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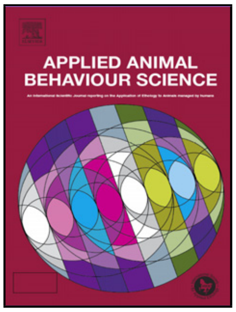

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Title:

Behavioural, endocrine and cardiac autonomic responses to a model of startle in horses

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Behavioural, endocrine and cardiac autonomic responses to a model of startle in horses

ABSTRACT

Startle is a fast response elicited by sudden acoustic, tactile or visual stimuli in a variety of animal species and in humans. The magnitude of startle response can be modulated by external and internal variables and can be a useful tool to study the sensory-motor integration in animals. Different stimuli have been used to induce startle in horses, which makes it difficult to compare the responses to these different approaches. The present study uses ultra-short-term heart rate variability (HRV) analysis to characterize the cardiac autonomic modulation, reactivity assessment and blood cortisol measurements to describe the behavioural and endocrine responses to a simple, easy to replicate, effective and safe method of startle (an umbrella is abruptly opened near the horse). The ultra-short-term (64 s) heart rate (HR) series were interpolated (4 Hz) and divided into 256 points segments then the spectra calculated (Fast Fourier Transform). The spectra were then integrated into low (LF; 0.01-0.07 Hz; Index of Cardiac Sympathetic Modulation) and high (HF; 0.07-0.50 Hz; Index of Cardiac Parasympathetic Modulation) frequency bands. Following the startle test, the HR (p=0.0101), the power of the LF band of the cardiac interval spectrum (p=0.0002) and the LF/HF ratio (p=0.0066) were found to be higher, whereas the power of the HF band of the cardiac interval spectrum was found to be lower (p=0.0002). Also, the horses showed a noticeable escape response, with latency of reaction varying from 0.28 to 1.28 s, duration of reaction ranging from 1.52 to 7.92 s and escape distance covered varying from 3.43 to 9.97 m. However, the endocrine measurements failed to reveal significant changes in the cortisol levels after the startle test. We conclude that the startle test used in the current study was effective to produce changes in behavioural parameters and cardiac autonomic modulation of the horses and can therefore be an appropriate tool for neurobiological studies. Furthermore, the use of ultra-short segments (64 s) for HRV analysis appears to be effective and promising for the detection of mental stress in horses.

HIGHLIGHTS:

Abrupt umbrella-opening produces startle in horses

It is a simple, replicable, effective and safe method of startle for horses

Umbrella-opening induces behavioural and autonomic responses in horses

Umbrella-opening does not change cortisol levels in horses

HRV analysis of ultra-short segments can be used to detect mental stress in horses

KEYWORDS:

Horses – startle response – umbrella-opening – autonomic response – cortisol – reactivity – behaviour – cardiac interval variability

ABBREVIATIONS

ACTH - adrenocorticotropic hormone

ASR - acoustic startle response

FFT - Fast Fourier Transform

HF - high frequency

HR - heart rate

HRV - heart rate variability

LF - low frequency

PSD - power spectral density

VLF -very low frequency

1. INTRODUCTION

Startle responses are defensive reflexes induced by unexpected and intense stimuli, which are characterized by coordinated eyelid closure and contraction of face, neck, foreleg and hind leg. Startle is also associated with increases in heart rate (HR) and arrest of other on-going behaviours. Although several kinds of stimuli (acoustic, tactile or visual) can induce startle in animals and humans, the acoustic startle response (ASR) has been investigated the most (Koch, 1999). The ASR is a proven, reliable and accurate approach to investigate the brain mechanisms of learning, memory, emotions and movement control since the magnitude of ASR can be increased or decreased by a variety of pathological conditions and experimental manipulations (Davis, 1990; Koch, 1999). For example, changes in emotional and perceptual homeostasis, i.e. conditioned and unconditioned aversive events, can enhance the magnitude of ASR (Bradley et al., 1990; Lang et al., 1990). Alternatively, the repeated application of startling stimuli, prior to the presentation of a prepulse (prepulse inhibition) or a pleasant emotional context (Lang et al., 1990; Schmid et al., 1995) may lead to attenuated startle responses (Koch et al., 1996).

Besides the behavioural response, startle induces autonomic and endocrine changes. Literature shows that the transitory increase (< 60 s) in HR induced by startle is consistently observed in different experimental animals and is mediated by the sympathetic and parasympathetic divisions of the autonomic nervous system (Baudrie et al., 1997; Vila et al., 2007). On the other hand, the startle-induced increase in corticosterone levels is not observed in all strains of rats (Glowa et al., 1992). A combined study of these responses is important for a better understanding of the physiological effects of startle tests on horses since the magnitude of autonomic, endocrine and behavioural responses to a stimulus cannot always be correlated.

