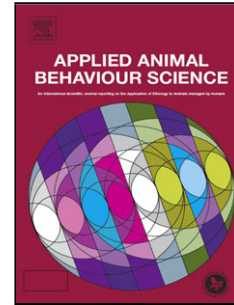


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1 Title:

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

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31 **Title:**

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

34 **ABSTRACT**

35 Startle is a fast response elicited by sudden acoustic, tactile or visual stimuli in a variety of animal species
36 and in humans. The magnitude of startle response can be modulated by external and internal variables and
37 can be a useful tool to study the sensory-motor integration in animals. Different stimuli have been used to
38 induce startle in horses, which makes it difficult to compare the responses to these different approaches.
39 The present study uses ultra-short-term heart rate variability (HRV) analysis to characterize the cardiac
40 autonomic modulation, reactivity assessment and blood cortisol measurements to describe the behavioural
41 and endocrine responses to a simple, easy to replicate, effective and safe method of startle (an umbrella is
42 abruptly opened near the horse). The ultra-short-term (64 s) heart rate (HR) series were interpolated (4 Hz)
43 and divided into 256 points segments then the spectra calculated (Fast Fourier Transform). The spectra
44 were then integrated into low (LF; 0.01-0.07 Hz; Index of Cardiac Sympathetic Modulation) and high (HF;
45 0.07-0.50 Hz; Index of Cardiac Parasympathetic Modulation) frequency bands. Following the startle test,
46 the HR ($p=0.0101$), the power of the LF band of the cardiac interval spectrum ($p=0.0002$) and the LF/HF
47 ratio ($p=0.0066$) were found to be higher, whereas the power of the HF band of the cardiac interval
48 spectrum was found to be lower ($p=0.0002$). Also, the horses showed a noticeable escape response, with
49 latency of reaction varying from 0.28 to 1.28 s, duration of reaction ranging from 1.52 to 7.92 s and escape
50 distance covered varying from 3.43 to 9.97 m. However, the endocrine measurements failed to reveal
51 significant changes in the cortisol levels after the startle test. We conclude that the startle test used in the
52 current study was effective to produce changes in behavioural parameters and cardiac autonomic
53 modulation of the horses and can therefore be an appropriate tool for neurobiological studies.
54 Furthermore, the use of ultra-short segments (64 s) for HRV analysis appears to be effective and promising
55 for the detection of mental stress in horses.

56 **HIGHLIGHTS:**

57 Abrupt umbrella-opening produces startle in horses

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

59 Umbrella-opening induces behavioural and autonomic responses in horses

60 Umbrella-opening does not change cortisol levels in horses

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

62

63 **KEYWORDS:**

64 Horses – startle response – umbrella-opening – autonomic response – cortisol – reactivity – behaviour –

65 cardiac interval variability

66 **ABBREVIATIONS**

67 ACTH - adrenocorticotropic hormone

68 ASR - acoustic startle response

69 FFT - Fast Fourier Transform

70 HF - high frequency

71 HR - heart rate

72 HRV - heart rate variability

73 LF - low frequency

74 PSD - power spectral density

75 VLF -very low frequency

76

76

77 **1. INTRODUCTION**

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

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

98 Startle responses are frequently observed in horses; furthermore, the analysis of startle reactions
99 in equines is important as it can be a useful tool to assess stress and welfare. Startle tests have been
100 combined with other measurements to predict temperament in horses. The literature suggests that a
101 horse's reaction to novelty, suddenness and to social isolation might be associated with a general trait of

102 “fearfulness” (Lansade et al., 2008). Excessive reactions of fear can hamper the use of horses, and can even
103 pose a risk to the animals themselves and people. Furthermore, exaggerated fearful reactions have also
104 been associated with an impaired learning ability of horses (Heird et al., 1986).

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

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

124 **2. METHODS**

125 *2.1. Animals*

126 Six Brazilian Sport horses (3 males and 3 females; 6-8 years old; 450-550 kg in weight), with
127 appropriate body condition scores (between 5.0 and 5.5) from the Brazilian Army Riding School were used

128 in the experimental protocols. The sample size used was based on previous studies and on the variability of
129 the parameters studied. These horses had been undergoing eventing training since they were 5 years old
130 and followed a 6-day-week training routine including galloping, jumping and dressage exercises. They were
131 housed in 4 x 4 m individual masonry box stalls, with water dispenser, feeder and wood shavings bedding.
132 The stall doors allow visual contact between horses. The horses were fed with concentrated coast-cross
133 hay and had free access to tap water.

