Equine Research

Cardiac activity and salivary cortisol concentration of leisure horses in response to the presence of an audience in the arena

Iwona Janczarek [a], Izabela Wilk [a,*], Anna Stachurska [a], Leszek Krakowski [b], Marta Liss [a]

[a] Faculty of Biology, Animal Sciences and Bioeconomy, Department of Horse Breeding and Use, University of Life Sciences in Lublin, Lublin, Poland
[b] Faculty of Veterinary Medicine, Department and Clinic of Animal Reproduction, University of Life Sciences in Lublin, Lublin, Poland

ABSTRACT

The aim of this study was to determine the influence of different forms of an audience's behavior on the heart work parameters and salivary cortisol level of leisure horses during work. Twelve horses were studied at rest, during standardized work, and at recovery. There was no audience in the arena in the control variant, whereas an audience (10 people) was situated in the middle of the arena in four other variants: standing still and silent in the first variant, standing still and talking in normal voice in the second variant, being silent but walking in both directions along the middle line of the arena in the third variant, and walking as above and simultaneously talking in the fourth variant. Heart rate, heart rate variability parameters: root mean square of successive beat-to-beat differences, low frequency of the power spectrum (LF), high frequency of the power spectrum (HF) and ratio of LF and HF signal (LF/HF), as well as salivary cortisol levels were measured. We found that audience presence and various forms of behavior during horse-riding leisure hours caused a significant decrease ($P < 0.05$) in the activity of the parasympathetic part of the autonomic nervous system, which reduces inhibiting functions in favor of functions activating the body. The horses' reaction to the audience was more intensive at faster gaits. When the audience was simultaneously walking and talking, there was a particularly distinct decrease in the activity of the parasympathetic system, and stress shown by an increase of cortisol secretion was also generated.

© 2018 Elsevier Inc. All rights reserved.

Introduction

Horses are thinking, emotional, and decision-making beings (Brandt, 2004). The positive and negative emotions that commonly occur in animals greatly influence behavior toward various situations, other individuals, or objects (Mellor, 2012). Anxiety and fear are some of the primary signs of negative emotions that usually result in undesirable forms of behavior (Keeling et al., 2009; Phelps and LeDoux, 2005). A consequence of repeated or long-lasting experiences caused by negative emotions is stress, which affects anatomic, behavioral, endocrine, and immunological responses of the body (Lazarus, 1993). The emotions and emotional excitability connected with the emotions are expressed by changes in cardiac activity: heart rate (HR) and heart rate variability (HRV) (von Borell et al., 2007). Faced with stress, the body defends itself by secreting considerable amounts of cortisol (Lassourd et al., 1996). Unfortunately, long-lasting enhanced secretion of this hormone may decrease individual fitness by immunosuppression and atrophy of tissues (Möstl and Palme, 2002; Munck et al., 1984). It is known that both inherited and environmental factors influence an animal's individuality (McBride and Mills, 2012). The role of people in affecting the individuality is currently considered to be the most important environmental factor (Maurstad et al., 2013). Studies have shown that other horses play only a secondary role in the life of today's horses (Hallberg, 2008). Long-term inappropriate application of negative reinforcement schedules may result in a chronic stress situation for the animal, potentially leading to reduced health (Broom and Johnson, 1993), high reactivity to acute stressors (Adell et al., 1988), or for some individuals, "learned helplessness" (behavioral depression) (Weiss et al., 1981).

Demands expressed ambiguously and inconsistently by riders and caretakers can establish undesirable behavior in a horse, then conflict, misunderstanding, and finally, behavioral anomalies and other stress symptoms (Hothersall and Casey, 2012). Other ways of expressing the

* Address for reprint requests and correspondence: Izabela Wilk, Department of Horse Breeding and Use, University of Life Sciences in Lublin, Akademicka 13, 20-950 Lublin, Poland. Tel: 48 (81) 4456503; Fax: 48 (81) 4456973.
E-mail address: izabela.wilk@up.lublin.pl (I. Wilk).

emotions are specific forms of behavior, which are sometimes considered to be malicious or aggressive (Hall et al., 2013). A lack of awareness about such behavior most often develops into inappropriate human ways of handling horses (Hausberger et al., 2008). Environmental factors such as the human-horse interaction and others that influence horses' emotional excitability should be examined carefully because they can decrease performance even in horses with outstanding aptitudes (von Borstel et al., 2011).

The crucial effect of people on horse's behavior begins at the birth of a foal; the best example of this is imprinting (Mosbach and Ramström, 1996). Later, horses are subject to initial schooling, breaking, and finally, specialized training (Waran and Casey, 2005). At each stage, the horse must deal with many people, including a rider, trainer, groom, farrier, and veterinarian (Goodwin, 1999). New people usually appear at different stages of life and this is sometimes associated with numerous changes of ownership and location (Janczarek and Kędzierski, 2011).

A specific type of contact between a person and a horse appears during leisure (Keaveney, 2008), when horses are ridden and handled by many familiar and unfamiliar people with different skill levels (VanDierendonck and Goodwin, 2005). These people may behave in ambiguous or unpredictable way. Another element which may increase emotional excitability among leisure horses is an audience. The audience is understood to be a group of people in close proximity that may behave in a way that causes various reactions in horses (Hallberg, 2008), such as negative emotions and even frustration, aggression, and many other signs of stress like physiological changes (Houpt and McDonnell, 1993). It has not yet been documented to what extent the audience influences emotional excitability and stress in horses, and consequently, their behavior. This seems to be particularly important in the case of leisure horses when maintaining a high level of safety is crucial. Audience presence may be more detrimental in competitions than in leisure; however, competitions are especially dedicated to the audience and they should not be run without an audience. In leisure, the presence of third parties is allowed and sometimes helpful but these people should be aware of the emotional reaction in horses. In addition, the third parties in leisure may be removed or asked to behave in a certain way. Our hypothesis was that an audience presence and different forms of an audience's behavior do not affect the emotional excitability of leisure horses. If that hypothesis was not correct, it would indicate ways to modify procedures to increase safety in leisure horse riding. The objective of the present study was to determine the influence of different forms of an audience's behavior on cardiac activity parameters and salivary cortisol levels in leisure horses.