Startle responses are frequently observed in horses; furthermore, the analysis of startle reactions in equines is important as it can be a useful tool to assess stress and welfare. Startle tests have been combined with other measurements to predict temperament in horses. The literature suggests that a horse’s reaction to novelty, suddenness and to social isolation might be associated with a general trait of "fearfulness" (Lansade et al., 2008). Excessive reactions of fear can hamper the use of horses, and can even pose a risk to the animals themselves and people. Furthermore, exaggerated fearful reactions have also been associated with an impaired learning ability of horses (Heird et al., 1986).

Different types of stimuli have been used to produce suddenness or startle in horses. The umbrella opening, a relatively common method, has been used in different ways. To study the existence of a ''fearfulness'' trait in horses and the effect of social isolation on the emotional reactivity, Lansade and colleagues used the umbrella opening when horses were eating (Lansade et al., 2008; Lansade et al., 2012). Other authors have induced the startle reaction by opening a coloured umbrella while the horses were walking to evaluate the influence of soy lecithin and corn oil diet on the behaviour (Holland et al., 1996). To study the effect of habituation and active human handling, an umbrella was manually opened when horses were released in an arena or when held on a lead rope by the handler (Górecka et al., 2007). HR and the heart rate variability (HRV) were analysed in young horses using a Novel Object test, in which an umbrella was lowered from the ceiling (Visser et al., 2002). Furthermore Anderson and colleagues with the aim to find appropriate methods of selecting horses for therapeutic riding programs used the umbrella opening between a series of other stimuli (a walking and vocalizing toy pig and a balloon popping near the horse). In this case the umbrella was opened by a handler standing in front of the animal (Anderson et al., 1999).

The variation in the methods used to produce startle makes the comparisons between the parameters studied difficult. Therefore the present study proposes the use of ultra-short-term HRV analysis to assess the cardiac autonomic responses to a simple, easy to replicate and effective method of startle - an umbrella is abruptly opened near the horse. Our hypothesis is this method of startle produces well-defined behavioural and autonomic responses in equines, and the ultra-short-term HRV analysis can be used to characterize this autonomic response.

2. METHODS

2.1. Animals

Six Brazilian Sport horses (3 males and 3 females; 6-8 years old; 450-550 kg in weight), with appropriate body condition scores (between 5.0 and 5.5) from the Brazilian Army Riding School were used in the experimental protocols. The sample size used was based on previous studies and on the variability of the parameters studied. These horses had been undergoing eventing training since they were 5 years old and followed a 6-day-week training routine including galloping, jumping and dressage exercises. They were housed in 4 x 4 m individual masonry box stalls, with water dispenser, feeder and wood shavings bedding. The stall doors allow visual contact between horses. The horses were fed with concentrated coast-cross hay and had free access to tap water.

All experimental procedures were approved by the Committee on Animal and Human Research and Ethics of the Federal Rural University of Rio de Janeiro/COMEP-UFRRJ/Brazil (protocol #230833.002064/2012-10).

2.2. Experimental Design

Early in the morning (0600–0700h) a heart monitor (RS 800 G3, Polar, Kempele, Finland) was strapped to the chest of the horses to record the HR, beat-by-beat and then the baseline blood samples (S1) were collected. The animals were then left to rest quietly for 20 minutes in their stalls. Next, each horse was taken individually to a covered arena (70 x 30m, known by the animals and often used for dressage exercises) and subjected to the startle test, the abrupt opening of an umbrella. Briefly, the horse was led by a known handler and positioned at a predetermined location, with its back to a low wall (70 cm high) that surrounds the arena and held loosely by its lead rope. The horse was left undisturbed until signs of quietness and inattention were seen (no attempts to escape or other significant movements). Then, a rainbow coloured umbrella (diameter of 70 cm) was suddenly opened and spun for 2 minutes by a person that was hidden behind the wall at a distance of approximately 1.5 m from the rump of the animal. The umbrella was positioned clearly in the visual field of the animal (an angle of approximately 45 degrees to the tail of the horse, Figure 1A). Following the test, the horse was kept in the arena, by the lead rope for an additional 3 minutes in order to record the behavioural responses on a videotape for analysis. After which the horse was returned to its stall and blood samples were collected at 30 and 60 minutes following the startle test (Figure 1B).

2.3. Behavioural Analysis of Reactivity

The horses were videotaped with a camera (SDR H20, Panasonic, Tokyo, Japan) positioned on a tripod in the arena at a distance of about 20 meters. The images were later processed and analysed by computer (ImageJ, U.S. National Institute of Health, http://rsb.info.nih.gov/nih-image). The behavioural analysis was done according to (Redondo et al., 2009); three parameters were assessed: 1) *Latency of reaction:* time between the beginning of the test and the first reaction of the animal; 2) *Duration:* total time spent in the motor response to the stimulus; and 3) *Covered distance:* displacement of the animal in response to the stimulus.

