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Research article

# Physiological stress responses in horses participating in novice endurance rides

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ABSTRACT

Horses participating in endurance events encounter enormous physical challenges. Heart rate (HR) and heart rate variability (HRV) have been reported before and after endurance rides, but these have not been determined during the rides. Moreover, the modulation in HRV and haematology in horses with different ride results (completed a course or disqualified due to irregular gait) have not been elucidated. Therefore, this study aimed to investigate changes in HR, HRV, and haematological parameters during novice endurance rides and to compare these parameters between horses that successfully completed the course (SC) or were disqualified for irregular gait (FTQ-GA). Beat-to-beat (RR) intervals of 16 healthy horses (aged 6–14 years) were recorded before and throughout the approximately 40 km endurance event. Blood samples were taken at the pre-ride inspection and after passing each veterinary inspection. HRV and haematology measures were determined from nine SC and seven FTQ-GA horses. Horses with different ride results demonstrated distinctive physiological stress responses. Increases in PCV, RBC, WBC and neutrophils after completing the ride were found only in SC horses, implying that they were ridden with greater effort than FTQ-GA horses. A reduction in HRV during warm-up, followed by a significant reduction during the first and second riding phases, was observed. HRV returned to baseline at the compulsory rest period of both phases. FTQ-GA horses experienced lower RR intervals, RR triangular index, modified deceleration capacity, very-low-frequency band, and parasympathetic nervous system index, coinciding with higher HR and sympathetic nervous system and stress indices than SC horses. These results indicated that endurance horses revealed a shift toward sympathetic activity during the ride. Lower parasympathetic activity in FTQ-GA horses suggests they were under more stress or discomfort than SC horses in novice endurance rides. These results have welfare implications, indicating the need for additional rest breaks in FTQ-GA horses.

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## 1. Introduction

Concerns about animal welfare are growing worldwide [1,2], including in equestrian sports [3–5]. Endurance riding is a strenuous activity in which the competitor must ensure their horse's safety over a long-distance race against the clock while overcoming challenges of weather and terrain [6]. A horse may be eliminated from the ride if it fails a veterinary examination because of lameness (irregular gait), metabolic irregularities, or other reasons indicating that it is unfit to compete; compulsory examinations occur before the start and during the ride [7–9]. Lameness appears to be the most common cause of elimination, followed by metabolic issues [10, 11]. The risk of elimination for these two reasons increases significantly in events with more than 80 and 100 participating horses, respectively [10]; longer events have lower completion rates than shorter ones [7,11,12]. In addition, some horses are ridden at an average speed of over 25 km/h in 120–160 km endurance rides, sometimes galloping during the last riding phase with speeds up to 30 km/h during intense competition [7]. For these reasons, the endurance horse is expected to encounter enormous physical challenges, which may affect the horse's welfare.

Haematological parameters are modulated following prolonged submaximal exercise in horses [13,14]. White blood cells (WBC), pack cell volume (PCV), and the enzyme creatine kinase (CK) increase significantly during and after endurance rides. These increments are thought to be associated with dehydration, stress, and muscular fatigue [13,15]. Furthermore, horses passing the veterinary inspection have lower heart rates and glucose and chloride concentrations than those failing it in an 80 km endurance ride [14]. In addition, sodium, chloride, and potassium concentrations decreased to different extents between horses completing a 160 km endurance ride and those eliminated [16]. Accordingly, changes in blood biochemical parameters differ in horses with different ride outcomes.

Heart rate variability (HRV) has frequently been used in conjunction with behaviour [17–19] and hormone levels [20–22] to assess autonomic nervous system (ANS) responses and, in turn, animal welfare. HRV is characterised by short-term fluctuations in duration between heartbeats in response to different stimuli [18,23]. This variation is influenced by hormonal factors and the rhythmic oscillations of the sympathetic and parasympathetic nerve impulses acting on the heart's sinoatrial node during the cardiac cycle [18, 24]. Several HRV variables are implemented to indicate ANS responses. Time domain variables, including the square root of the mean squared differences between successive beat-to-beat (RR) intervals (RMSSD), the relative number of successive RR interval pairs that differ more than 50 ms (pNN50), and the high frequency (HF) power spectrum represent parasympathetic (vagal) activity [18,23]. Decreases in these variables indicate a reduced role of the vagal component [23,25]. Likewise, the standard deviation of RR intervals (SDNN) indicates long-term variation in the cardiac cycle and is influenced by both sympathetic and parasympathetic activities [23]. Moreover, depending on the measurement condition, the SDNN is correlated to the very-low-frequency spectrum (VLF), low-frequency spectrum (LF), and total power frequency spectrum [26]. The triangular interpolation of normal-to-normal intervals (TINN) and RR triangular index time variables reflect overall variation in heart rates [18,27].

Declines in SDNN, VLF, LF, TINN, and RR triangular index have been observed during physical exercise, indicating reduced HRV [28]. Consequently, changes in HRV have been adapted to evaluate various conditions in horses, including interactions between cognition and emotion in response to mental effort [29], the effects of equine-assisted therapy [30], and reactions to fearful stimuli [31]. Moreover, HRV changes can be utilised to assess stress responses during exercise [20,32] and competition in equestrian events, including jumping [33,34], dressage [35], eventing [36], and endurance riding [37].

Although changes in HR, HRV [37] and haematological parameters [13,14] have been reported before and after endurance rides, literature is scarce concerning real-time HR and HRV modulation in horses during endurance rides. Moreover, comparisons in HR, HRV, and haematology changes in horses with different ride outcomes, particularly in horses that complete the course or fail it following veterinary examination, have never been published. Therefore, this study aimed to investigate changes in HR, HRV, and haematology parameters in horses participating in endurance rides and to compare these parameters between horses who completed the ride and those who were disqualified due to irregular gait. It was hypothesised that horses would show increased physiological stress during the event and that these stress responses would differ between horses who completed the course and those who failed to do so due to their irregular gait.

## 2. Material and methods

### 2.1. Animals

Eighteen healthy Arabian horses (nine geldings, two stallions, and seven mares; age 6–14 years; weight 398–430 kg) were studied. The horses participated in one of two novice-qualified rides ranging between 40 and 79 km, which took place according to the FEI rules [38] in Thailand. Ten horses took part in a ride in Roi-et province (latitude:15.852820786375426, longitude:103.86323745202183) in February 2022, and eight horses participated in a ride in Suphanburi province (latitude:14.161352240433521, longitude:100.08267481089804) in March 2022. All horses were trained and had been participating in novice endurance rides before being recruited for this study.

Horses were housed in 4 × 4 m separate stables within their barn. Commercial pellets and hay were provided three times daily, while clean water was freely available within each stable. Equine influenza vaccination was administered according to the FEI veterinary rules before the rides [39]. Routine deworming was also performed at 60–90 day intervals. On the night before the pre-inspection, they were moved to designated 4 × 4 m stables at the venue. The inclusion criteria for horses in this study were as follows: (1) successful completion of at least one novice endurance event within a year before the beginning of the study, and (2) passing the pre-ride inspection according to the FEI endurance rules [6]. Unfortunately, two horses failed the pre-ride inspection because of gait abnormalities. Thus, only 16 horses were included in this study. The Board Directors of the Thailand Equestrian Federation (TEF) approved the experimental protocol conducted at the TEF-recognised events.

