, Pecchia L. Ultra-short term HRV features as surrogates of

325   short term HRV: A case study on mental stress detection in real life. BMC Medical Informatics and Decision

326   Making. 2019;19(1):5-11.

327   19.   Ille N, Aurich J, Erber R, Aurich C. Comparison of heart rate and heart rate variability obtained by heart

328   rate monitors and simultaneously recorded electrocardiogram signals in nonexercising horses. Journal of Veterinary

329   Behavior: Clinical Applications and Research. 2014;9(6):341-6.

330   20.   Sackman JE, Houpt KA. Equine personality: Association with breed, use, and husbandry factors. Journal of

331   Equine Veterinary Science. 2019;72:47-55.

332   21.   Lloyd AS, Martin JE, Bornett-Gauci HLI, Wilkinson RG. Horse personality: Variation between breeds.

333   Applied Animal Behaviour Science. 2008;112(3-4):369-83.

334   22.   Roberts K, Hemmings AJ, Moore-Colyer M, Parker MO, McBride SD. Neural modulators of temperament:

335   A multivariate approach to personality trait identification in the horse. Physiology and Behavior. 2016;167:125-31.

336   23.   Hausberger M, Scolan NL. Interplay between environmental and genetic factors in temperament /

337   personality traits in horses (*Equus caballus*). 2004;118(4):434-46.

338   24.   Schwarzwald CC, Vet M, Kedo M, Birkmann K, Vet M, Hamlin RL. Relationship of heart rate and

339   electrocardiographic time intervals to body mass in horses and ponies *. Journal of Veterinary Cardiology.

340   2012;14(2):343-50.

341   25.   Yeates JW, Main DCJ. Assessment of positive welfare: A review. Veterinary Journal. 2008;175(3):293-

342   300.

343   26.   A CVH-l, A DS, B IW. Effect of stallion age on the expression of LH and FSH receptors and aromatase

344   P450 in equine male reproductive tissues. 2016;450:2016-26.

345   27.   Graf P, Von Borstel UK, Gauly M. Practical considerations regarding the implementation of a temperament

346   test into horse performance tests: Results of a large-scale test run. Journal of Veterinary Behavior: Clinical

347   Applications and Research. 2014;9(6):329-40.

348 28.   Von Borstel UK, Euent S, Graf P, König S, Gauly M. Equine behaviour and heart rate in temperament tests

349 with or without rider or handler. Physiology & behavior. 2011;104(3):454-63.

350 29.   Visser EK, van Reenen CG, Hopster H, Schilder MBH, Knaap JH, Barneveld A, et al. Quantifying aspects

351 of young horses' temperament: consistency of behavioural variables. Applied Animal Behaviour Science.

352 2001;74(4):241-58.

353 30.   Wolff A, Hausberger M, Le Scolan N. Experimental tests to assess emotionality in horses. Behavioural

354 Processes. 1997;40(3):209-21.

355 31.   Baragli P, Banti L, Vitale V, Sighieri C. Effect of aging on behavioural and physiological responses to a

356 stressful stimulus in horses (*Equus caballus*). Behaviour. 2014;151(11):1513-33.

357 32.   Shaffer F, Ginsberg JP. An overview of heart rate variability metrics and norms. Frontiers in Public Health.

358 2017;5(September):1-17.

359 33.   Munsters CC, Visser KE, van den Broek J, van Oldruitenborgh-Oosterbaan MMS. The influence of

360 challenging objects and horse-rider matching on heart rate, heart rate variability and behavioural score in riding

361 horses. The Veterinary Journal. 2012;192(1):75-80.

362 34.   Wilk I, Janczarek I, Zastrzeżyńska M. Assessing the suitability of Thoroughbred horses for equestrian

363 sports after their racing careers. 2016. p. 43-9.

364 35.   Young T, Creighton E, Smith T, Hosie C. A novel scale of behavioural indicators of stress for use with

365 domestic horses. Applied Animal Behaviour Science. 2012;140(1-2):33-43.