2.4. Cortisol Analysis

Blood samples from the jugular vein were collected in SST Vacutainer® tubes. Following the collection, the blood was centrifuged for 10 minutes at 3200 rpm. The serum (~ 3 mL) was collected in plastic tubes and kept at -20°C. Serum cortisol concentrations were determined, in duplicate, by a double antibody radioimmunoassay method using a commercial kit (RD Coated Tube Cortisol I125 RIA, Costa Mesa, CA, USA). The sensitivity of the assay was 0.17 µg/dL and the intra assay coefficient of variation was 6.59%.

2.5. Heart Rate Variability Analysis

Cardiac intervals were continuously sampled using a heart monitor (RS 800 G3, Polar, Kempele, Finland). Following acquisition, the data were transmitted from the heart monitor to custom computer software (Polar Pro Trainer 5, Polar, Kempele, Finland) through an infrared interface. The recordings were then processed and a time series of cardiac interval values were generated. Next, the time series of cardiac interval from the moments: *basal stall* (horses in their stalls before the startle test), *basal arena* (horse in the arena, immediately before the test), *startle* and *post-startle* (horses in their stalls, 30 minutes after the startle test) were submitted to HRV analysis.

The heart rate variability analysis was performed using custom computer software (CardioSeries v2.4 - http://www.danielpenteado.com) designed to perform time-frequency analysis of cardiovascular variability, and which allowed precise adjustment of the parameters related to this kind of analysis (e.g.

interpolation rate, segment length and boundaries of frequency bands). Beat-by-beat series of cardiac interval values were converted to data points every 250 ms using cubic spline interpolation (4 Hz). The interpolated series were divided into half-overlapping sequential sets of 256 data points (64 s), which were detrended and tested for stationarity. The existence of slow trends in time series can affect spectra calculation and the power of frequency bands (Berntson et al., 1997). Before spectral calculation, the time series were detrended by subtracting the linear trend (obtained by linear regression calculation) from data points (Nait-Ali, 2009).

The cardiovascular variability analysis requires at least a weakly stationary data series (i.e. mean and stable covariance over time) (Berntson et al., 1997; Porta et al., 2004). Stationary data series can be verified by means of stationarity tests (i.e. enhanced reproducibility of the results among users and laboratories) (Porta et al., 2004; Magagnin et al., 2011), as well as through visual inspection of data series (van de Borne et al., 1997; Porta et al., 2001; Dias et al., 2010). In our study, a well-experienced researcher visually inspected the segments of interpolated time series searching for transients that could affect the calculation of the power spectral density (PSD). To confirm that the visual inspection of the time series was properly performed, a Hanning window was used to attenuate side effects and the spectrum was calculated for all segments using a direct Fast Fourier Transform (FFT) algorithm for discrete time series. All segments were visually inspected for abnormal spectra. Lastly, the results from the time series and spectra inspections were taken together for the PSD calculation; non-stationary data were not considered (Oliveira et al., 2012). The spectra were integrated in the low frequency band (LF; 0.01-0.07 Hz) and high frequency band (HF; 0.07-0.50 Hz) (Physick-Sheard et al., 2000). The normalised values were achieved by calculating the percentage of LF and HF power with regard to the total power of the spectrum minus the very low frequency band (VLF; <0.01 Hz) power (van de Borne et al., 1997; Billman, 2011). The LF/HF ratio was calculated in order to assess the sympathovagal balance, (Physick-Sheard et al., 2000; Rietmann et al., 2004; Matsuura et al., 2010; Ohmura et al., 2012). Before choosing the frequency band setting in the current study two other ranges of frequency bands were tested: LF: 0.01-0.15/HF: 0.15-0.50 Hz and LF: 0.04-0.15/HF: 0.15-0.50 Hz and two segment lengths: *ultra-short* (64 s, interpolation rate of 4 Hz and segments with 256 points) and *short* (128 s, interpolation rate of 4 Hz and segments with 512 points). The use of ultra-short segments (64 sec) and distinctive frequency bands (LF: 0.01 to 0.07 Hz and HF: 0.07 to 0.50 Hz) seemed to be more advantageous since only *ultra-short* segments showed a significant increase in the LF/HF ratio induced by startle. Furthermore, the setting LF: 0.04-0.15/HF: 0.15-0.50 Hz was not able to show significant increases in the LF/HF ratio induced by startle while the setting: LF: 0.01 to 0.15/HF: 0.15 to 0.50 Hz showed highly variable values of the LF/HF ratio.

2.6. Statistical Analysis

HRV parameters were analysed by one-way analysis of variance (ANOVA) for repeated measures, followed by Newman-Keuls post-test. The cortisol levels were analysed by Friedman test followed by Dunn's Multiple Comparison Test since this data did not show normal distribution in the Kolmogorov-Smirnov test. Behavioural data after startle were shown as descriptive statistics and the correlation among the LF/HF, cortisol levels and behavioural data were assessed by the Spearman test. Differences were considered statistically significant if $P<0.05$. The results are presented as mean ± standard error of mean.