### 2.2. Experimental design and data collection

#### 2.2.1. Endurance ride

The novice ride in Suphanburi province was divided into two phases, encompassing two riding loops of 20.74 and 21.40 km; the entire course was 42.14 km. The ride in Roi-et province consisted of two loops of 20.37 km each, accounting for a course length of 40.74 km. The minimum and maximum speeds of the ride in both venues were set according to the endurance rules [6] at 10 and 16 km/h, respectively. The experimental horses took part in a mass start, along with the other horses in the event. Speed was recorded by a sports watch (Polar Vantage 2; Polar Electro Oy, Kempele, Finland) throughout the riding periods. Environmental humidity and temperature were measured in both venues from 06.00 h to 11.00 h at 15-min intervals during the riding periods using a temperature and humidity data logger (TM-305U; Tenmars Electronics, Taipei, Taiwan). Mean (±SEM) environmental humidity and temperature were 79.67 ± 6.96 % and 28.95 ± 0.70 °C at the Suphanburi venue, and 69.50 8.31 % and 28.11 ± 0.99 °C at the Roi-et venue. Nine horses successfully completed (SC) the course (five geldings and four mares; age 9.0 ± 0.9 years; weight 418.1 ± 4.9 kg). However, seven horses failed the rides due to irregular gait (FTQ-GA) (one stallion, two geldings, four mares; age 8.3 ± 1.2 years; weight 413.5 ± 3.8 kg).

#### 2.2.2. Haematology

Blood samples (8 mL each, obtained by jugular venipuncture) were collected three times: before the ride and immediately after passing the veterinary inspection at the first and second riding phases. Of each sample, 4 mL was placed in a serum collection tube, of which the inner wall was coated with spray-dried microscopic silica particles to facilitate the clotting process and serum separation. The serum sample was used to analyse creatine kinase (CK) using an activated UV test (Mindray BS 800 chemistry analyser, Germany). The remaining 4 mL was preserved in a tube containing potassium ethylenediaminetetraacetic acid (EDTA) for complete blood count determination using an automated haematology analyser (Advia®2120i; Siemens Healthineers, Erlangen, Germany). Haematological parameters were determined, including PCV, haemoglobin (Hb), RBCs, WBCs, neutrophils, and neutrophil/lymphocyte ratio.

#### 2.2.3. RR interval recording

Horses were equipped with heart rate monitoring devices from 60 min before the start of the ride. In brief, horses were fitted around the chest with an equine belt for trotters (Polar Electro Oy, Kempele, Finland), with a heart rate sensor (Polar H10) located on the chest's left side (Supplementary File 1: Fig. S1). The device was wirelessly connected to the sports watch (Polar Vantage 2) for recording RR intervals. The device remained on the horses for the entire riding period, except during the veterinary inspection. Since the riders were blinded from the relevant information about the endurance horse (including HR, speed and distance), the RR recording sports watch (which showed the information on the screen) was placed in a sealed cross-body bag that the athletes carried throughout the ride (Supplementary File 2: Fig. S2). The RR intervals were determined for the horses from 60 min before the start of the first riding phase until the end of their ride.

#### 2.2.4. HRV data acquisition

RR interval information from the sports watch was uploaded to the Polar Flow program (https://flow.polar.com/) for HRV determination. HRV parameters were calculated from RR interval data using Kubios Premium software (Kubios HRV Scientific; https://www.kubios.com/hrv-premium/) and then exported as a MATLAB MAT-file (Supplementary File 3: Fig. S3). The premium version provides the automatic artefact correction algorithm, which computes more accurate HRV variables than the standard version and is validated to correct artefacts and ectopic beats in the interbeat interval (IBI) data [40]. Automatic noise detection was fixed at the medium level. Smoothness priors were used to remove IBI time series nonstationarities before HRV analysis. The cutoff frequency was set at 0.035 Hz, according to the user guideline manual (https://www.kubios.com/downloads/Kubios_HRV). The HRV parameters included (1) time domain results: RR interval, HR, SDNN, RMSSD, pNN50, TINN, RR triangular index, stress index, and modified heart rate deceleration capacity computed as a two-point difference (DCmod); (2) frequency domain results: VLF (by default 0–0.04 Hz), LF (by default 0.04–0.15 Hz), high-frequency spectrum (HF; by default 0.15–0.4 Hz), respiratory rate (RESP), LF/HF ratio, and total power; (3) nonlinear results: standard deviation of the Poincaré plot perpendicular to the line of identity (SD1) and standard deviation of the Poincaré plot along the line of identity (SD2); and (4) ANS indexes: parasympathetic nervous system (PNS) index and sympathetic nervous system (SNS) index. The HRV parameters were evaluated according to the timeline as follows: ***Pre-ride***, horses were equipped with riding gear 15 min before the ride; ***W-rd***, horses warmed up approximately 45 min before starting the first riding phase; ***P1-rd***, horses entered the course to complete the loop of the first riding phase; ***P1-chd***, horses were compulsorily halted for up to 15 min during the first riding phase; ***P1-crd***, horses compulsorily rested for 40 min after the first riding phase; ***P2-rd***, horses re-entered the course to complete the loop of the second riding phase; and ***P2-chd***, horses were compulsorily halted for up to 20 min during the second riding phase.

#### 2.2.5. Statistical analysis

Data were analysed using GraphPad Prism version 10.2.1 (GraphPad Software Inc, San Diego, USA). Because of missing data, a mixed-effects model (restricted maximum likelihood) with Greenhouse–Geisser correction was applied to determine the effects of the group, time, and group-by-time interaction on modifying the horses' HRV parameters and haematological profiles. Tukey's multiple comparisons test was applied to evaluate the modulation of HRV parameters, with the group compared with the pre-ride period and between groups at the given time points. Tukey's test was also used to determine changes in haematological and biochemical profiles during the ride. The Shapiro–Wilk test was implemented to evaluate the normal distribution in two-group comparisons. If the data were normally distributed, an independent-samples *t*-test was used to assess differences in overall HRV variables between SC and FTQ-GA horses during the entire ride; otherwise, the Mann–Whitney test was applied. Independent-samples *t*-tests were also used to evaluate differences in environmental humidity and temperature due to the normal distribution of the data. All variables were expressed as means ± SEM, and statistical significance was set at $P < 0.05$.

## 3. Results

### 3.1. Average speed of horses during the ride

The average speeds of SC and FTQ-GA horses are shown in Table 1. There was no difference in average speed between horses passing or failing the first veterinary inspection ($P = 0.9941$). Moreover, the average speed was unchanged in horses passing the first and second veterinary inspections ($P = 0.7324$). However, the speed was reduced in horses failing the second veterinary inspection compared with those passing the first and second inspections ($P = 0.0197$ and $P = 0.0047$, respectively).

### 3.2. Haematology

Effects of time and group-by-time were detected on modifications in WBC ($P = 0.0293$ and $P = 0.0490$) and neutrophils ($P = 0.0148$ and $P = 0.0020$), while group ($P = 0.0213$), time ($P = 0.0097$) and group-by-time ($P = 0.0177$) effects influenced change in the neutrophil/lymphocyte ratio. In contrast, modulation in lymphocytes and CK were under the effects of group ($P = 0.0298$) and time ($P = 0.0136$), respectively. For PCV and RBC, there were only significant group-by-time effects (PCV: $P = 0.0254$; RBC: $P = 0.0293$) (Table 2).

Increases in PCV ($P < 0.01$), RBC ($P < 0.01$), WBC ($P < 0.01$), neutrophils ($P < 0.001$), and lymphocytes ($P < 0.05$) were observed after the second riding phase only in SC horses. The neutrophil/lymphocyte (Neu/Lymp) ratio was not different in FTQ-GA horses but increased after the second riding phase in SC horses ($P < 0.01$) compared with the pre-ride. Meanwhile, after the second riding phase, the Neu/Lymp ratio was higher in SC than in FTQ-GA horses. Hb and CK of all horses in the endurance ride were higher after the second riding phase than in the pre-ride ($P < 0.05$ and $P < 0.01$, respectively) (Table 2).