134 All experimental procedures were approved by the Committee on Animal and Human Research and
135 Ethics of the Federal Rural University of Rio de Janeiro/COMEP-UFRRJ/Brazil (protocol
136 #230833.002064/2012-10).

137

138 *2.2. Experimental Design*

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

154

155 2.3. Behavioural Analysis of Reactivity

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

163

164 2.4. Cortisol Analysis

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

170

171 2.5. Heart Rate Variability Analysis

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

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

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

189 The cardiovascular variability analysis requires at least a weakly stationary data series (i.e. mean
190 and stable covariance over time) (Berntson et al., 1997; Porta et al., 2004). Stationary data series can be
191 verified by means of stationarity tests (i.e. enhanced reproducibility of the results among users and
192 laboratories) (Porta et al., 2004; Magagnin et al., 2011), as well as through visual inspection of data series
193 (van de Borne et al., 1997; Porta et al., 2001; Dias et al., 2010). In our study, a well-experienced researcher
194 visually inspected the segments of interpolated time series searching for transients that could affect the
195 calculation of the power spectral density (PSD). To confirm that the visual inspection of the time series was
196 properly performed, a Hanning window was used to attenuate side effects and the spectrum was
197 calculated for all segments using a direct Fast Fourier Transform (FFT) algorithm for discrete time series. All
198 segments were visually inspected for abnormal spectra. Lastly, the results from the time series and spectra
199 inspections were taken together for the PSD calculation; non-stationary data were not considered (Oliveira
200 et al., 2012). The spectra were integrated in the low frequency band (LF; 0.01-0.07 Hz) and high frequency
201 band (HF; 0.07-0.50 Hz) (Physick-Sheard et al., 2000). The normalised values were achieved by calculating
202 the percentage of LF and HF power with regard to the total power of the spectrum minus the very low
203 frequency band (VLF; <0.01 Hz) power (van de Borne et al., 1997; Billman, 2011). The LF/HF ratio was
204 calculated in order to assess the sympathovagal balance, (Physick-Sheard et al., 2000; Rietmann et al.,
205 2004; Matsuura et al., 2010; Ohmura et al., 2012). Before choosing the frequency band setting in the
206 current study two other ranges of frequency bands were tested: LF: 0.01-0.15/HF: 0.15-0.50 Hz and LF:
207 0.04-0.15/HF: 0.15-0.50 Hz and two segment lengths: *ultra-short* (64 s, interpolation rate of 4 Hz and
208 segments with 256 points) and *short* (128 s, interpolation rate of 4 Hz and segments with 512 points). The

209 use of ultra-short segments (64 sec) and distinctive frequency bands (LF: 0.01 to 0.07 Hz and HF: 0.07 to
210 0.50 Hz) seemed to be more advantageous since only *ultra-short* segments showed a significant increase in
211 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
212 show significant increases in the LF/HF ratio induced by startle while the setting: LF: 0.01 to 0.15/HF: 0.15
213 to 0.50 Hz showed highly variable values of the LF/HF ratio.

214

215 2.6. Statistical Analysis

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

222 3. RESULTS

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

229 In the current study, horses subjected to the startle test showed an increase in HR ($F_{2,8}=0.4017$,
230 $P=0.0101$), in the power of the LF band of the cardiac interval spectrum ($F_{2,8}=0.8073$, $P=0.0002$) and in the
231 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
232 spectrum ($F_{2,8}=0.8073$, $P=0.0002$) (Figures 2 and 3).