3. RESULTS

In response to the umbrella opening, the horses showed a standard escape response, characterized by a small jump followed by a quick movement away from the open umbrella. After this reaction, the animals remained looking at the umbrella that was spun for 2 minutes after its opening. After that, the horses exhibited little motion in the remaining time that they were observed, but remained alert to the environment. Some animals even approached the handler and umbrella. The behavioural startle response is shown in Table 1.

In the current study, horses subjected to the startle test showed an increase in HR ($F_{2,8}=0.4017$, $P=0.0101$), in the power of the LF band of the cardiac interval spectrum ($F_{2,8}=0.8073$, $P=0.0002$) and in the LF/HF ratio ($F_{2,8}=0.9695$, $P=0.0066$), but a decrease in the power of the HF band of the cardiac interval spectrum ($F_{2,8}=0.8073$, $P=0.0002$) (Figures 2 and 3).

In contrast to the remarkable cardiac autonomic responses observed following startle, the Friedman test followed by Dunn's Multiple Comparison Test did not detected any significant difference in the cortisol levels (p= 0.521), Figure 4.

In the present study, no correlation was found among the cortisol levels 30 minutes after startle, the ratio LF/HF and the distance, latency and time of reaction in the behavioural analysis (data not shown).

4. DISCUSSION

The startle test in this study was able to produce an escape response associated with an increase in the HR, in the power of the LF band of the cardiac interval spectrum and in the LF/HF ratio, but a decrease in the power of the HF band of the cardiac interval spectrum, while no changes were found in the cortisol levels.

Our results confirmed, in horses, the marked cardiac autonomic imbalance typically observed following startle stimulus in other species (Baudrie et al., 1997; Vila et al., 2007). Studies in the literature show that startle is associated with a pronounced tachycardic response, mainly mediated by sympathetic activation (Graham, 1979). However, studies in rats and humans have shown that the startle-induced changes in HR are mediated by both sympathetic and parasympathetic activation (Baudrie et al., 1997; Vila et al., 2007). In humans, the cardiac response to startle lasts nearly 70 seconds and is characterized by two distinct tachycardic phases: the short-latency phase with a peak observed 4 seconds following the startle stimulus; and, the long-latency phase with a peak observed 35 seconds following the startle stimulus (Vila et al., 2007). Furthermore, Vila and colleagues (2007) described a mild response to startle characterized by a tachycardic-bradycardic-tachycardic-bradycardic response pattern. The first tachycardic/bradycardic response cycle is mediated mainly by the parasympathetic system (inhibition followed by activation) and the second tachycardic/bradycardic response cycle is mediated essentially by sympathetic and parasympathetic modulation working reciprocally (Vila et al., 2007). Baudrie and colleagues (1997) using different autonomic blockades, demonstrated that the startle–induced HR changes in rats also lasts only a few seconds and combines the sympathetic and parasympathetic activations (Baudrie et al., 1997).

Recently, HRV analysis has been extensively used to assess cardiovascular autonomic modulation in both experimental and clinical settings (Malliani et al., 1991; Task-Force, 1996; Castiglioni et al., 2013). However, HRV analysis has not been widely used in studies of startle or other kinds of acute mental stress. The short-lasting changes in the ANS observed following startle can restrict the use of HRV analysis techniques, since the literature recommends that HRV analysis should be performed in a beat-by-beat time series of at least 5 minutes (Task-Force, 1996). Following a mental stress stimulus a combined activation of both sympathetic and parasympathetic systems is observed (Vila et al., 2007). In this situation, i.e. autonomic activation following mental stress stimulus, the use of long beat-by-beat time series for HRV analysis could hamper the distinct assessment of the sympathetic and parasympathetic cardiovascular modulation. Few studies have used the HRV analysis to measure mental stress. Salahudding and colleagues (2007) used ultra-short-term HRV analysis to assess mental stress in subjects during a *Stroop* colour word test. Data analysis was conducted using time series with a length ranging from 10 s to 150 s and these authors suggested that segments shorter than 50 s could be reliably used to monitor cardiac autonomic responses to mental stress stimulus (Salahuddin et al., 2007). Studies in the literature have also shown that time series 10 s long, i.e. ultra short-term, could be used to evaluate autonomic activation in exercise (Ostojic et al., 2010) and for early risk stratification following acute ST-elevation in myocardial infarction patients (Karp et al., 2009). In the current study, 64 s long segments were used for HRV analysis in order to meet the requirements for the Fast Fourier Transform (FFT) technique, i.e. segments should be long enough to allow the quantification of low frequency components. Longer segments (128 s) were also tested but the HRV analysis revealed no differences among values obtained before (in the stall and in the arena), immediately after and 30 minutes after the startle test (data not shown). The current study showed that the startle-induced changes in cardiac autonomic modulation could not be seen using 128 s long segments, but were clearly observed when HRV analysis was performed using 64 s long segments.