### 3.3. Heart rate variability

#### 3.3.1. Time domain results

Group, time, and group-by-time effects caused changes in SDNN ($P = 0.0399$, $P < 0.0001$, and $P = 0.0122$, respectively), pNN50 (*P*

**Table 1**
Average speed (mean ± SEM) of horses that passed the veterinary inspection or failed the inspection for irregular gait in each riding phase.

| Horses | Average speed (km/h) | | | | Riding outcomes |
|---|---|---|---|---|---|
| | 1st riding phase | | 2nd riding phase | | |
| | Pass | Fail | Pass | Fail | |
| **Suphanburi venue** | | | | | |
| No. 1 | 11.69 | – | 13.40 | – | **SC** |
| No. 2 | 12.22 | – | 13.92 | – | **SC** |
| No. 3 | – | 11.43 | – | – | **FTQ-GA** |
| No. 4 | 11.94 | – | 13.68 | – | **SC** |
| No. 5 | 9.90 | – | 12.07 | – | **SC** |
| No. 6 | 11.34 | – | 11.5 | – | **SC** |
| No. 7 | 11.89 | – | 13.64 | – | **SC** |
| No. 8 | 12.39 | – | 13.49 | – | **SC** |
| **Roi-et venue** | | | | | |
| No. 1 | 11.11 | – | – | 10.83 | **FTQ-GA** |
| No. 2 | 13.05 | – | 10.80 | – | **SC** |
| No. 3 | – | 12.74 | – | – | **FTQ-GA** |
| No. 4 | – | 11.20 | – | – | **FTQ-GA** |
| No. 5 | – | 11.58 | – | – | **FTQ-GA** |
| No. 6 | 12.74 | – | – | 8.46 | **FTQ-GA** |
| No. 7 | 12.29 | – | – | 9.39 | **FTQ-GA** |
| No. 8 | 12.30 | – | 9.52 | – | **SC** |
| Mean ± SEM | $11.91 \pm 0.2^{a}$ | $11.74 \pm 0.34^{ab}$ | $12.45 \pm 0.5^{a}$ | $9.56 \pm 0.69^{b}$ | **SC = 9**<br>**FTQ-GA = 7** |

Distinct letters (**a** and **b**) indicate significant differences between groups. **SC**: successful completion; **FTQ-GA**: failure to qualify following irregular gait.

**Table 2**
Haematological profiles (mean ± SEM) of SC horses and FTQ-GA horses in the novice endurance ride.

| Haematological profiles | Ride results | Ride periods | | | Interaction *P*-value |
|---|---|---|---|---|---|
| | | Pre-ride | post-1st inspection | Post-2nd inspection | |
| **PCV (%)** | **SC** | 34.67 ± 0.89$^{a}$ | 37.04 ± 1.29$^{a}$ | 40.40 ± 1.49$^{b}$ | 0.0254* |
| | **FTQ-GA** | 40.25 ± 1.76 | 39.42 ± 1.51 | 38.17 ± 1.96 | |
| **Hb (g/dL)** | **SC and FTQ-GA** | 12.88 ± 0.97$^{a}$ | 13.40 ± 0.35$^{ab}$ | 13.85 ± 0.59$^{b}$ | 0.0846 |
| **RBC (x10$^{6}$/μL)** | **SC** | 7.57 ± 0.26$^{a}$ | 8.11 ± 0.30$^{a}$ | 8.85 ± 0.35$^{b}$ | 0.0326* |
| | **FTQ-GA** | 8.59 ± 0.37 | 8.50 ± 0.35 | 8.22 ± 0.46 | |
| **WBC (/μL)**‡ | **SC** | 7266.67 ± 672.48$^{a}$ | 7688.89 ± 771.08$^{a}$ | 9922.22 ± 672.02$^{b}$ | 0.0293* |
| | **FTQ-GA** | 9316.67 ± 552.82 | 8600.00 ± 619.14 | 9266.67 ± 554.78 | |
| **Neu (/μL)**‡ | **SC** | 5103.33 ± 644.76$^{a}$ | 5417.44 ± 446.68$^{a}$ | 8694.44 ± 596.66$^{b}$ | 0.0148* |
| | **FTQ-GA** | 6214.00 ± 506.20 | 5427.50 ± 638.97 | 6348.67 ± 1048.90 | |
| **Lymp (/μL)**† | **SC** | 1969.78 ± 263.97$^{a}$ | 2062.78 ± 368.56$^{a}$ | 1132.00 ± 220.51$^{b}$ | 0.8384 |
| | **FTQ-GA** | 2905.33 ± 115.31 | 2898.00 ± 642.20 | 2573.00 ± 553.79 | |
| **Neu/Lymp ratio**† ‡ | **SC** | 3.06 ± 0.64$^{a}$ | 3.06 ± 0.39$^{a}$ | 9.58 ± 1.58$^{b,x}$ | 0.0177* |
| | **FTQ-GA** | 2.16 ± 0.13 | 2.81 ± 1.09 | 2.99 ± 1.23$^{y}$ | |
| **CK (IU/L)**‡ | **SC and FTQ-GA** | 195.23 ± 8.98$^{a}$ | 235.87 ± 11.87$^{a}$ | 380.17 ± 7.07$^{b}$ | 0.9879 |

*, †, and ‡ Indicate the statistical significance of the group-by-time, group, and time effects, respectively. Distinct letters indicate differences within groups (**a, b** and **c**) and between groups (**x** and **y**). **SC**: successful completion; **FTQ-GA**: failure to qualify following irregular gait; **PCV**: packed cell volume; **Hb**: haemoglobin; **RBC**: red blood cells; **WBC**: white blood cells; **Neu**: neutrophils; **Lymp**: lymphocytes; **CK**: creatine kinase.