Material and methods

Horses and their work regime up to 3 days before the experiment

The study included 12 adult warmblood horses, riding-school geldings, maintained in the same stable and surrounded by the same horses in neighboring boxes for at least 12 months. They were typically ridden in a group of five to six horses at a walk, trot, and canter for 1 hour per day, 6 days a week, in an open or covered arena. There were only riders and one trainer familiar with the horses in the arena. The riders groomed their horses for a total of approximately 30 minutes before and after the work. The horses were in a paddock for 1 to 3 hours every day, depending on the weather conditions.

Experimental procedure

The experiment was conducted between 10:00 h and 12:00 h to minimize possible effects of circadian rhythm in cardiac activity and cortisol concentrations (Irvine and Alexander, 1994; Kuwahara et al. 1999). Five research variants that differed according to the presence

Table 1
Description of research variants

Research variant	Description of audience factor in particular research variants
Research variant 0 (RV0)	No audience in the arena except for riders on the horses, a trainer, and a researcher
Research variant 1 (RV1)	An additional 10 silent people standing still in the middle of the arena
Research variant 2 (RV2)	An additional 10 people standing still and talking in normal voice in the middle of the arena
Research variant 3 (RV3)	An additional 10 silent people walking in both directions along the middle line of the arena
Research variant 4 (RV4)	An additional 10 people walking in both directions along the middle line of the arena and talking in normal voice

Table 2
Sources of variation

Sources of variation	df	F	P
HR			
Research variant	2	1335.31	0.0000
Type of effort	8	3.10	0.0037
Research variant × type of effort	4	8.88	0.0000
RMSSD			
Research variant	4	6.89	0.0002
Type of effort	2	249.10	0.0000
Research variant × type of effort	8	4.68	0.0001
LF			
Research variant	4	6.07	0.0005
Type of effort	2	67.61	0.0000
Research variant × type of effort	8	2.83	0.0073
HF			
Research variant	4	4.26	0.0049
Type of effort	2	123.07	0.0000
Research variant ×	8	3.84	0.0006
LF/HF			
Research variant	4	3.51	0.0301
Type of effort	2	3.92	0.0230
Research variant × type of effort	8	2.88	0.0324
Cortisol			
Research variant		11.225	0.0000
Cortisol concentration	2	7.462	0.0009
Research variant × cortisol concentration	8	5.996	0.0000

df, degrees of freedom; F, value of F-Snedecor test; LF, low frequency of the power spectrum; p, probability value; RMSSD, root mean square of successive beat-to-beat differences.
Factors are significant at $P < 0.05$ and highly significant at $P < 0.01$.

Table 3
HR (beats per minute) in horses in research variants

Research variant/type of effort	Rest	Work			Recovery
		Walk	Trot	Canter	
Research variant 0					
Mean	31.25a	58.08a	82.58a	108.42a	56.00a
SD	1.06	3.65	4.89	3.42	3.41
Research variant 1					
Mean	32.33a	59.67a	88.25a	110.42a	61.75b
SD	0.98	3.37	5.38	5.68	3.14
Research variant 2					
Mean	32.58a	59.33a	95.83b	118.92a	61.50b
SD	1.00	5.25	9.38	6.54	6.20
Research variant 3					
Mean	32.25a	60.67a	88.25a	117.58b	61.08b
SD	0.87	6.04	6.03	10.07	4.42
Research variant 4					
Mean	31.83a	64.50b	94.50b	119.75b	63.92b
SD	1.27	7.18	11.41	6.66	6.14

HR, heart rate; SD, standard deviation.
Means in columns marked with different letters statistically differ at $P < 0.05$.
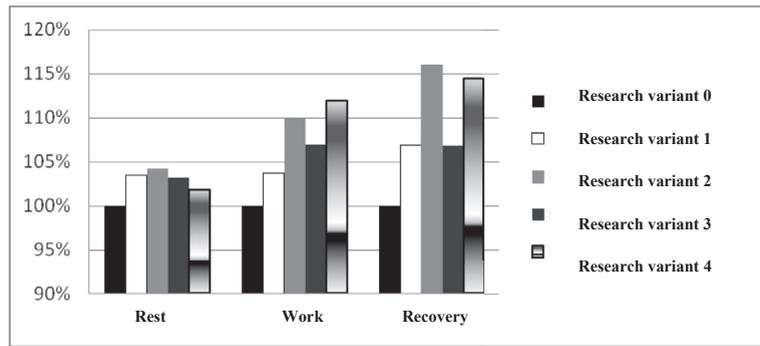
Rest					Work					Recovery				
RV0	RV1	RV2	RV3	RV4	RV0	RV1	RV2	RV3	RV4	RV0	RV1	RV2	RV3	RV4
a	a	a	a	a	a	a	bc	ac	bc	a	a	b	a	b

Different letters within a variant show statistical differences between means at p<0.05.