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

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

238 **4. DISCUSSION**

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

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

258 Recently, HRV analysis has been extensively used to assess cardiovascular autonomic modulation in
259 both experimental and clinical settings (Malliani et al., 1991; Task-Force, 1996; Castiglioni et al., 2013).
260 However, HRV analysis has not been widely used in studies of startle or other kinds of acute mental stress.
261 The short-lasting changes in the ANS observed following startle can restrict the use of HRV analysis
262 techniques, since the literature recommends that HRV analysis should be performed in a beat-by-beat time
263 series of at least 5 minutes (Task-Force, 1996). Following a mental stress stimulus a combined activation of
264 both sympathetic and parasympathetic systems is observed (Vila et al., 2007). In this situation, i.e.
265 autonomic activation following mental stress stimulus, the use of long beat-by-beat time series for HRV
266 analysis could hamper the distinct assessment of the sympathetic and parasympathetic cardiovascular
267 modulation. Few studies have used the HRV analysis to measure mental stress. Salahuddin and colleagues
268 (2007) used ultra-short-term HRV analysis to assess mental stress in subjects during a *Stroop* colour word
269 test. Data analysis was conducted using time series with a length ranging from 10 s to 150 s and these
270 authors suggested that segments shorter than 50 s could be reliably used to monitor cardiac autonomic
271 responses to mental stress stimulus (Salahuddin et al., 2007). Studies in the literature have also shown that
272 time series 10 s long, i.e. ultra short-term, could be used to evaluate autonomic activation in exercise
273 (Ostojic et al., 2010) and for early risk stratification following acute ST-elevation in myocardial infarction
274 patients (Karp et al., 2009). In the current study, 64 s long segments were used for HRV analysis in order to
275 meet the requirements for the Fast Fourier Transform (FFT) technique, i.e. segments should be long
276 enough to allow the quantification of low frequency components. Longer segments (128 s) were also tested
277 but the HRV analysis revealed no differences among values obtained before (in the stall and in the arena),
278 immediately after and 30 minutes after the startle test (data not shown). The current study showed that
279 the startle-induced changes in cardiac autonomic modulation could not be seen using 128 s long segments,
280 but were clearly observed when HRV analysis was performed using 64 s long segments.

281 Although there are some issues about the use of heart rate monitors (HRM) Polar® in horses
282 (Parker et al., 2009), several studies have used this low cost, practical and non-invasive tool to collect
283 cardiac interval data in this species (Physick-Sheard et al., 2000; Visser et al., 2002; Rietmann et al., 2004;
284 Schmidt et al., 2010). Ille and colleagues in a recent publication showed that HRMs Polar® are adequate

285 tools for experiments where an ECG tracing is not needed and the use of this system is acceptable to assess
286 HR and HRV as physiological stress parameters in horses (Ille et al., 2014). Another important
287 methodological aspect of the HRV studies in horses is the range of frequency bands used in the spectral
288 analysis. In the present study we tested three frequency band settings: LF:0.01-0.07/HF: 0.07-0.50 (Physick-
289 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
290 (generally used in humans). The range LF:0.01-0.07/HF: 0.07-0.50 was chosen because it was able to show
291 an increase in the LF/HF ratio induced by startle with less variable data than the other settings. Therefore,
292 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)
293 appears to be helpful in detecting mental stress in horses.

294 In contrast to the remarkable cardiac autonomic responses observed following startle in horses in
295 this study, the cortisol levels only had a tendency to increase 30 minutes after the startle test. Studies in
296 the literature show that stress responses to startle vary widely among animal species. Parker and
297 colleagues (2011) showed increased levels of adrenocorticotrophic hormone (ACTH) and cortisol in monkeys
298 subjected to acoustic startle stimulus (Parker et al., 2011). In addition, different responses are observed
299 among rat strains. Following startle, the corticosterone levels were found unchanged in Lewis/N rats but a
300 2-fold increase was observed in Sprague-Dawley rats and a 5-fold increase in F344/N rats (Glowa et al.,
301 1992). The nature and the intensity of the stimulus should be considered in this analysis, since the opening
302 of an umbrella may not be a stimulus strong enough to increase the cortisol levels. It is important to
303 mention that the lifestyle of a horse could affect its response to a startle test. The athletic horses used in
304 the present study were familiar with different kinds of stimuli as they were regularly subjected to physical
305 training sessions and competitions, making them more resilient and less responsive to mild stimuli (Visser
306 et al., 2003; Górecka et al., 2007). However, further research should be conducted in order to better
307 address the effect of startle on cortisol levels in different animal species and in experimental settings.