**Table 3**
Time domain variables (mean ± SEM) of SC horses and FTQ-GA horses in the novice endurance ride.

| HRV variables | Ride results | Ride periods | | | | | | | Interaction *P*-value |
|---|---|---|---|---|---|---|---|---|---|
| | | Pre-rd | W-rd | P1-rd | P1-chd | P1-crd | P2-rd | P2-chd | |
| **Mean RR (ms)**‡ | **SC and FTQ-GA** | 1288.69 ± 38.02 | 815.55 ± 26.88$^{d}$ | 542.55 ± 45.12$^{d}$ | 962.56 ± 50.56$^{c}$ | 1158.78 ± 128.78 | 555.78 ± 19.44$^{d}$ | 929.94± 53.94$^{a}$ | 0.0964 |
| **Mean HR (bpm)**‡ | **SC** | 49.22 ± 3.21 | 78.89 ± 5.22$^{b}$ | 102.89 ± 3.11$^{d,x}$ | 60.56 ± 3.24 | 47.11 ± 1.57$^{x}$ | 104.56 ± 2.16$^{d}$ | 62.50 ± 3.42 | 0.0407* |
| | **FTQ-GA** | 45.71 ± 2.04 | 76.29 ± 7.93$^{a}$ | 122.29 ± 5.32$^{d,y}$ | 66.00 ± 1.53$^{d}$ | 58.33 ± 2.40$^{y}$ | 113.00 ± 8.19$^{a}$ | 69.00 ± 3.00 | |
| **SDNN (ms)**†‡ | **SC** | 65.88 ± 8.02 | 38.24 ± 5.39$^{a}$ | 19.97 ± 1.62$^{b,x}$ | 31.76 ± 2.89$^{b,x}$ | 60.80 ± 5.98 | 18.64 ± 1.78$^{b}$ | 29.78 ± 5.62$^{b}$ | 0.0122* |
| | **FTQ-GA** | 69.27± 6.73 | 50.07 ± 6.60 | 14.17 ± 1.27$^{b,y}$ | 50.79 ± 4.92$^{y}$ | 64.37 ± 6.50 | 19.20 ± 4.02$^{a}$ | 71.30 ± 13.50 | |
| **RMSSD (ms)**†‡ | **SC** | 52.60 ± 8.43 | 21.38 ± 3.58$^{a}$ | 8.39 ± 0.91$^{b,x}$ | 24.78 ± 2.51$^{a}$ | 50.37 ± 5.11 | 8.59 ± 0.92$^{b}$ | 24.74 ± 4.39$^{a}$ | 0.0810 |
| | **FTQ-GA** | 65.04 ± 7.40 | 32.80 ± 7.19$^{a}$ | 5.76 ± 0.71$^{c,y}$ | 34.46 ± 4.75$^{a}$ | 50.57 ± 5.68 | 10.70 ± 3.64$^{a}$ | 61.30 ± 13.50 | |
| **pNN50 (%)**†‡ | **SC** | 23.30 ± 4.61 | 5.08 ± 1.52$^{a}$ | 0.35 ± 0.13$^{b}$ | 5.42 ± 1.23$^{a}$ | 22.31 ± 2.85 | 0.39 ± 0.14$^{b}$ | 5.88 ± 2.76$^{a}$ | 0.0098* |
| | **FTQ-GA** | 29.96 ± 3.66 | 9.83 ± 3.45$^{a}$ | 0.13 ± 0.05$^{c}$ | 11.92 ± 3.42$^{a}$ | 21.67 ± 3.05 | 0.86 ± 0.67$^{a}$ | 32.88 ± 13.71 | |
| **TINN (ms)**†‡ | **SC** | 337.89± 43.83 | 205.56 ± 30.14 | 203.78 ± 21.92 | 173.33 ± 17.80$^{b,x}$ | 363.89± 31.38$^{x}$ | 201.22 ± 19.87 | 146.50± 25.37$^{b,x}$ | 0.0559 |
| | **FTQ-GA** | 450.00± 58.17 | 287.43 ± 41.01 | 177.00 ± 11.77$^{a}$ | 274.43 ± 19.05$^{y}$ | 443.00± 11.15$^{y}$ | 188.67 ± 43.73 | 345.00 ± 27.00$^{y}$ | |
| **RR triangular index**†‡ | **SC** | 13.52 ± 2.00 | 7.10 ± 1.83 | 3.61 ± 0.27$^{b,x}$ | 7.10 ± 0.52$^{x}$ | 13.35 ± 1.37 | 3.50 ± 0.28$^{b}$ | 6.92 ± 1.35$^{a,x}$ | 0.0479* |
| | **FTQ-GA** | 14.50 ± 1.17 | 10.51 ± 1.00 | 2.68 ± 0.22$^{c,y}$ | 10.06 ± 1.06$^{a,y}$ | 13.14 ± 1.58 | 3.23 ± 0.34 | 16.01 ± 1.86$^{y}$ | |
| **Dcmod (ms)**‡ | **SC and FTQ-GA** | 60.15 ± 5.46 | 23.74 ± 5.82$^{c}$ | 5.12 ± 1.05$^{d}$ | 29.57 ± 6.33$^{b}$ | 50.77 ± 0.43 | 6.72 ± 0.44$^{c}$ | 41.59 ± 15.21$^{a}$ | 0.3944 |

*, †, and ‡ Indicate the statistical significance of the group-by-time, group, and time effects, respectively. **a**, **b**, **c**, and **d** indicate statistical significance at $P < 0.05$, $P < 0.01$, $P < 0.001$, and $P < 0.0001$ compared to the control (Pre-rd). Different letters (**x** and **y**) indicate statistical differences between comparison pairs at given time points. **Pre-rd**: pre-ride; **W-rd**: warm-up; **P1-rd**: 1st phase riding; **P1-chd**: 1st phase compulsory halt; **P1-crd**: 1st phase compulsory rest; **P2-rd**: 2nd phase riding; **P2-chd**: 2nd phase compulsory halt; **SC**: successful completion; **FTQ-GA**: failure to qualify following irregular gait; **HRV**: heart rate variability; **RR**: beat-to-beat interval; **HR**: heart rate; **SDNN**: standard deviation of normal-to-normal RR intervals; **RMSSD**: root mean square of successive RR interval differences; **pNN50**: relative number of successive RR interval pairs that differ by more than 50 msec; **TINN**: triangular interpolation of normal-to-normal intervals; **DCmod**: modified deceleration capacity computed as a two-point difference.

$= 0.0128$, $P < 0.0001$, and $P = 0.0098$, respectively), and RR triangular index ($P = 0.0312$, $P < 0.0001$, and $P = 0.0479$, respectively). Time ($P < 0.0001$) and group-by-time ($P = 0.0407$) affected changes in mean HR. Separate effects of group and time also affected the modification of RMSSD ($P = 0.0331$ and $P < 0.0001$, respectively) and TINN ($P = 0.0113$ and $P < 0.0001$, respectively). Although there was a trend in the effect of group-by-time on mean RR modification ($P = 0.0964$) and an independent group effect on DCmod ($P = 0.0634$), only time was considered to influence the changes in mean RR and DCmod ($P < 0.0001$ for all) (Table 3).

The mean RR of all horses decreased at the W-rd, P1-rd and P1-chd ($P < 0.001$–$0.0001$); it then returned to baseline at the P1-crd. The mean RR declined again during the P2-rd ($P < 0.0001$) and P2-chd ($P < 0.05$). Mean HR increased during the W-rd and P1-rd in SC horses ($P < 0.01$ and $P < 0.0001$, respectively) and FTQ-GA horses ($P < 0.05$ and $P < 0.0001$, respectively). The mean HR in SC horses decreased during P1-chd and P1-crd. However, in FTQ-GA horses, it remained higher during the P1-chd ($P < 0.0001$) and decreased later during the P1-crd. A rise in mean HR was found again during the P2-rd in both SC ($P < 0.0001$) and FTQ-GA horses ($P < 0.05$). In SC horses, SDNN decreased in virtually all periods (W-rd, P1-rd, P1-chd, P2-rd, and P2-chd, $P < 0.05$-$0.01$), except for P1-crd ($P > 0.05$). Conversely, it decreased only during the P1-rd ($P < 0.01$) and P2-rd ($P < 0.05$) in FTQ-GA horses. RMSSD was also lower in SC horses during the W-rd, P1-rd, P1-chd, P2-rd, and P2-chd ($P < 0.05$-$0.01$), except for P1-crd. In contrast, in FTQ-GA horses, it was lower during W-rd, P1-rd, P1-chd, and P2-rd ($P < 0.05$–$0.001$), but not P1-crd and P2-chd.