Figure 1. Percentage changes in the HR in research variants versus RV0. HR, heart rate; RV0, research variant 0.

and behavior of an audience were performed: one control variant without the audience (RV0) and four variants with the audience present (RV1, RV2, RV3, and RV4; Table 1). The audience consisted of 10 persons, each time the same, who were unfamiliar with the horses. The horses were randomly divided into two groups of six horses and next, six riders were randomly assigned, a rider to one horse from each group, for the entire experiment. Within each research variant, the two 6-horse groups were tested one by one, so that each horse experienced each of the variants one time. Successive variants were conducted at 3-day intervals. There were similar weather conditions during each variant ($16 \pm 2°C$ air temperature; $68 \pm 3\%$ relative humidity; 1012 ± 4 hPa barometric pressure; 0.5 ± 0.2 m/s wind velocity). Both 3 days before the beginning of the experiment and during breaks between successive variants, the horses were only in the paddock for 120 minutes per day. The six riders who mounted the horses in the experiment were of the same sex and had similar age, body weights, and level of equestrian experience. The horses were ridden in the arena independently, in equal distances. Each time, the horses' work was standardized according to the following outline: mounting immediately after entering the arena, 10 minutes of walking (5 minutes to the left and 5 minutes to the right), 7 minutes of trotting to the left, 3 minutes of walking, changing rein, 7 minutes of trotting to the right, 3 minutes of walking, changing rein, 1 minute of cantering to the left at 350 m/minute, 1 minute of trotting, changing rein, 1 minute of cantering to the right at 350 m/minute, 2 minutes of trotting, changing rein, 20 minutes of walking (10 minutes to the right and 10 minutes to the left).

Cardiac activity was measured using a PolarRS800CX telemetric transmitter (Polar Electro Oy, Kempele, Finland). The gauge used was adapted to register HR and HRV (von Borell et al., 2007). The equipment included an electrode that detected the heart echo and a receiver with micro memory that constantly registered the heart activity. The electrode was dampened with an ultrasonography gel and then attached to an elastic belt around the horse's chest, on the left side of the horse's body in the heart region, that is, approximately 10 cm above the olecranon. At the beginning of the recording, the receiver stopwatch was turned on in synchrony with a manual stopwatch, which enabled a constant reading of the data. Considering the type of effort, the data were collected at rest (while the stable was quiet in the morning; 20 minutes measurement), at work (from the beginning of the first walk after mounting till the end of the last trot; a continuous 35-minute measurement, later divided into 16 minutes of walking, 17 minutes of trotting, and 2 minutes of cantering), and at recovery (during the last walk; 20-minute measurement).

Table 4
RMSSD (ms) in horses in research variants

Research variant/type of effort	Rest	Work			Recovery
		Walk	Trot	Canter	
Research variant 0					
Mean	88.38a	40.47a	13.66a	13.58a	44.71a
SD	8.79	12.23	4.09	4.24	6.57
Research variant 1					
Mean	90.53a	39.48a	11.93ac	11.42ac	34.34b
SD	12.70	6.73	3.60	4.49	11.60
Research variant 2					
Mean	92.28a	40.58a	10.03bc	9.38bc	30.75b
SD	8.08	16.30	4.12	4.18	8.71
Research variant 3					
Mean	95.87a	34.28a	10.22 bc	9.94bc	32.63b
SD	17.63	12.47	6.07	4.41	10.52
Research variant 4					
Mean	87.17a	19.21b	7.49b	10.28bc	27.95b
SD	20.91	9.21	3.04	3.03	7.25

RMSSD, root mean square of successive beat-to-beat differences; SD, standard deviation.
Means in columns marked with different letters statistically differ at $P < 0.05$.
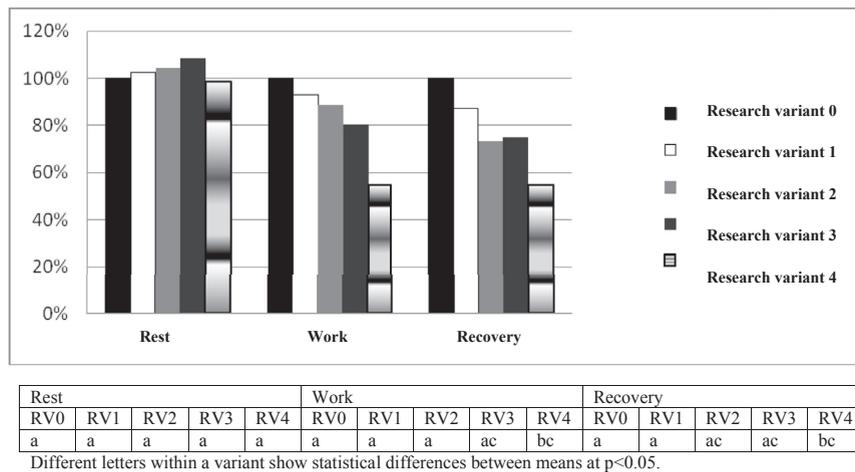
Rest					Work					Recovery				
RV0	RV1	RV2	RV3	RV4	RV0	RV1	RV2	RV3	RV4	RV0	RV1	RV2	RV3	RV4
a	a	a	a	a	a	a	a	ac	bc	a	a	ac	ac	bc

Different letters within a variant show statistical differences between means at p<0.05.

Figure 2. Percentage changes in RMSSD in research variants versus RV0. RMSSD, root mean square of successive beat-to-beat differences; RV0, research variant 0.

The data were transmitted with a specialized peripheral IrDA type wireless device to a base computer and then analyzed using Kubios HRV software (Kuopio, Finland) and Polar ProTrainer 5.0 software (Kempele, Finland). Trend components were removed from the data, and artifact correction was made following established methods (Tarvainen et al. 2009). The data were used to analyze the following parameters: HR frequency, that is, the number of heart beats per minute; root mean square of successive beat-to-beat differences (RMSSD) (ms)—the square root of the mean of squares of the successive differences between adjacent R-R intervals in the QRS curve in the electrocardiogram record; LF (ms^2)—low frequency spectrum $(0.040–0.150$ Hz), which expresses the activity of the sympathetic system; HF (ms^2)—high frequency spectrum $(0.150–0.400$ Hz), which expresses the activity of the parasympathetic system; and LF/HF—percentage ratio of low frequency to high frequency components of a continuous series of beats (power spectrum), which indicates the balance of the autonomic nervous system (ANS) (Tarvainen et al., 2014).