Although there are some issues about the use of heart rate monitors (HRM) Polar® in horses (Parker et al., 2009), several studies have used this low cost, practical and non-invasive tool to collect cardiac interval data in this species (Physick-Sheard et al., 2000; Visser et al., 2002; Rietmann et al., 2004; Schmidt et al., 2010). Ille and colleagues in a recent publication showed that HRMs Polar® are adequate tools for experiments where an ECG tracing is not needed and the use of this system is acceptable to assess HR and HRV as physiological stress parameters in horses (Ille et al., 2014). Another important methodological aspect of the HRV studies in horses is the range of frequency bands used in the spectral analysis. In the present study we tested three frequency band settings: LF:0.01-0.07/HF: 0.07-0.50 (Physick-Sheard et al., 2000), LF: 0.01-0.15/HF: 0.15-0.50 (Rietmann et al., 2004) and LF: 0.04-0.15/HF: 0.15-0.50 (generally used in humans). The range LF:0.01-0.07/HF: 0.07-0.50 was chosen because it was able to show an increase in the LF/HF ratio induced by startle with less variable data than the other settings. Therefore, the use of ultra-short segments (64 s) with frequency bands of 0.01 to 0.07 (LF) and 0.07 to 0.50 (HF) appears to be helpful in detecting mental stress in horses.

In contrast to the remarkable cardiac autonomic responses observed following startle in horses in this study, the cortisol levels only had a tendency to increase 30 minutes after the startle test. Studies in the literature show that stress responses to startle vary widely among animal species. Parker and colleagues (2011) showed increased levels of adrenocorticotropic hormone (ACTH) and cortisol in monkeys subjected to acoustic startle stimulus (Parker et al., 2011). In addition, different responses are observed among rat strains. Following startle, the corticosterone levels were found unchanged in Lewis/N rats but a 2-fold increase was observed in Sprague-Dawley rats and a 5-fold increase in F344/N rats (Glowa et al., 1992). The nature and the intensity of the stimulus should be considered in this analysis, since the opening of an umbrella may not be a stimulus strong enough to increase the cortisol levels. It is important to mention that the lifestyle of a horse could affect its response to a startle test. The athletic horses used in the present study were familiar with different kinds of stimuli as they were regularly subjected to physical training sessions and competitions, making them more resilient and less responsive to mild stimuli (Visser et al., 2003; Górecka et al., 2007). However, further research should be conducted in order to better address the effect of startle on cortisol levels in different animal species and in experimental settings.

As found in the present study, the lack of correlation between endocrine, behavioural and autonomic parameters was also observed in captive European starlings (Nephew et al., 2003). Schommer and colleagues (2003) showed a dissociation of Hypothalamus-Pituitary-Adrenal Axis and the Sympathetic-Adrenal-Medullary System response patterns in subjects submitted to repeated psychosocial stress

(Schommer et al., 2003). The dissociation between endocrine, autonomic and behavioural responses to stress suggests that the mechanisms involved in these responses can be regulated independently and reinforces the importance of evaluating various physiological parameters in response to a given stimulus.

The method used in this study to produce startle in horses has some notable advantages compared to other approaches (Holland et al., 1996; Lansade et al., 2008; Keeling et al., 2009; Redondo et al., 2009; Lansade et al., 2012). Firstly, the method did not require any sophisticated technology, since the umbrella was opened manually. Secondly, since the horses were standing there was no influence of locomotor activity on cardiovascular measurements. Thirdly, horses were not able to see the subject holding the umbrella, keeping the startle responses exclusively related to umbrella opening and not to the presence of a human being. Moreover, the fact that the subject holding the umbrella is protected by a low wall makes the method safe. Furthermore, in the current study the methods used for the analysis of autonomic, endocrine and behavioural responses of the horses allowed an accurate and less subjective assessment of the parameters.

5. CONCLUSIONS

Although there are some similarities in the responses to startle, the mechanisms involved in startle reaction can differ widely among species. The development and further improvement of a neurobiological model of startle in horses is of great importance, so that it can be used to predict behavioural and physiological responses to stress situations commonly experienced by horses. In addition, the mental stress models that are usually employed in experimental studies, involve the association of different kinds of visual and acoustic stimuli with a wide diversity of types, magnitudes and durations that affect neural processing and subsequent physiological responses. Since the startle model employed in the current study can be easily reproduced and is effective in evaluating behavioural and autonomic responses to startle, we suggest that this model can be effectively used for neurological studies in horses. Furthermore, the use of ultra-short segments (64 s) for HRV analysis appears to be effective and promising for the detection of mental stress in horses.

6. COMPETING INTERESTS

The authors declare that they have no competing interests.

7. ACKNOWLEDGEMENTS

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Figure Captions

Figure 1: Diagram illustrating the position of the horse and the handler during the startle test (A). Timeline representation of the experimental design (B).

Figure 2: Representative spectra (Power Spectral Density, PSD) of all assessed moments. Basal Stall (A), Basal Arena (B), Startle (C) and After Startle (D). Smaller inner spectra highlight the low-frequency (LF) band.