**Table 4**
Frequency domain and nonlinear variables (mean ± SEM) of SC horses and FTQ-GA horses in the novice endurance ride.

| HRV variables | Ride results | Ride periods | | | | | | | Interaction *P*-value |
|---|---|---|---|---|---|---|---|---|---|
| | | Pre-rd | W-rd | P1-rd | P1-chd | P1-crd | P2-rd | P2-chd | |
| Frequency domain parameters | | | | | | | | | |
| **VLF ($ms^2$)‡** | **SC and FTQ-GA** | 1189.46 ± 38.32 | 573.29 ± 153.29 | 97.33 ± 35.90$^{c}$ | 196.14 ± 83.14$^{b}$ | 787.67 ± 47.33 | 90.78 ± 20.78$^{b}$ | 173.06 ± 50.44$^{a}$ | 0.8961 |
| **LF ($ms^2$)‡** | **SC** | 2627.56 ± 702.13 | 1273.57 ± 334.19 | 289.22 ± 50.60$^{a,x}$ | 400.33 ± 69.57$^{a,x}$ | 2415.22 ± 549.34 | 270.00 ± 54.01$^{a}$ | 621.88 ± 237.62$^{a}$ | 0.0350* |
| | **FTQ-GA** | 2391.86 ± 578.01 | 1722.86 ± 573.41 | 155.14 ± 24.65$^{a,y}$ | 1980.57 ± 493.96$^{y}$ | 1944.00 ± 521.76 | 281.33 ± 111.82 | 3078.50 ± 1603.50 | |
| **HF ($ms^2$)†‡** | **SC** | 810.667 ± 267.49 | 190.56 ± 63.10 | 29.67 ± 7.28 | 144.22 ± 26.04 | 576.00 ± 121.78 | 31.22 ± 7.43 | 264.38 ± 124.77 | 0.0092* |
| | **FTQ-GA** | 1034.57 ± 173.08 | 542.29 ± 208.02 | 15.43 ± 3.84$^{b}$ | 602.71 ± 202.29 | 962.67 ± 199.07 | 67.67 ± 39.96 | 1682.00 ± 237.00 | |
| **LF/HF ratio‡** | **SC and FTQ-GA** | 4.15 ± 1.72 | 7.74 ± 1.88$^{b}$ | 13.16 ± 1.37$^{b}$ | 3.97 ± 0.48 | 3.08 ± 1.07 | 8.47 ± 1.70 | 2.31 ± 0.58 | 0.2565 |
| **Total power ($ms^2$)‡** | **SC and FTQ-GA** | 4621.88 ± 44.45 | 2471.28 ± 520.70 | 342.02 ± 110.31$^{c}$ | 1760.48 ± 1102.38$^{b}$ | 3737.22 ± 5.11 | 426.11 ± 13.22$^{b}$ | 2997.19 ± 1987.81 | 0.1065 |
| **RESP (Hz)‡** | **SC and FTQ-GA** | 0.213 ± 0.010 | 0.217 ± 0.008 | 0.304 ± 0.029$^{c}$ | 0.232 ± 0.010 | 0.219 ± 0.002 | 0.279 ± 0.016$^{c}$ | 0.242 ± 0.007 | 0.0652 |
| **Nonlinear parameters** | | | | | | | | | |
| **SD1 (ms)†‡** | **SC** | 37.26 ± 5.97 | 15.11 ± 2.54$^{a}$ | 5.94 ± 0.64$^{b,x}$ | 17.56 ± 1.78$^{a}$ | 35.62 ± 3.61 | 6.06 ± 0.65$^{b}$ | 17.53 ± 3.11$^{a}$ | 0.0816 |
| | **FTQ-GA** | 46.04 ± 5.23 | 23.21 ± 5.08$^{a}$ | 4.09 ± 0.50$^{c,y}$ | 24.39 ± 3.37$^{a}$ | 35.77 ± 4.01 | 7.57 ± 2.56$^{a}$ | 43.40 ± 9.60 | |
| **SD2 (ms)†‡** | **SC** | 84.91 ± 10.15 | 51.78 ± 7.31 | 27.58 ± 2.21$^{b,x}$ | 40.96 ± 3.83$^{b,x}$ | 78.14 ± 7.75 | 25.64 ± 2.34$^{b}$ | 38.11 ± 7.50$^{b}$ | 0.0075* |
| | **FTQ-GA** | 86.27 ± 8.44 | 66.47 ± 8.43 | 19.61 ± 1.74$^{b,y}$ | 67.57 ± 6.22$^{y}$ | 83.67 ± 8.32 | 26.03 ± 5.20$^{a}$ | 91.15 ± 16.65 | |
| **SD2/SD1 ratio‡** | **SC and FTQ-GA** | 2.25 ± 0.33 | 3.54 ± 1.82$^{c}$ | 4.87 ± 0.10$^{d}$ | 2.68 ± 0.21 | 2.28 ± 0.07 | 3.90 ± 0.12$^{a}$ | 2.14 ± 0.02 | 0.4306 |

*, †, and ‡ Indicate the statistical significance of the group-by-time, group, and time effects, respectively. a, b, c, and d indicate statistical significance at $P < 0.05$, $P < 0.01$, $P < 0.001$, and $P < 0.0001$, respectively, compared to the control (Pre-rd). Different letters (**x** and **y**) indicate statistical differences between comparison pairs at given time points. **Pre-rd**: pre-ride; **W-rd**: warm-up; **P1-rd**: 1st phase riding; **P1-chd**: 1st phase compulsory halt; **P1-crd**: 1st phase compulsory rest; **P2-rd**: 2nd phase riding; **P2-chd**: 2nd phase compulsory halt; **SC**: successful completion; **FTQ-GA**: failure to qualify following irregular gait; **VLF**: HRV very-low-frequency band, by default 0–0.04 Hz; **LF**: HRV low-frequency band, by default 0.04–0.15 Hz; **HF**: HRV high-frequency band, by default 0.15–0.4 Hz; **RESP**: respiration rate; **SD1**: standard deviation of the Poincaré plot perpendicular to the line of identity; **SD2**: standard deviation of the Poincaré plot along the line of identity.

The change in pNN50 was similar to the RMSSD modulation as it reduced in all periods ($P < 0.05$–$0.01$) except for the P1-crd in SC horses. However, in FTQ-GA horses, it declined during most ride periods ($P < 0.05$–$0.001$), except for the P1-crd and P2-chd. TINN reduced during the P1-chd and P2-chd in SC horses ($P < 0.01$ for both periods). In contrast, it decreased during the P1-rd ($P < 0.05$) in FTQ-GA horses. The RR triangular index declined during the P1-rd ($P < 0.01$), P2-rd ($P < 0.01$), and P2-chd ($P < 0.05$) in SC horses. In contrast, it decreased during P1-rd ($P < 0.001$) and P1-chd ($P < 0.05$) in FTQ-GA horses. DCmod decreased virtually every period, except for the P1-crd, in both SC and FTQ-GA horses ($P < 0.05$–$0.0001$) (Table 3).

Comparing the variables between given time points, the mean HR in SC horses was lower than FTQ-GA horses during P1-rd and P1-crd ($P < 0.05$ for both periods). SDNN and RMSSD were higher in SC horses than in FTQ-GA horses during P1-rd ($P < 0.05$ for both variables). On the contrary, the SDNN of SC horses was lower than FTQ-GA horses during P1-chd ($P < 0.05$). TINN was lower in SC horses than in FTQ-GA horses during the P1-chd ($P < 0.01$), P1-crd ($P < 0.05$), and P2-chd ($P < 0.05$) (Table 3).

#### *3.3.2. Frequency domain results*

Group, time, and group-by-time affected HF power spectrum modification ($P = 0.0054$, $P < 0.0001$, and $P = 0.0092$, respectively), while time ($P < 0.0001$) and group-by-time ($P = 0.0350$) affected changes in the LF power spectrum. Only time caused a modification in the VLF power spectrum, LF/HF ratio, total power spectrum, and RESP ($P < 0.0001$ for all) (Table 4).

The VLF power spectrum decreased in SC and FTQ-GA horses during P1-rd, P1-chd, P2-rd, and P2-chd ($P < 0.05$–$0.001$). However, differences in frequency domain parameters were found in the LF and HF power spectra. The LF power spectrum declined during P1-rd, P-chd, P2-rd, and P2-chd in SC horses ($P < 0.05$ for all). However, it decreased only during the P1-rd in FTQ-GA horses ($P < 0.05$). The HF power spectrum did not change in any period in SC horses, although it showed a trend to decrease during P1-rd ($P = 0.0761$) and P2-rd ($P = 0.0789$). In FTQ-GA horses, the HF power spectrum was reduced during the P1-rd ($P < 0.01$). LF/HF ratio increased during W-rd and P1-rd ($P < 0.01$ for both periods) and showed an insignificant increase during P2-rd ($P = 0.0629$) in SC and FTQ-GA horses. The SC and FTQ-GA horses showed a decrease in the total power spectrum during the P1-rd, P1-chd, and P2-rd ($P < 0.01$–$0.001$). An increase in RESP was seen in SC and FTQ-GA horses during the P1-rd and P2-rd ($P < 0.001$ for both periods) (Table 4).