To determine the level of cortisol secretion, that is, the stress indicator, saliva samples were collected three times during each research variant: at rest (while the stable was quiet in the morning), at work (after the last trot), and at recovery (20 minutes after the last walk). The samples were collected with a small sponge cube moistened with a 1% solution of acetic acid. The sponge was inserted into the horse's mouth using long nippers and then the horse's tongue, sides of cheeks, and palate were rubbed with the sponge. The sponge soaked in saliva was placed in a centrifuge test tube and separated from the bottom with a plastic 2-cm long tube. The plastic tube in the test tube protected against renewed soaking of the material by the sponge. Until all the measurements were finished, samples were kept at $−12°C$, which made it possible to remove the viscosity of the saliva. All the test tubes were successively centrifuged. The cortisol concentration was determined by enzyme immunoassay, using an appropriate set of reagents. The concentration (mg/dl) was measured using a reader produced in Labsystem (Krakow, Poland) and endowed with GENESIS V 3.00 software (Paris, France).

Statistical analysis

The null hypotheses were that an audience presence and different forms of an audience's behavior as well as the type of horse effort do not affect the emotional excitability of leisure horses. Two-factorial analysis of variance for repeated measures was performed using a SAS software package (v. 6.0 SAS, Cary, USA) (O'Rourke and Hatcher, 2013). Two factors and an interaction between the factors were considered: (1) the research variant (RV0, RV1, RV2, RV3, and RV4) and (2) the type of effort (for analysis of

Table 5
LF (ms^2) in horses in research variants

Research variant/type of effort	Rest	Work			Recovery
		Walk	Trot	Canter	
Research variant 0					
Mean	1656.93a	1629.96a	385.42a	348.90a	933.97a
SD	1063.47	979.93	221.57	223.02	407.99
Research variant 1					
Mean	1691.98a	1344.12a	460.15a	306.05a	1235.50ab
SD	819.99	872.60	254.81	269.90	818.18
Research variant 2					
Mean	2433.34b	1580.91a	179.10b	122.16b	1162.05a
SD	1268.30	786.55	238.86	101.32	566.81
Research variant 3					
Mean	2487.85b	1320.05a	168.33b	167.96b	1054.45a
SD	1217.61	823.69	208.94	124.75	630.44
Research variant 4					
Mean	2377.69b	1552.23a	158.31b	201.94b	863.34ac
SD	1058.78	1002.69	120.84	138.85	511.08

LF, low frequency of the power spectrum; SD, standard deviation.
Means in columns marked with different letters statistically differ at $P < 0.05$.
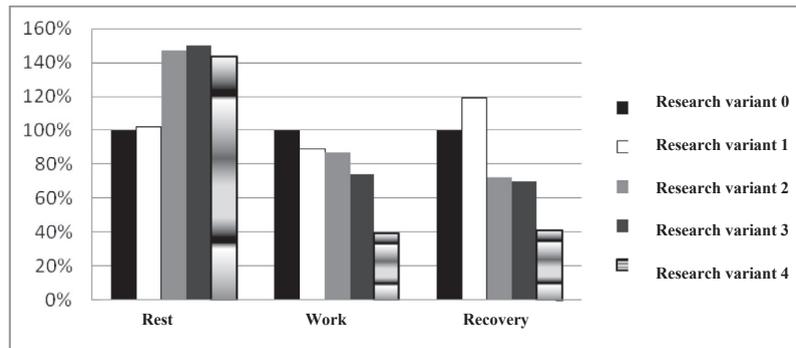
Rest					Work					Recovery				
RV0	RV1	RV2	RV3	RV4	RV0	RV1	RV2	RV3	RV4	RV0	RV1	RV2	RV3	RV4
a	a	b	b	b	a	a	a	a	b	a	ac	a	a	bd

Different letters within a variant show statistical differences between means at p<0.05.

Figure 3. Percentage changes in LF in research variants versus RV0. LF, low frequency of the power spectrum; RV0, research variant 0.

cardiac activity: at rest, at work while walking, trotting, and cantering, and at recovery; for analysis cortisol concentration: at rest, at work in total and at recovery). In the statistical model, the research variant was analyzed as repeated measures. The significance of differences between the means was determined by multiple comparison T-Tukey's test. The statistical significance of differences was assumed at $\alpha = 0.05$. In addition, percentage changes of heart activity parameters and cortisol concentration in RV1, RV2, RV3, and RV4 versus RV0 were calculated individually for each horse and then averaged. In the case of cortisol analysis, the work data were considered as a whole, that is, without separating them into particular gaits. The significance of differences between mean percentage values was determined using Parker's test (Parker, 1978).

Results

The considered factors and interaction between them were significant in the case of all parameters at the significance level $P < 0.01$, and only for LF/HF, the level was $P < 0.05$ (Table 2). There were no significant differences in the HR at rest between the research variants (Table 3). Differences appeared at work and recovery. A considerably higher value was observed while walking in RV4 compared with other variants. While trotting, higher values were observed in RV2 and RV4, whereas the cantering value was

also higher in RV3. The HR recovery level was significantly elevated in all variants compared with RV0. The HR in RV2 and RV4 as a percent of HR in RV0 was considerably higher at work and recovery compared with RV0 (Figure 1).

Significant differences were found in RMSSD at work and recovery in successive variants (Table 4). While walking, the parameter was lower in RV4 than in other variants. While trotting, statistically lower values appeared in RV2, RV3, and RV4, although in RV1, this parameter was similar to RV2 and RV3. Results for cantering were similar; however, the RV1 parameter was like the RV2, RV3, and RV4 parameters ($P > 0.05$). During recovery, RMSSD in all research variants was lower than that in RV0. A significant decrease in RMSSD at work and recovery was observed in percentage RV4 compared with RV0 (Figure 2).

Resting LF was lower ($P < 0.05$) in RV0 and RV1 than in the other variants (Table 5). There were no significant differences between means for walking. For trotting and cantering, lower LF was observed in RV2, RV3, and RV4. There was a significant ($P < 0.05$) difference in LF between higher RV1 and lower RV4 during recovery. Resting LF in percent of the value in RV0 was higher in RV2, RV3, and RV4 than in RV0 (Figure 3). At work, a significant increase of the percentage LF compared to RV0 was observed only in RV4. The results were different during the recovery, when a considerable decrease in the percentage LF was noted for RV4.