Figure 3: Effect of startle on the heart rate (HR, Panel A), ratio between the power of the low and high frequency bands (LF/HF, Panel B), LF power (LF, Panel C) and HF power (HF, Panel D) of the pulse interval spectrum. Data obtained from basal stall (horses in their stalls before the startle test), basal arena (horse in the arena, immediately before the test), startle and a er startle. * different from basal stall, P<0.05; † different from basal arena, P<0.05 and ‡ different from a er startle, P<0.05.

Figure 4: Serum cortisol levels in basal conditions (Basal), 30 minutes following the startle test (30min) and 60 minutes following the startle test (60min).

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Table 1: Behavioural responses to startle in horses.

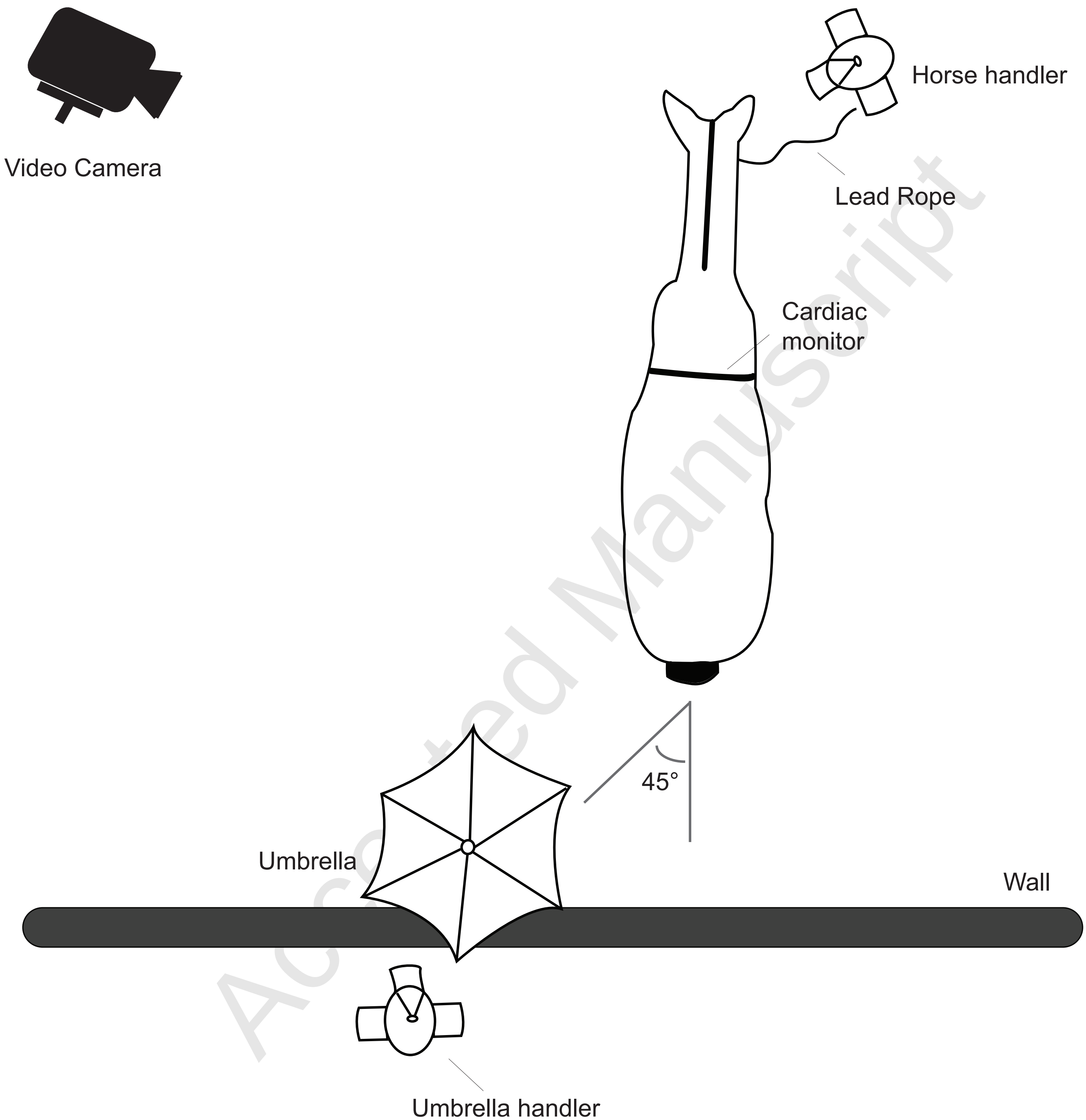
	Average	Range (minimum-maximum)
Latency (s)	0.71	0.28 to 1.28
Duration (s)	3.97	1.52 to 7.92
Distance (m)	5.16	3.43 to 9.96

Latency: time until the animal reaction.

Duration: total time spent in the response.

Distance: displacement of the animal.