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

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

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

325 5. CONCLUSIONS

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

337

338 6. COMPETING INTERESTS

339 The authors declare that they have no competing interests.

340

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345 Figure Captions

346

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

349

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

352

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

358

359 **Figure 4:** Serum cortisol levels in basal conditions (Basal), 30 minutes following the startle test (30min) and
360 60 minutes following the startle test (60min).

361

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469

470

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470 **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

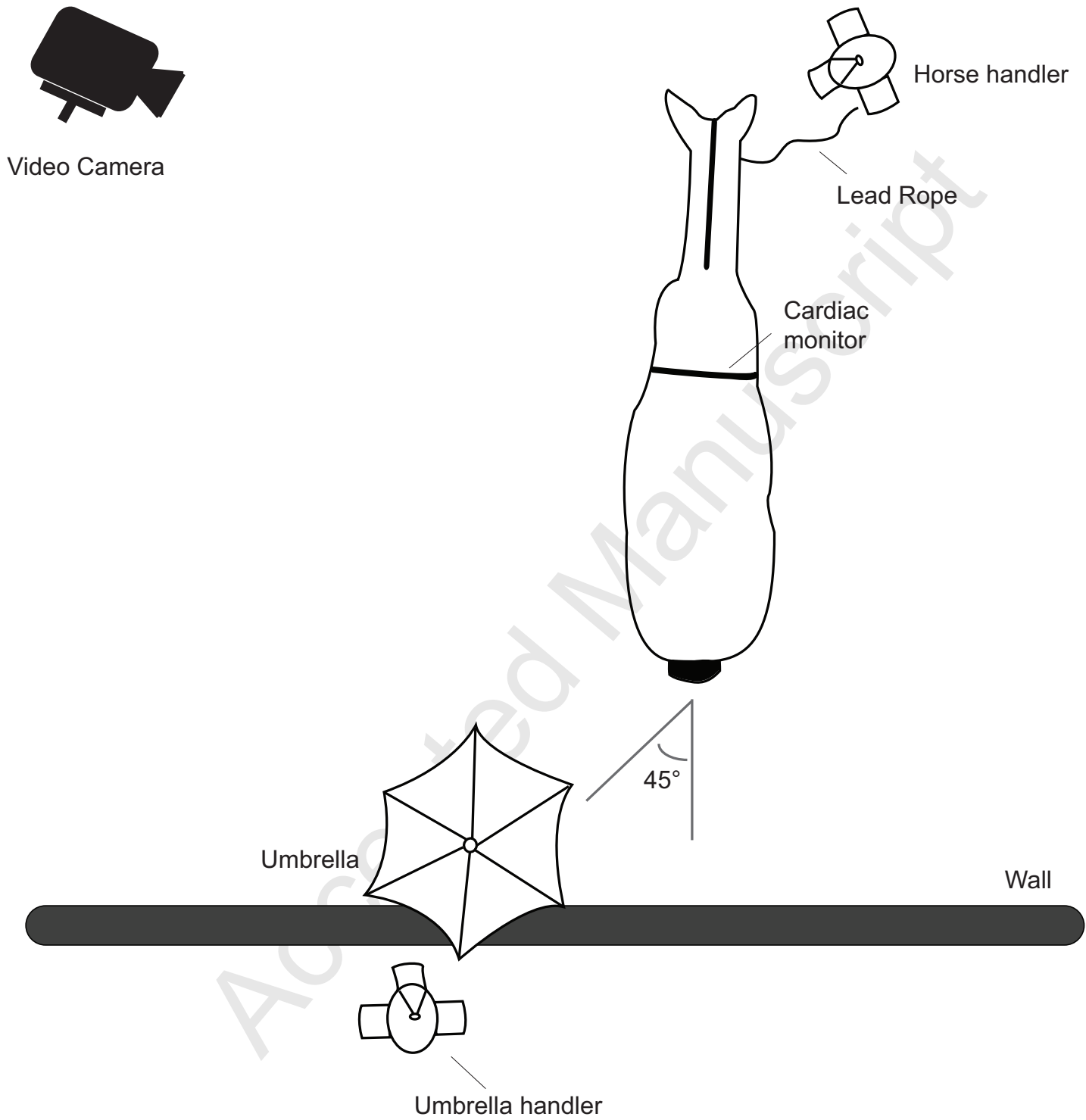
471 *Latency: time until the animal reaction.*472 *Duration: total time spent in the response.*473 *Distance: displacement of the animal.*