Table 6
HF (ms²) in horses in research variants

Research variant/type of effort	Rest	Work			Recovery
		Walk	Trot	Canter	
Research variant 0					
Mean	1251.26a	301.75a	56.22a	67.20a	489.95a
SD	442.30	210.77	36.85	56.96	344.59
Research variant 1					
Mean	1201.92a	288.18a	44.29a	51.10a	345.29ad
SD	363.93	122.62	26.40	42.41	282.64
Research variant 2					
Mean	1326.60a	495.82b	29.62b	31.96b	273.57bd
SD	324.83	351.03	25.50	24.64	154.38
Research variant 3					
Mean	1941.22b	311.87a	47.02a	37.17b	388.31ad
SD	375.90	179.95	67.64	30.56	319.18
Research variant 4					
Mean	1969.41b	111.45c	19.91b	31.54b	191.10c
SD	482.21	103.34	22.58	27.40	83.81

HF, high frequency of the power spectrum; SD, standard deviation.
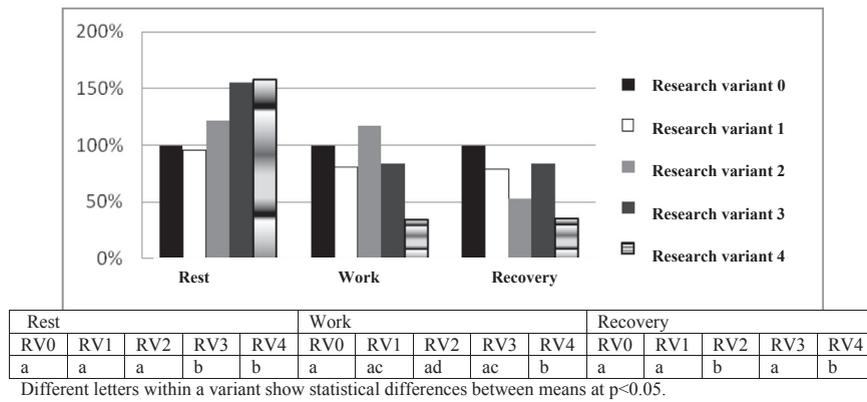Means in columns marked with different letters statistically differ at $P < 0.05$.

Rest					Work					Recovery				
RV0	RV1	RV2	RV3	RV4	RV0	RV1	RV2	RV3	RV4	RV0	RV1	RV2	RV3	RV4
a	a	a	b	b	a	ac	ad	ac	b	a	a	b	a	b

Different letters within a variant show statistical differences between means at p<0.05.

Figure 4. Percentage changes in HF in research variants versus RV0. HF, high frequency of the power spectrum; RV0, research variant 0.

Statistically significant differences in HF were found between the experimental variants for all measurements (Table 6). At rest, the parameter was higher in RV3 and RV4 than in other variants. While walking, an elevated HF level was observed in RV2, whereas the lowest level was found in RV4. While trotting, lower values were observed in RV2 and RV4 than in other variants. The HF was significantly lower in RV2, RV3, and RV4 when cantering. During recovery, the parameter was higher in RV0, RV1, and RV3 than in RV2 and especially than in RV4. A higher resting HF expressed in percent of RV0 was found in RV3 and RV4 (Figure 4). At work, the parameter increased in RV2 and decreased in RV4. At recovery, it was lower in RV2 and RV4.

Differences between the variants in resting and work LF/HF were usually insignificant (Table 7). Significant differences were only found between RV2 and RV3 while walking and at recovery, when the parameter was lower in RV0 than in the RVs. The resting LF/HF expressed in percent of the RV0 value was higher in RV2 than in RV4 (Figure 5). At work, the parameter did not change. During recovery, it did not increase either, although RV1 and RV2 were higher than RV3.

Only one significant difference was found in mean cortisol concentrations in the horses' saliva between the variants (Table 8). Namely, the concentration was higher at work in RV4 than in the other cases. A significant decrease in the resting salivary cortisol concentration in the saliva expressed as percent of the RV0 level was noted in RV2 and RV3 (Figure 6). At work, an increase of the

parameter occurred in RV4, whereas there were no significant differences in the parameter at recovery.

## Discussion

Parameter changes induced by audience

The fact that physiological parameters measured in the successive research variants at rest were usually not found to be different indicates that the procedure and measurement techniques were adequately accurate to determine the possible influence of the factors of interest on the horses' emotional excitability. Audience presence in the arena during work and recovery resulted in elevated levels of HR compared with when the audience was absent. It is known that HR may increase due to both positive and negative emotions (Brosschot and Thayer, 2003). Keeling et al. (2009) found that HR in horses increased in reaction to a person behaving impulsively. Rietmann et al. (2004) reported a positive correlation between HR level and undesirable horse behavior.

When the audience was present in the arena, many changes of HRV parameters appeared in the horses during a low-intensity exercise compared with when the audience was absent. The reactions, usually RMSSD and HF decrease, showed lowered activity of the parasympathetic ANS branch. The changes were particularly apparent when the audience simultaneously walked and talked. According to Hockenhull (2010), leisure horses are subject to

Table 7
LF/HF (%) in horses in research variants

Research variant/type of effort	Rest	Work			Recovery
		Walk	Trot	Canter	
Research variant 0					
Mean	136.72a	564.97a	972.64a	864.84a	249.65a
SD	46.28	296.27	575.34	750.86	180.00
Research variant 1					
Mean	146.74a	538.59a	1110.38a	1054.88a	511.68b
SD	50.86	370.09	501.95	719.13	182.16
Research variant 2					
Mean	156.77a	492.12ab	1053.33a	853.81a	591.38b
SD	40.66	273.93	604.12	699.90	181.07
Research variant 3					
Mean	133.68a	796.47ac	841.60a	819.72a	406.53b
SD	42.48	453.38	519.82	565.26	178.40
Research variant 4					
Mean	128.48a	656.64a	1027.29a	1026.07a	489.19b
SD	46.61	386.55	581.55	662.91	174.90