A. Model of starle by opening umbrella in horses



B. Experimental Design

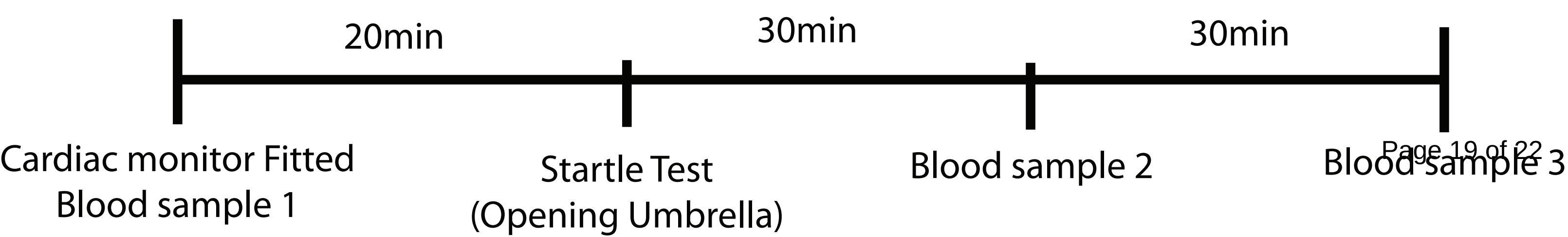


Figure 2

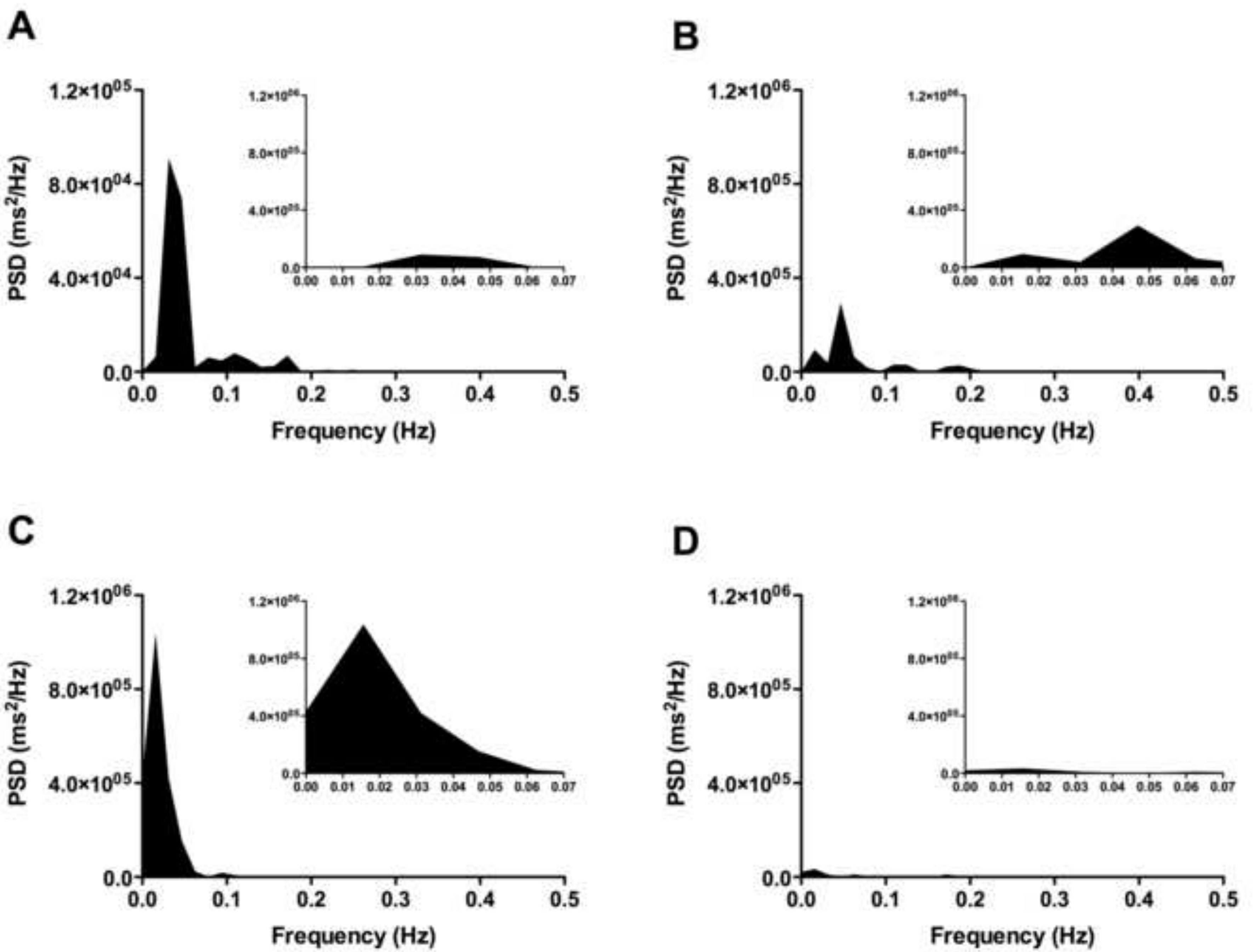

A
B
C
D
PSD (ms²/Hz)
Frequency (Hz)
1.2×10⁰⁵
8.0×10⁰⁴
4.0×10⁰⁴
1.2×10⁰⁶
8.0×10⁰⁵
4.0×10⁰⁵
0.0
0.1
0.2
0.3
0.4
0.5
0.00
0.01
0.02
0.03
0.04
0.05
0.06
0.07