The LF power spectrum in SC horses was higher during P1-rd ($P < 0.05$) but lower during P1-chd ($P < 0.05$) than in FTQ-GA horses. The HF in SC horses showed a non-significant trend towards lower values during P1-chd ($P = 0.0642$) and P2-chd ($P = 0.0525$) than in FTQ-GA horses (Table 4).

#### *3.3.3. Nonlinear results*

Group, time, and group-by-time influenced SD2 modulation ($P = 0.0444$, $P < 0.0001$, and $P = 0.0075$, respectively). Even though the interaction effect between group and time was non-significant ($P = 0.0816$), significant effects of group ($P = 0.0333$) and time ($P < 0.0001$) were observed in SD1 modification. The SD2/DS1 ratio changed due to the main effect of time ($P < 0.0001$) (Table 4).

A marked decrease in SD1 occurred in virtually all periods in both groups ($P < 0.05$–$0.001$), except for P1-crd in SC horses and P1-crd and P2-chd in FTQ-GA horses. SD2 decreased during the P1-rd, P1-chd, P2-rd, and P2-chd SC horses ($P < 0.01$ for all periods). In contrast, it decreased only during P1-rd ($P < 0.01$) and P2-rd ($P < 0.05$) in FTQ-GA horses. An increase in SD2/SD1 ratio was observed in SC and FTQ-GA horses during the W-rd ($P < 0.001$), P1-rd ($P < 0.0001$), and P2-rd ($P < 0.05$) (Table 4).

SD1 and SD2 were higher in SC horses than in FTQ-GA horses during P1-rd ($P < 0.05$). On the other hand, SD2 was lower in SC horses than in FTQ-GA horses during the P1-chd ($P < 0.01$) (Table 4).

#### *3.3.4. Autonomic nervous system index*

There were group-by-time and time effects on changes in the stress index ($P = 0.0131$ and $P < 0.0001$, respectively) and SNS index

**Table 5**
Autonomic nervous system (ANS) indexes (mean ± SEM) of SC horses and FTQ-GA horses in the novice endurance ride.

| HRV variables | Ride results | Ride periods | | | | | | | Interaction *P*-value |
|---|---|---|---|---|---|---|---|---|---|
| | | Pre-rd | W-rd | P1-rd | P1-chd | P1-crd | P2-rd | P2-chd | |
| **Stress index‡** | **SC** | 6.11 ± 0.77 | 14.37 ± 2.28[a] | 15.89 ± 1.10[c] | 11.92 ± 1.28[b,x] | 5.58 ± 0.46 | 15.72 ± 0.96[c] | 13.13 ± 1.20[b,x] | 0.0131* |
| | **FTQ-GA** | 4.83 ± 0.49 | 9.50 ± 1.53 | 18.26 ± 0.89[d] | 8.34 ± 0.64[b,y] | 5.40 ± 0.46 | 17.77 ± 2.84[a] | 6.05 ± 0.65[y] | |
| **SNS index‡** | **SC** | −1.49 ± 0.31 | 1.78 ± 0.70[a] | 3.69 ± 0.38[d,x] | 0.11 ± 0.38[a] | −1.73 ± 0.15 | 3.76 ± 0.27[d] | 0.39 ± 0.32[a] | 0.0149* |
| | **FTQ-GA** | −1.98 ± 0.16 | 0.82 ± 0.76[a] | 5.72 ± 0.56[d,y] | −0.06 ± 0.15[c] | −1.03 ± 0.24 | 4.81 ± 1.4[a] | −0.32 ± 0.31 | |
| **PNS index‡** | **SC and FTQ-GA** | 2.06 ± 0.37 | −1.02 ± 0.29[d] | −3.00 ± 0.34[d] | −0.22 ± 0.12[d] | 1.24 ± 0.58 | −2.80 ± 0.09[d] | 0.05 ± 0.25[b] | 0.0998 |

*, †, and ‡ Indicate the statistical significance of the group-by-time, group, and time effects, respectively. **a**, **b**, **c**, and **d** indicate statistical significance at $P < 0.05$, $P < 0.01$, $P < 0.001$, and $P < 0.0001$, respectively, compared to the control (pre-ride). Different letters (**x** and **y**) indicate statistical differences between comparison pairs at given time points. **Pre-rd**: pre-ride; **W-rd**: warm-up; **P1-rd**: 1st phase riding; **P1-chd**: 1st phase compulsory halt; **P1-crd**: 1st phase compulsory rest; **P2-rd**: 2nd phase riding; **P2-chd**: 2nd phase compulsory halt; **SC**: successful completion; **FTQ-GA**: failure to qualify following irregular gait; **PNS**: parasympathetic nervous system; **SNS**: sympathetic nervous system.

($P = 0.0149$ and $P < 0.0001$, respectively). Despite a trend to show a group-by-time effect ($P = 0.0998$), only time significantly affected a modification of the PNS index ($P < 0.0001$) (Table 5).

The PNS index decreased in virtually all periods except for P1-crd in SC and FTQ-GA horses ($P < 0.0001$ for W-rd, P1-rd, P1-chd, and P2-rd; $P < 0.01$ for P2-chd). A rise in the SNS index was also noted in virtually all periods, except for P1-crd in SC horses ($P < 0.05$ for W-rd, P1-chd, and P2-chd; $P < 0.0001$ for P1-rd and P2-rd). In contrast, the SNS index increased during W-rd ($P < 0.05$), P1-rd ($P < 0.0001$), P1-chd ($P < 0.001$), and P2-rd ($P < 0.05$), but not for P1-crd and P2-chd in FTQ-GA horses. In SC horses, the stress index increased in all periods (P < 0.05–0.001) except for P1-crd. Conversely, the stress index in FTQ-GA horses increased only during P1-rd ($P < 0.0001$), P1-chd ($P < 0.01$), and P2-rd ($P < 0.05$) (Table 5).

The stress index was higher in SC horses than in FTQ-GA horses during the P1-chd ($P < 0.05$) and P2-chd ($P < 0.01$). At the same time, the SNS index was lower in SC horses than in FTQ horses during P1-rd ($P < 0.01$) (Table 5).

### 3.3.5. *Comparison of overall HRV between SC and FTQ-GA horses*

The comparison of overall HRV between SC and FTQ-GA horses during the entire ride is shown in Table 6. Horses completing the ride demonstrated higher multiple HRV parameters than those eliminated following irregular gait, including mean RR ($P < 0.01$), RR triangular index ($P < 0.05$), and DCmod ($P < 0.05$). In contrast, they showed lower mean HR than those eliminated from the ride ($P < 0.01$). Horses completing the ride also exhibited higher VLF power spectra and PNS indices than those failing it (VLF: $P < 0.05$; PNS index: $P < 0.01$). Meanwhile, stress and SNS indices in horses failing the ride were higher than those completing it (stress index: $P < 0.05$; SNS index: $P < 0.01$).