HF, high frequency of the power spectrum; LF, low frequency of the power spectrum; SD, standard deviation.
Means in columns marked with different letters statistically differ at $P < 0.05$.
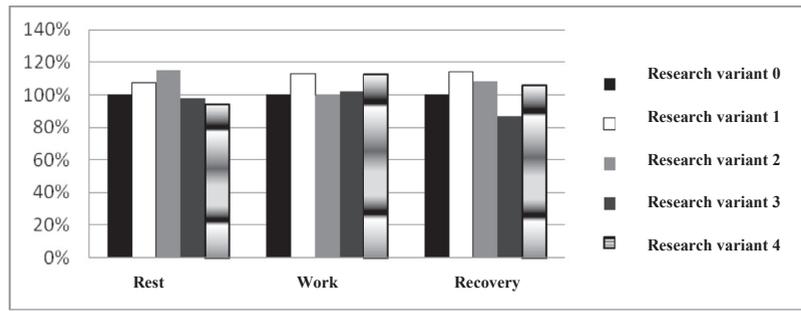
Rest					Work					Recovery				
RV0	RV1	RV2	RV3	RV4	RV0	RV1	RV2	RV3	RV4	RV0	RV1	RV2	RV3	RV4
a	a	ab	a	ac	a	a	a	a	a	a	ab	ab	ac	a

Different letters within a variant show statistical differences between means at p<0.05.

Figure 5. Percentage changes in LF/HF in research variants versus RV0. HF, high frequency of the power spectrum; LF, low frequency of the power spectrum; RV0, research variant 0.

monotonous physical effort of low or sporadically medium intensity. They should not undergo an excitation in work because this may threaten the safety of riders (Wilk et al., 2013). Taking into account that the parasympathetic system is responsible for inhibiting functions of the body, it may be suggested that a decrease in its activity is undesirable in leisure riding. Hence, the audience effect tested in this study turned out to be undesirable. The previously mentioned lowered balance of the ANS was also caused by a decrease in parasympathetic activity in our study. The observed LF decrease during trot and canter and fluctuations during recovery, in RVs compared with RV0, seem to be less important because they may be caused by both parts of the ANS. An increase in LF amplitude appears for varying reasons, such as central sympathetic stimulation under psychological stress or physical effort (Berntson and Cacioppo, 2004; Sztajzel, 2004). The sympathetic activation associated with stressful circumstances is a transient process that results in rapidly elapsing functional changes (McCraty et al., 1995). The higher values of LF/HF at recovery in the RVs compared to RV0 showed an effect of the audience presence on lowering the sympathovagal balance. The ANS disturbances indicate that excessive excitement of a horse may positively correlate with undesirable behavior (Schmidt et al., 2010). The cortisol level in the saliva distinctly showed a negative influence of the audience simultaneously talking and walking. This result suggests that such behavior of the audience is frustrating and stressful for the horses.

Cardiac activity and cortisol level in different gaits and at recovery

The experiment was conducted under circumstances similar to typical horse-riding leisure hours in the arena, although the horses' work was maximally standardized. The reaction to the effort expressed in the parameters studied was consistent: HR and LF/HF increased, and RMSSD, LF and HF decreased with a fast gait, whereas at recovery, the parameters approached the resting levels. Significant HR and HRV differences between the research variants when the audience was present, and when the audience was absent, were fewest while walking. They were more frequent while trotting and most frequent while cantering. Thus, with increasingly faster gaits, the horse's reaction to the audience increased. According to Snow et al., (1992), adrenaline secretion rises during canter, which activates the horse's emotional excitability. Consequences of the elevated hormone concentration may apparently be compounded by the audience, even if its behavior is quiescent. Similarly, recovery in leisure horses can be considerably disturbed by the presence of an audience. It was clearly seen that the horses' heart activity parameters moved toward resting levels more quickly when the audience was absent: HR and LF/HF lowered, whereas RMSSD, LF, and HF increased. Mengoli et al. (2014) found that a familiar and friendly environment positively influenced horses' cognitive abilities and behavior after physical effort. A study by Tafalla and Evans (1997) showed that excessive excitability and lack

Table 8
Cortisol concentration (mg/dL) in horses in research variants

Research variant/type of effort	Rest	Work	Recovery
Research variant 0			
Mean	2.28a	1.84a	1.82a
SD	0.97	1.06	1.11
Research variant 1			
Mean	1.64a	2.11a	2.08a
SD	1.39	1.47	1.50
Research variant 2			
Mean	1.28a	1.74a	1.65a
SD	0.35	0.60	0.88
Research variant 3			
Mean	1.22a	1.86a	1.79a
SD	0.44	0.89	0.90
Research variant 4			
Mean	1.51a	3.36b	1.92a
SD	0.95	1.17	0.99

SD, standard deviation.
Means in columns marked with different letters statistically differ at $P < 0.05$.
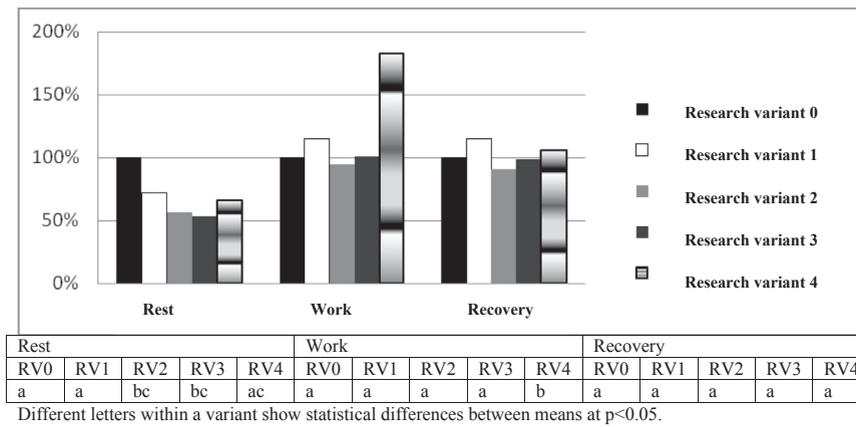
Rest					Work					Recovery				
RV0	RV1	RV2	RV3	RV4	RV0	RV1	RV2	RV3	RV4	RV0	RV1	RV2	RV3	RV4
a	a	bc	bc	ac	a	a	a	a	b	a	a	a	a	a

Different letters within a variant show statistical differences between means at $p < 0.05$.