366 36.   Janczarek I, Stachurska A, Wilk I, Krakowski L, Przetacznik M, Zastrzeżyńska M, et al. Emotional

367 excitability and behaviour of horses in response to stroking various regions of the body. Animal Science Journal.

368 2018;89(11):1599-608.

369 37.   Wilk I, Janczarek I. Relationship between behavior and cardiac response to round pen training. Journal of

370 Veterinary Behavior: Clinical Applications and Research. 2015;10(3):231-6.

371 38.   Christensen JW, Zharkikh T, Ladewig J. Do horses generalise between objects during habituation? Applied

372 Animal Behaviour Science. 2008;114(3-4):509-20.

373

374

15

Tables and Figures

375

383

384

385

386   **Table 1** Ethogram of behaviors recorded in the novel object test.

Behavioral parameter	Definition
Behavioral score	Rated behavioral reactivity by the experimenter during the test according to the behavioral reactivity scale Table 2.
Escape duration (s)	The time from stimulus initiation to the end of escaping responses. The end point was decided as the point in time when the test horse first stood still, and did not exhibit physical signs of fearfulness over 15 seconds (e.g., stiff neck muscles and head moving along with the stimulus).

387   Abbreviation: s, seconds.

388

389 **Table 2** Behavioral score during the novel object test.

Behavioral score	Description
5	Horse suddenly jumps away more than 2 meters from the novel object, typically followed by trotting, galloping, alertness, and possibly snorting; keeps far away from the novel object despite handler encouragement
4	Horse is alert and suddenly jumps away less than 2 meters from the novel object, typically followed by trotting, galloping, further alertness, and possibly snorting
3	Horse escapes and exhibits vigilant behavior (elevated neck, head, and ears oriented toward the test stimulus), typically followed by trotting, galloping, alertness, and possibly snorting
2	Horse escapes but is easily restrained
1	Horse does not react to the novel object and approaches it without hesitation

390

391 **Table 3** Cardiac parameters in response to the novel object.

Cardiac parameters (unit)	Definition
BHR (bpm)	Basal heart rate measured before 5 s of plastic-bag introduction
MHR (bpm)	Maximum heart rate during the novel object test
Time to MHR (s)	Delay to reach maximum heart rate from the beginning of the test
SDNN (ms)	Standard deviation of the beat-to-beat intervals
RMSSD (ms)	Square root of the mean squared differences of successive beat-to-beat intervals
LF/HF	Ratio of low frequency (LF) to high frequency (HF) components of a continuous series of beats (power spectrum)

392 Abbreviations: bpm, beats per minute; s, seconds; ms, milli seconds.

18

393 **Table 4** Means ± SD of behavioral and cardiac parameters, calculated for breed and sex.

			Behavior		Heart rate			Heart rate variability		
		N	Behavioral score	Escape duration (s)	BHR (bpm)	MHR (bpm)	Time to MHR (s)	SDNN (ms)	RMSSD (ms)	LF/HF
Breed	Jeju crossbred	12	2.75 ± 1.01 [a]	98.58 ± 48.23 [a]	44.42 ± 5.79 [a]	132.42 ± 13.31 [a]	37.00 ± 12.96 [a]	86.98 ± 33.11 [a]	68.50 ± 21.18 [a]	1.76 ± 0.79 [a]
	Thoroughbred	15	3.73 ± 0.93 [b]	118.27 ± 75.36 [a]	40.27 ± 4.19 [ab]	146.07 ± 23.74 [a]	40.87 ± 19.81 [a]	66.79 ± 21.72 [a]	49.12 ± 16.81 [b]	4.24 ± 3.94 [a]
	Warmblood	12	3.58 ± 0.95 [ab]	118.33 ± 52.82 [a]	39.42 ± 4.86 [b]	137.58 ± 15.54 [a]	57.67 ± 31.92 [a]	66.78 ± 19.35 [a]	50.48 ± 13.45 [ab]	3.88 ± 3.62 [a]
Sex	Female	22	3.45 ± 1.08 [a]	109.05 ± 62.49 [a]	41.95 ± 5.24 [a]	141.50 ± 16.62 [a]	41.82 ± 18.24 [a]	69.28 ± 29.66 [a]	52.60 ± 20.51 [a]	3.72 ± 3.91 [a]
	Gelding	17	3.29 ± 1.02 [a]	116.35 ± 61.07 [a]	40.41 ± 5.41 [a]	136.35 ± 22.28 [a]	48.76 ± 29.99 [a]	77.81 ± 21.78 [a]	59.25 ± 17.24 [a]	2.90 ± 2.42 [a]