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A. Model of starle by opening umbrella in horses



B. Experimental Design

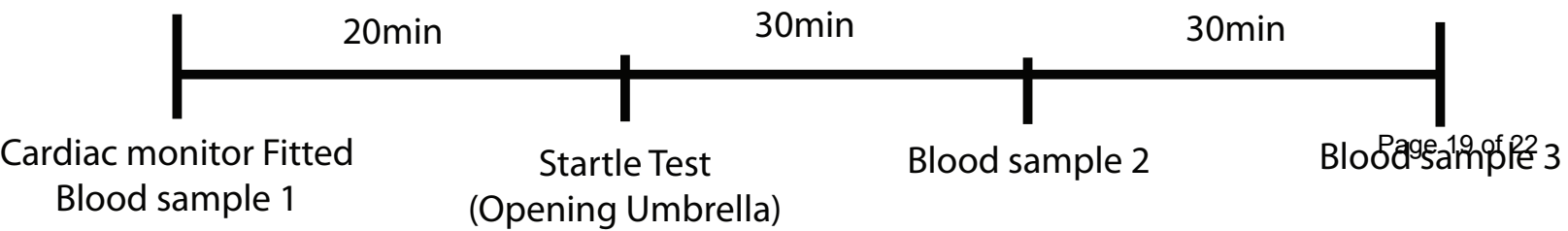
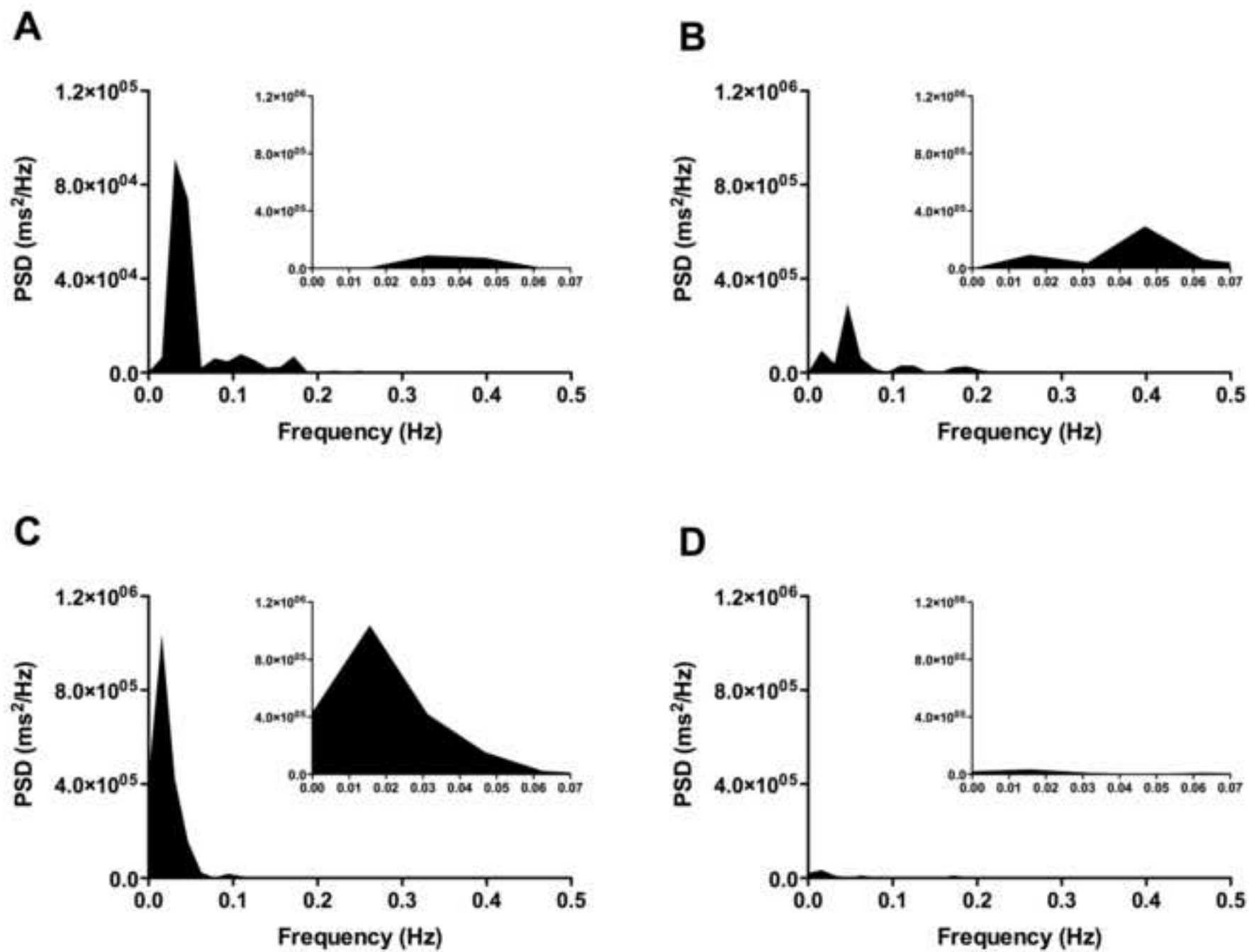