Figure 6. Percentage changes in cortisol concentration in research variants versus RV0. RV0, research variant 0.

of silence after an effort interrupted and lengthened the recovery time.

Effect of audience voice and movement

According to Timney and Macuda (2001), horses are hypersensitive to some sounds. A horse is able to discriminate between the voices of a familiar and an unfamiliar human (Lampe and Andre, 2012). Horses appear less distressed and calmer when a human is speaking in a pleasant voice and show more distress when a stern voice of a low tone is heard (Merkies et al., 2013). In the present study, the most significant differences in particular parameters appeared when the audience was simultaneously walking and talking (17 differences). There were fewer differences when it was only talking (15), still fewer when it was only walking (12), and the fewest when it was standing still and silent (7). Moreover, both at work and recovery, HR expressed in percent of this parameter in RV0 was statistically similar when the audience was only talking and when the audience was simultaneously walking and talking and statistically different from when it was only walking. This suggests that there is a tendency for people's voices to have a more adverse effect than their movement. However, this pattern should be better documented in future studies because a negative effect of the audience on the cortisol level was found only when the audience was simultaneously walking and talking. In addition, whether leisure horses can get accustomed to the audience and its behavior should be studied.

Conclusion

Having an audience present during horse-riding leisure hours in the arena causes a decrease in the activity of the parasympathetic part of the ANS, which reduces inhibiting functions in favor of functions activating the body. Consequently, an increase in emotional excitability undesirable in horse-riding leisure may appear. The horses' reaction to audience presence in the arena is more intensive in faster gaits. When the audience is simultaneously walking and talking, a decrease in the activity of the parasympathetic system is particularly distinct, and stress shown by an increase in cortisol secretion is also generated.

Ethical considerations

Animal care and experimental procedures were in accordance with the European Committee Regulations on Protection of Experimental Animals and were approved by the Local Ethic Review Committee for Animal Experiments.

Conflict of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

References

Adell, A., Garcia-Marquez, C., Armario, A., Gelpi, E., 1988. Chronic stress increases serotonin and noradrenaline in rat brain and sensitizes their responses to a further acute stress. J. Neurochem. 50 (6), 1678–1681.

Berntson, G.G., Cacioppo, J.T., 2004. Heart rate variability: stress and psychiatric conditions. In: Dynamic Electrocardiography, pp. 57–64.

Brandt, K., 2004. A language of their own: an interactionist approach to human-horse communication. Soc. Anim. 12, 299–316.

Broom, D.M., Johnson, K.G., 1993. Stress and Animal Welfare. Chapman & Hall, London.

Brosschot, J.F., Thayer, J.F., 2003. Heart rate response is longer after negative emotions than after positive emotions. Int. J. Psychophysiol. 50, 181–187.

Goodwin, D., 1999. The importance of ethology in understanding the behavior of the horse. Equine Vet. J. 28, 15–19.

Hall, C., Huws, N., White, C., Taylor, E., Owen, H., McGreevy, P., 2013. Assessment of ridden horse behavior. J. Vet. Behav.: Clin. Appl. Res. 8 (2), 62–73.

Hallberg, L., 2008. Walking the Way of the Horse: Exploring the Power of the Horse-Human Relationship. iUniverse, Bloomington, USA.

Hausberger, M., Roche, H., Henry, S., Visser, E.K., 2008. A review of the human–horse relationship. Appl. Anim. Behav. Sci. 109, 1–24.

Hockenhull, J.S., 2010. The Epidemiology of Behaviour Problems and Risk Factors for Poor Welfare in UK Leisure Horses (Doctoral dissertation, University of Liverpool, UK).

Hothersall, B., Casey, R., 2012. Undesired behavior in horses: a review of their development, prevention, management and association with welfare. Equine Vet. Educ. 24, 479–485.

Houpt, K.A., McDonnell, S.M., 1993. Equine stereotypies. Comp. Cont. Educ. Pract. Vet. 15, 1265–1271.

Irvine, C.H.G., Alexander, S.L., 1994. Factors affecting the circadian rhythm in plasma cortisol concentrations in the horse. Domest. Anim. Endocrinol. 11 (2), 227–238.

Janczarek, I., Kędzierski, W., 2011. Emotional response of young race horses to a transfer from a familiar to an unfamiliar environment. Anim. Sci. Pap. Rep. 29, 205–212.

Keaveney, S.M., 2008. Equines and their human companions. J. Bus. Res. 61, 444–454.

Keeling, L.J., Jonare, L., Lanneborn, L., 2009. Investigating horse–human interactions: the effect of a nervous human. Vet. J. 181, 70–71.

Kuwahara, M., Hiraga, A., Kai, M., Tsubone, H., Sugano, S., 1999. Influence of training on autonomic nervous function in horses: evaluation by power spectral analysis of heart rate variability. Equine Vet. J. 31 (S30), 178–180.

Lampe, J.F., Andre, J., 2012. Cross-modal recognition of human individuals in domestic horses (*Equus caballus*). Anim. Cogn. 15 (4), 623–630.