394 Different letters within breed or sex group in a single column indicate significance at $p < 0.05$.
395 Abbreviations: SD, standard deviation; bpm, beats per minute; s, seconds; ms, milli seconds; BHR, basal heart rate; MHR, maximum heart rate; SDNN, the standard
396 deviation of N-N intervals; RMSSD, root mean square of successive differences; LF/HF, the ratio of low frequency to high frequency components of continuous series
397 of beats (power spectrum).

398 **Table 5** Correlations in parameters studied in the novel object test with regard to the age of horse.

	Behavior		Heart rate			Heart rate variability		
	Behavioral score	Escape duration (s)	BHR (bpm)	MHR (bpm)	Time to MHR (s)	SDNN (ms)	RMSSD (ms)	LF/HF
age	0.165	0.118	0.225	0.187	0.267	**-0.488**	**-0.416**	0.073

399 Correlations significant at $p < 0.05$ are marked in bold.
400 Abbreviations: bpm, beats per minute; s, seconds; ms, milli seconds; BHR, basal heart rate; MHR, maximum heart
401 rate; SDNN, the standard deviation of N-N intervals; RMSSD, root mean square of successive differences; LF/HF,
402 the ratio of low frequency to high frequency components of continuous series of beats (power spectrum).

403 **Table 6** Correlations between behavioral and cardiac parameters in the novel object test, grouped by breed and sex.

Behavioral parameters			N	Heart rate			Heart rate variability		
				BHR (bpm)	MHR (bpm)	Time to MHR (s)	SDNN (ms)	RMSSD (ms)	LF/HF
Behavioral score	Breed	Jeju crossbred	12	0.132	**0.807**	0.311	0.126	0.070	-0.462
		Thoroughbreds	15	0.173	**0.690**	0.160	**-0.644**	-0.429	0.129
		Warmbloods	12	0.361	**0.646**	0.168	-0.140	-0.146	0.569
	Sex	Female	22	0.101	**0.638**	0.145	-0.248	-0.298	0.186
		Gelding	17	-0.097	**0.775**	0.374	-0.470	-0.355	0.286
Escape duration (s)	Breed	Jeju crossbred	12	-0.130	-0.096	0.075	0.468	0.500	-0.261
		Thoroughbreds	15	0.338	0.076	0.089	**-0.699**	**-0.614**	0.270
		Warmbloods	12	-0.054	0.434	0.537	-0.430	-0.152	0.064
	Sex	Female	22	-0.136	0.319	0.026	**-0.451**	**-0.516**	0.220
		Gelding	17	0.192	-0.032	0.472	-0.029	0.271	0.087

404 Correlations significant at $p < 0.05$ are marked in bold.
405 Abbreviations: N, the total number of horses used for a particular group; bpm, beats per minute; s, seconds; ms,
406 milli seconds; BHR, basal heart rate; MHR, maximum heart rate; SDNN, the standard deviation of N-N intervals;
407 RMSSD, root mean square of successive differences; LF/HF, the ratio of low frequency to high frequency
408 components of continuous series of beats (power spectrum).