Figure 2



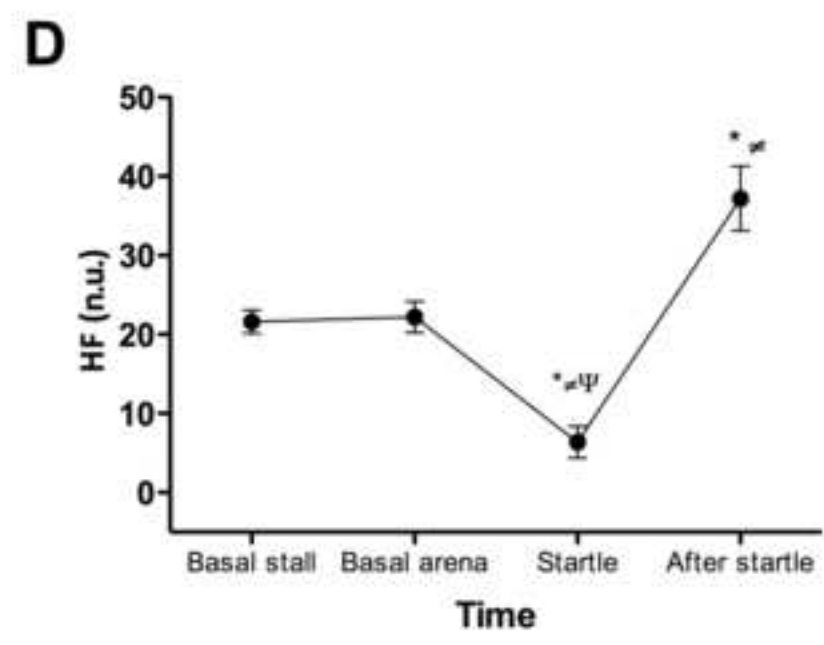
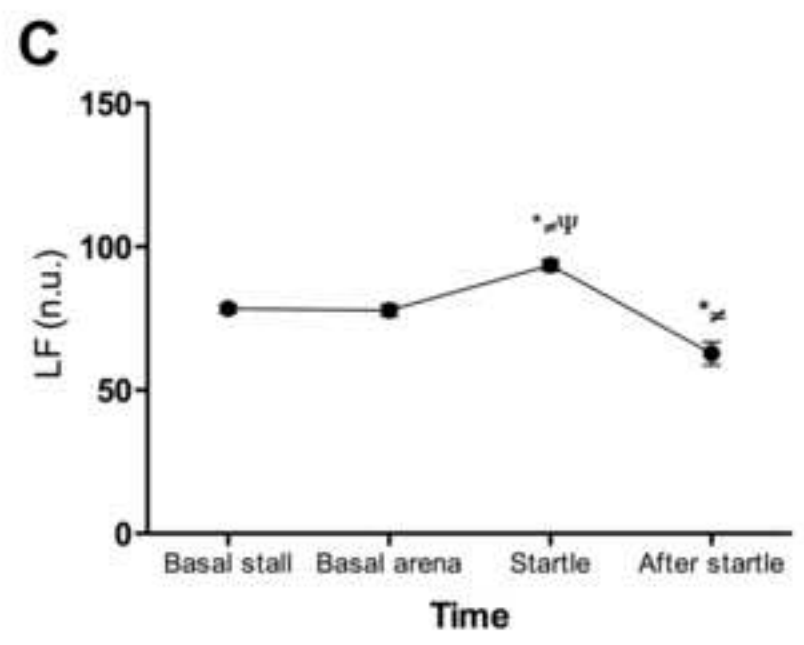