Lassourd, V., Gayrard, V., Laroute, V., Alvinerie, M., Benard, P., Courtot, D., Toutain, P.L., 1996. Cortisol disposition and production rate in horses during rest and exercise. Am. J. Physiol. 271, R25–R33.

Lazarus, R.S., 1993. From psychological stress to the emotions: a history of changing outlooks. Annu. Rev. Psychol. 44 (1), 1–22.

Maurstad, A., Davis, D., Cowles, S., 2013. Co-being and intra-action in horse—human relationships: a multi-species ethnography of be(com)ing human and be(com)ing horse. Soc. Anthropol. 21, 322—335.

McBride, S.D., Mills, D.S., 2012. Psychological factors affecting equine performance. BMC Vet. Rese. 8 (1), 180.

McCraty, R., Atkinson, M., Tiller, W.A., Rein, G., Watkins, A.D., 1995. The effects of emotions on short-term power spectrum analysis of heart rate variability. Am. J. Cardiol. 76, 1089—1093.

Mellor, D.J., 2012. Animal emotions, behavior and the promotion of positive welfare states. N. Z. Vet. J. 60, 1—8.

Mengoli, M., Pageat, P., Lafont-Lecuelle, C., Monneret, P., Giacalone, A., Sighieri, C., Cozzi, A., 2014. Influence of emotional balance during a learning and recall test in horses (Equus caballus). Behav. Processes 106, 141—150.

Merkies, K., MacGregor, H., Ouimette, M., Bogart, E., Miraglia, K., 2013. Does `the human voice have a calming effect on horses? J. Equine Vet. Sci. 33, 368.

Mosbach, K., Ramström, O., 1996. The emerging technique of molecular imprinting and its future impact on biotechnology. Nat. Biotechnol. 14, 163—170.

Möstl, E., Palme, R., 2002. Hormones as indicators of stress. Domestic Anim. Endocrinol. 23 (1), 67—74.

Munck, A., Guyre, P.M., Holbrook, N.J., 1984. Physiological functions of glucocorticoids in stress and their relation to pharmacological actions. Endocr. Rev. 5 (1), 25—44.

O'Rourke, N., Hatcher, L., 2013. A Step-by-Step Approach to Using SAS for Factor Analysis and Structural Equation Modelling. SAS Institute, North Crolina, USA.

Parker, R.E., 1978. Wprowadzenie Do Statystyki Dla Biologów. PWN Warszawa, Warsaw, Poland, p. 133. in Polish.

Phelps, E.A., LeDoux, J.E., 2005. Contributions of the amygdala to emotion processing: from animal models to human behavior. Neuron 48, 175—187.

Rietmann, T.R., Stuart, A.E.A., Bernasconi, P., Stauffacher, M., Auer, J.A., Weishaupt, M.A., 2004. Assessment of mental stress in warmblood horses: heart rate variability in comparison to heart rate and selected behavioral parameters. Appl. Anim. Behav. Sci. 88, 121—136.

Schmidt, A., Aurich, J., Möstl, E., Müller, J., Aurich, C., 2010. Changes in cortisol release and heart rate and heart rate variability during the initial training of 3-year-old sport horses. Hormon. Behav. 58 (4), 628—636.

Snow, D.H., Harris, R.C., MacDonald, I.A., Forster, C.D., Marlin, D.J., 1992. Effects of high-intensity exercise on plasma catecholamines in the Thoroughbred horse. Equine Vet. J. 24, 462—467.

Sztajzel, J., 2004. Heart rate variability: a noninvasive electrocardiographic method to measure the autonomic nervous system. Swiss Med. Wkly. 134, 514—522.

Tafalla, R.J., Evans, G.W., 1997. Noise, physiology, and human performance: the potential role of effort. J. Occupat. Health Psychol. 2 (2), 148.

Tarvainen, M.P., Niskanen, J.P., Lipponen, J.A., Ranta-Aho, P.O., Karjalainen, P.A., 2009. Kubios HRV—a software for advanced heart rate variability analysis. In: 4th European Conference of the International Federation for Medical and Biological Engineering. Springer Berlin Heidelberg, Germany, pp. 1022—1025.

Tarvainen, M.P., Niskanen, J.P., Lipponen, J.A., Ranta-Aho, P.O., Karjalainen, P.A., 2014. Kubios HRV—heart rate variability analysis software. Comput. Meth. Prog. Bio. 113, 210—220.

Timney, B., Macuda, T., 2001. Vision and hearing in horses. J. Am. Vet. Med. Assoc. 218, 1567—1574.

VanDierendonck, M.C., Goodwin, D., 2005. Social contact in horses: implications for human-horse interactions. In: de Jonge, F.H., van den Bos, R. (Eds.), The Human-animal Relationship. Van Gorcum, pp. 65—81.

von Borell, E., Langbein, J., Després, G., Hansen, S., Leterrier, C., Marchant-Forde, J., Valance, D., 2007. Heart rate variability as a measure of autonomic regulation of cardiac activity for assessing stress and welfare in farm animals—a review. Physiol. Behav. 92, 293—316.

von Borstel, U.K., Pasing, S., Gauly, M., 2011. Towards a more objective assessment of equine personality using behavioral and physiological observations from performance test training. Appl. Anim. Behav. Sci. 135, 277—285.

Waran, N.K., Casey, R., 2005. Horse Training. The Domestic Horse: The Origins, Development and Management of its Behavior. Cambridge University Press, Cambridge, pp. 184—195.

Weiss, J.M., Goodman, P.A., Losito, B.G., Corrigan, S., Charry, J.M., Bailey, W.H., 1981. Behavioral depression produced by an uncontrollable stressor: relationship to norepinephrine, dopamine, and serotonin levels in various regions of rat brain. Brain Rese. Rev. 3 (2), 167—205.

Wilk, I., Janczarek, I., Klimek, B., 2013. A project of the method for assessing horse suitability for leisure riding. Annal. UMCS. Sec. EE. XXXI (3), 53—64.