## 4. Discussion

We investigated the changes in haematology and HRV parameters in horses who participated in novice endurance rides and compared these variables between horses who completed the course or did not do so because of irregular gait. The significant findings from this study were as follows: (1) Horses demonstrated a reduced HRV during warm-up, riding, and compulsory halt periods in both riding phases but returned to baseline during the compulsory rest period. (2) Modulation in haematological profiles (PCV, RBC, WBC, neutrophils, and the neutrophil/lymphocyte ratio), mean HR, and various HRV parameters (SDNN, pNN50, RR triangular index, LF power spectrum, HF power spectrum, SD2, stress index, and SNS index) differed between SC and FTQ-GA horses. (3) Increases in those haematological parameters occurred only after the second riding phase in SC horses. (4) Mean HR increased considerably during both riding phases and was higher in FTQ-GA than SC horses during riding and the compulsory rest periods of the first phase. (5) Various HRV variables (including mean RR interval, mean HR, RR triangular index, DCmod, VLF power spectrum, and PNS index) were lower

**Table 6**
Comparisons in overall HRV (mean ± SEM) between SC horses and FTQ-GA horses during the entire ride.

| HRV variables | Ride results | | *P*-value |
|---|---|---|---|
| | SC | FTQ-GA | |
| **Mean HR (bpm)** | 92.67 ± 1.83 | 113.10 ± 5.63 | 0.0018* |
| **Mean RR (ms)** | 650.00 ± 13.49 | 538.90 ± 28.59 | 0.0020* |
| **SDNN (ms)** | 25.54 ± 1.65 | 21.09 ± 2.32 | 0.1301 |
| **RMSSD (ms)** | 17.0 ± 61.35 | 12.97 ± 2.19 | 0.1191 |
| **pNN50 (%)** | 2.25 ± 0.28 | 1.47 ± 0.49 | 0.1669 |
| **TINN (ms)** | 351.80 ± 25.60 | 330.10 ± 25.63 | 0.6213 |
| **RR triangular index** | 3.84 ± 2.56 | 2.93 ± 0.21 | 0.0200* |
| **DCmod (ms)** | 10.18 ± 0.68 | 6.97 ± 1.23 | 0.0294* |
| **VLF ($ms^2$)** | 229.20 ± 30.45 | 125.60 ± 30.16 | 0.0324* |
| **LF ($ms^2$)** | 641.30 ± 100.50 | 387.90 ± 83.38 | 0.0834 |
| **HF ($ms^2$)** | 124.20 ± 21.38 | 117.10 ± 41.33 | 0.8733 |
| **LF/HF ratio** | 5.18 ± 0.39 | 5.59 ± 1.52 | 0.7678 |
| **Total power ($ms^2$)** | 994.70 ± 147.00 | 630.9 ± 151.8 | 0.1111 |
| **RESP (Hz)** | 0.27 ± 0.01 | 0.31 ± 0.02 | 0.4517 |
| **SD1 (ms)** | 12.08 ± 0.96 | 9.14 ± 1.55 | 0.1138 |
| **SD2 (ms)** | 34.77 ± 2.19 | 28.34 ± 2.97 | 0.0964 |
| **SD2/SD1 ratio** | 2.91 ± 0.08 | 3.310.26 | 0.1200 |
| **Stress index** | 11.29 ± 0.55 | 13.09 ± 0.61 | 0.0459* |
| **PNS index** | −2.01 ± 0.08 | −2.80 ± 0.22 | 0.0023* |
| **SNS index** | 2.12 ± 0.18 | 4.01 ± 0.54 | 0.0024* |

* Indicates significant differences between groups; **SC**: successful completion; **FTQ-GA**: failure to qualify following irregular gait; **RR**: beat-to-beat interval; **HR**: heart rate; **SDNN**: standard deviation of normal-to-normal RR intervals; **RMSSD**: root mean square of successive RR interval differences; **pNN50**: relative number of successive RR interval pairs that differ by more than 50 msec; **TINN**: triangular interpolation of normal-to-normal intervals; **DC**: deceleration capacity of heart rate computed as a four-point difference; **DCmod**: modified DC computed as a two-point difference; **VLF**: HRV very-low-frequency band, by default 0–0.04 Hz; **LF**: HRV low-frequency band, by default 0.04–0.15 Hz; **HF**: HRV high-frequency band, by default 0.15–0.4 Hz; **RESP**: respiration rate; **SD1**: standard deviation of the Poincaré plot perpendicular to the line of identity; **SD2**: standard deviation of the Poincaré plot along the line of identity **PNS**: parasympathetic nervous system; **SNS**: sympathetic nervous system.

 

in FTQ-GA than in SC horses during the entire ride. These results indicate that horses demonstrated reduced vagal activity – coinciding with increased sympathetic activity – during exertion in the endurance rides. Compared with horses completing the ride, an overall reduction in several HRV parameters indicated a greater reduction in vagal activity – causing a shift toward more sympathetic dominance – in horses that were eliminated due to irregular gait.

The rides at the two venues were planned for dates with suitable weather conditions to minimise any compromise to horse welfare, according to the FEI rules [6,41]. Although the experiments were conducted in two different places, the health conditions of horses were similar before the rides. This conclusion was supported by no differences in haematological, HR, or HRV variables between SC and FTQ-GA horses at the pre-ride measurement (Tables 2–5). Moreover, environmental conditions (humidity: $P = 0.4145$; temperature: $P = 0.3347$) and total ride distances did not differ between the venues. In addition, this study was conducted in novice endurance rides, in which the speed of horses was limited to a maximum of 16 km/h according to the FEI rules. Based on these conditions, horses were supposed to be of similar status and ridden in the same condition in the two events.

Concerning the riding speed and related race outcome, previous reports demonstrate that horses eliminated because of gait abnormality display higher speed in the first phase of the race in 80–160 km competitions [42,43]. The risk of gait abnormality is likely associated with high-speed riding on the track [42]. However, this notion contrasted with the present study's result, as the horses passing or failing the veterinary inspection showed similar speeds in the first riding phase. A possible reason for this disparity is that the former reports investigated Concours de Raid d'Endurance International (CEI) star-level events, which involve a race against time; the higher speeds involved in such events might have resulted in speed-related gait abnormalities. On the other hand, the present study was carried out in novice qualification rides, wherein the speed is restricted to no more than 16 km/h [6], meaning that speed-related gait abnormalities were unlikely to be involved in horses in the first riding phase. Although there was no difference in average speed in horses passing the inspection of both riding phases, the horses failing the second phase's inspection because of irregular gait displayed lower riding speeds compared with their speeds in the first riding phase (Supplementary File 4: Fig. S4) or of the horses that passed the inspections in both the first and the second riding phase (Table 1). This result suggests that the reduced speed was likely associated with the gait irregularity for which the horses were eliminated from the course, and that limb discomfort persisting during the second riding phase caused the horses to decrease their speed to cope with the discomfort.

In this study, horses demonstrated increased physiological stress during exertion, primarily indicated by increased Hct and CK levels after the ride ended. As reported previously, haematological parameters – including Hct, RBC, Hb and leucocytes – increase post-exercise in thoroughbred racehorses [44] and endurance horses [16]. Likewise, increased Hct and Hb were detected in horses midway and at the end of 100 km endurance rides [45]. Accordingly, increases in Hct in the present study may be attributed to dehydration during the ride [45,46], as well as catecholamine release following exercise, leading to the release of splenic erythrocytes and consequent enhancement of red blood cell parameters [44]. In addition to Hct, a rise in CK levels after the ride suggested increased muscle permeability due to muscle stress during exercise in horses in both groups, paralleling a previous report [47]. However, several haematological parameters demonstrated different responses between horses with distinct riding outcomes.