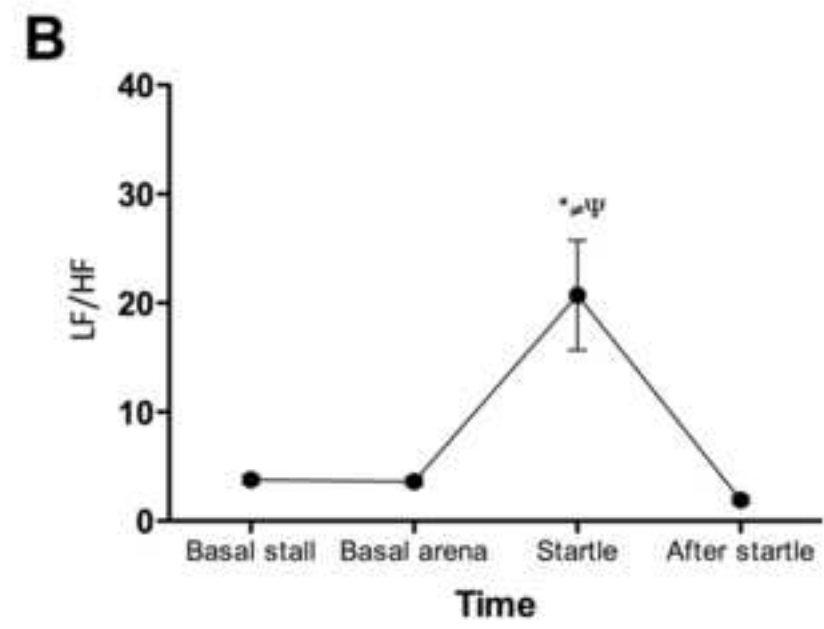
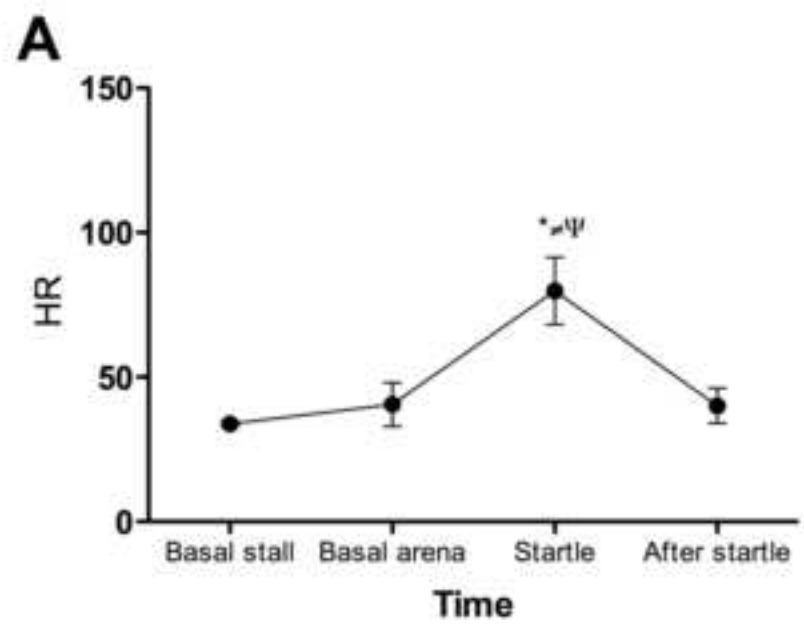


Figure 4