Figure 3

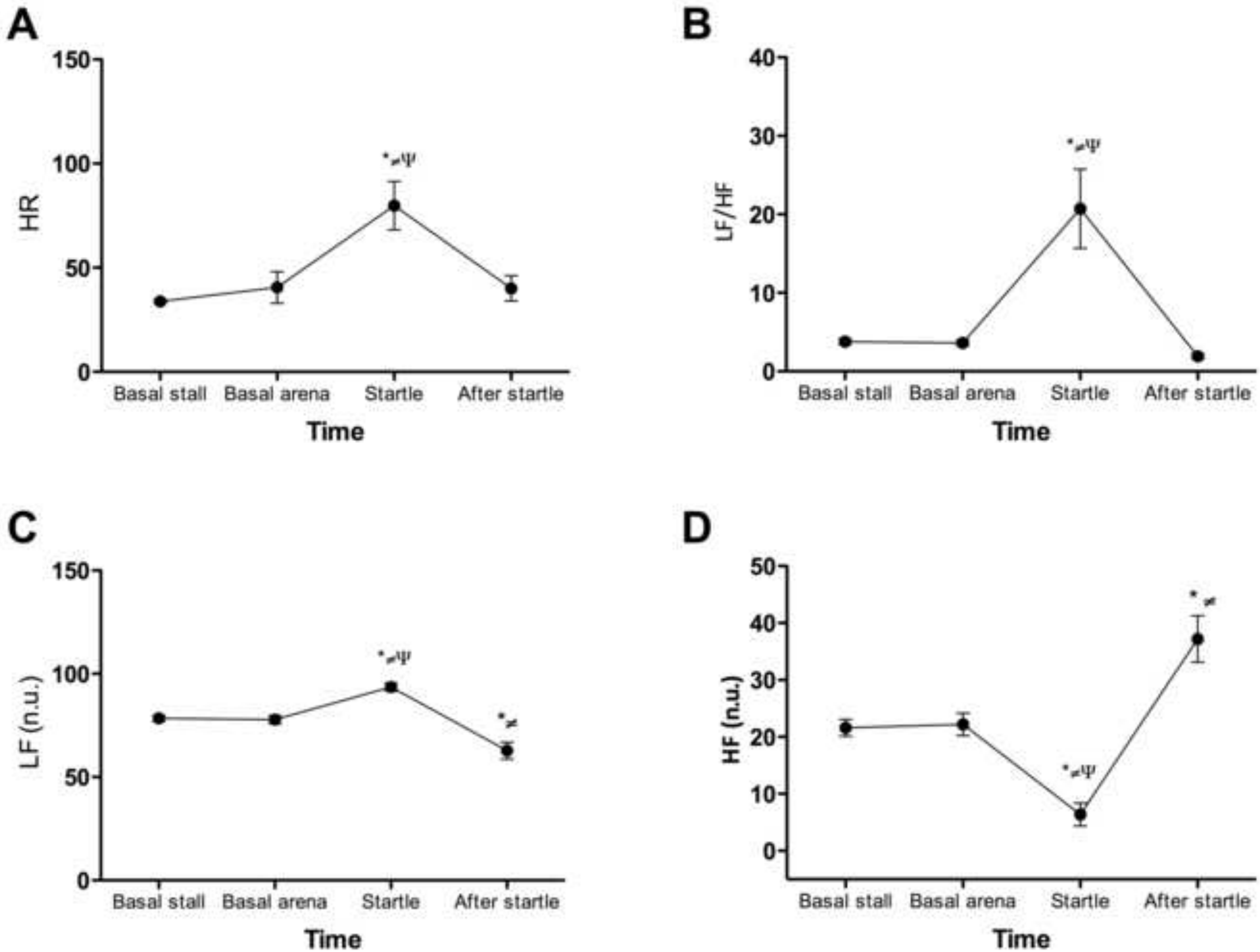

A
150
100
50
0
HR
*≠Ψ
Basal stall
Basal arena
Startle
After startle
Time
B
40
30
20
10
0
LF/HF
*≠Ψ
Basal stall
Basal arena
Startle
After startle
Time
C
150
100
50
0
LF (n.u.)
*≠Ψ
*≠
Basal stall
Basal arena
Startle
After startle
Time
D
50
40
30
20
10
0
HF (n.u.)
*≠
*≠Ψ
Basal stall
Basal arena
Startle
After startle
Time

Figure 4