In this study, increases in PCV, RBC, WBC, neutrophils, and lymphocytes were only detected in SC horses, not FTQ-GA horses. These results contrast with previous reports showing no differences in PCV, Hb, or RBC in horses finishing or failing a ride [14,16]. It is plausible that the previous reports compared the modulation in haematological parameters between horses finishing the ride and all horses that failed it with physical and metabolic disorders rather than only due to their gait. In addition, the previous studies experimented with horses participating in 80 km [14] or 160 km [16] endurance rides, which could exert more substantial effects on body homeostasis than those experienced by the horses in this study. Accordingly, these factors might cause a disparity in haematological results between studies. In the present study, the higher average speeds in SC than FTQ-GA horses may be the underlying cause of increased dehydration in SC horses. This circumstance led to haemoconcentration and greater elevation of haematological parameters post-exercise. In addition, pronounced catecholamine release under sympathetic influence might have compounded haemoconcentration in SC horses and further increased the PCV and RBC.

Apart from haematological parameters, a marked decrease in multiple HRV parameters – including time domain variables (RR interval, SDNN, RMSSD, pNN50, RR triangular index and DCmod), frequency domain variables (VLF, LF and total power spectra), nonlinear variables (SD1 and SD2), and the PNS index – subsequently supported the evidence of increased stress during the exercise in endurance horses, similar to reports described elsewhere [48–50]. In addition, increases in the LF/HF and SD2/SD1 ratios, the parameters that indicate sympathovagal balance [23,51], also supported the evidence of increased sympathetic activity and, in turn, greater stress during exertion in the endurance ride. However, corresponding to haematological modification, autonomic modulation also differed between horses with different riding outcomes.

FTQ-GA horses ran with more stress than SC horses during the first riding phase. This finding was indicated by a higher HR, coinciding with a lower RMSSD and SD1, in FTQ-GA horses, indicating a shift toward more sympathetic activity in these horses during the first riding period. The lower SDNN, LF power spectrum, and SD2 in FTQ-GA horses also supported the notion of more pronounced sympathetic activity in these horses during the first riding phase. Despite no different in HF between the groups during the first riding period, a significantly decreased HF power spectrum in FTQ-GA horses can confirm, in part, a greater reduction in vagal activity in FTQ-GA than in SC horses in this period. However, SDNN, LF power spectrum, and SD2 returned to the baseline values in FTQ-GA horses but decreased in SC horses. This circumstance led to higher HRV – reflecting more vagal activity – in FTQ-GA horses during the first phase's compulsory halt period. It is plausible that SC horses, which ran with similar speed but more comfort than FTQ-GA horses during the first riding phase, may still be alert for further riding during the compulsory halt period. In contrast, the FTQ-GA horses were expected to transiently relax after riding with discomfort in the first riding phase, causing higher HRV than SC horses in this compulsory halt period. A higher HR in FTQ-GA horses during the compulsory rest period could support the evidence of high stress in FTQ-GA horses after the first riding phase. In the second riding period, FTQ-GA horses ran at a lower speed than in the first phase. They might have reduced their speed to compensate for discomfort during the second riding phase. This circumstance led to reduced mean HR – corresponding with increased HRV – in FTQ-GA horses and, thereby, no difference in the parameters between groups during the second riding phase (Tables 3 and 5). Nevertheless, the higher TINN and RR triangular index, coinciding with a lower stress index, were marked in FTQ-GA horses compared with SC horses, indicating predominant vagal activity and transient relaxation in FTQ-GA horses during the second phase's compulsory halt period, similar to those observed during the first phase's compulsory halt period.

Regarding the overall HRV in the novice endurance rides, lower HRV variables (RR interval, RR triangular index, VLF power spectrum, Dcmod, and PNS index) – corresponding to higher HR, stress index and SNS index – reflected that FTQ-GA horses demonstrated a lower vagal activity than SC horses during the rides. These results suggested that FTQ-GA horses suffered more stress or discomfort. DCmod is a measure of vagal modulation of the heart rate and is computed as the two-point difference [52]. A decrease in the DC index is associated with postmyocardial infarction, leading to sudden cardiac arrest and death in humans [53]. Changes in the VLF power spectrum accompany physical activity, thermoregulation, renin-angiotensin activation, and vasomotor tone [54]. PNS activity may also affect the modification of the VLF power spectrum [55]. A low VLF power spectrum has been reported to be associated with arrhythmic death in humans [56]. Accordingly, the reductions in overall VLF and DCmod in FTQ-GA horses implied reduced capacity of vagal modulation compared with SC horses and may, at least in part, indicate negative influences on the heart in the FTQ-GA horses. Further investigations are needed to determine the acceptable values of HRV variables as criteria for indicating gait abnormality in endurance horses. Although differences in HRV modulation were observed between horses with SC and FTQ-GA outcomes, questions arise as to whether horses who have different outcomes following higher-category or longer-distance events also display similar modifications in HRV. In addition, an investigation of changes in HRV in horses eliminated from endurance rides because of metabolic issues might be insightful.

The main limitation of the present study is that blood samples could be collected only before the ride and during the compulsory rest period of the first and second riding phases. This limitation occurred because the riders were concerned that the horses might be affected if blood samples were withdrawn during active periods of the ride. Since the data were collected from horses participating in competitive events, the number of those with different outcomes was not evenly distributed across both venues. In addition, compulsory halt periods could not be evenly scheduled for all horses because of the time constraints of the ride. Thus, the comparisons of autonomic regulation during the ride and changes in HRV parameters during the compulsory halt periods should be interpreted with care. In addition, HRV parameters after the second riding phase could not be obtained because horses were returned to their stables for post-riding care or to undergo veterinary examination for gait abnormalities. Hence, the autonomic responses during the rest period after the second riding phase were scarce.

## 5. Conclusions

Horses showed reduced HRV during exertion in novice endurance rides. A lower HRV – mirroring reduced vagal activity – in FTQ-GA horses during the first riding period and the entire rides suggested that FTQ-GA horses experienced more stress or discomfort than SC horses. These results could raise awareness about potential stress or discomfort in horses being eliminated from the event due to irregular gait. More research must be conducted to investigate whether or not an additional out-of-competition period is required for horses failing to qualify following irregular gait, in order to preserve horse welfare in endurance rides.

## Ethics approval

The use of animals in this study was approved by the Kasetsart University's Institute of Animal Care and Use Committee (ACKU65-VET-003).

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## Data availability statement

HRV data of horses are available at https://www.doi.org/10.6084/m9.figshare.25877353.

## CRediT authorship contribution statement

**Onjira Huangsaksri:** Writing – original draft, Visualization, Validation, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Kanokpan Sanigavatee:** Writing – original draft, Visualization, Validation, Software, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Chanoknun Poochipakorn:** Writing – original draft, Visualization, Validation, Software, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Thita Wonghanchao:** Writing – original draft, Visualization, Validation, Software, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Mona Yalong:** Writing – original draft, Visualization, Validation, Software, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Khunanont Thongcham:** Writing – original draft, Visualization, Validation, Software, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Chanikarn Srirattanamongkol:** Writing – original draft, Visualization, Validation, Software, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Siwakorn Pornkittiwattanakul:** Visualization, Resources, Investigation, Formal analysis, Data curation, Conceptualization. **Thanawan Sittiananwong:** Visualization, Resources, Investigation, Formal analysis, Data curation, Conceptualization. **Burasarin Ithisariyanont:** Visualization, Resources, Investigation, Formal analysis, Data curation, Conceptualization. **Chatrapee Pakpokai:** Visualization, Resources, Investigation, Formal analysis, Data curation, Conceptualization. **Supanut Ninyeeruae:** Visualization, Resources, Investigation, Formal analysis, Data curation, Conceptualization. **Metha Chanda:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

## Declaration of generative AI and AI-assisted technologies in the writing process

The authors did not use any artificial intelligence-assisted technologies in the writing process.

## Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Metha Chanda reports article publishing charges was provided by Kasetsart University Faculty of Veterinary Medicine Kamphaengsaen Campus. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

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