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HPA Axis Dynamics in Standardbred Trotters: Differential ACTH and Cortisol Responses to Training Versus Racing Across Age and Sex

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ABSTRACT

The hypothalamic-pituitary-adrenal (HPA) axis is a key neuroendocrine pathway responsible for the adaptive stress response triggered by physical exertion. Exercise acts as a major challenge to physiological balance, stimulating the concurrent secretion of ACTH and cortisol depending on the exercise features in sport horses. Considering this, the present study aimed to assess fluctuations in circulating adrenocorticotropic hormone and cortisol levels in Standardbred trotters following both training and race efforts, while accounting for age and sex differences. Specifically, it sought to quantify the extent of ACTH and cortisol elevation during maximal exertion in racing and to contrast hormonal responses under two exercise intensities: routine training and competitive performance. Ten healthy Standardbred horses (three mares and seven stallions) participated in two distinct conditions—non-competitive training and subsequent official racing. Among them, four were 2 years old and six were 3 years old. Both training and racing produced significant effects on ACTH ($p < 0.01$) and cortisol ($p < 0.01$) levels. Relative to training, racing caused markedly higher ACTH concentrations at rest ($p < 0.001$), and at 5 ($p < 0.01$) and 30 minutes ($p < 0.001$) post-exercise, while resting cortisol levels were lower ($p < 0.01$) following the race. After racing, both 2- and 3-year-olds displayed increased ACTH levels at 5 and 30 minutes ($p < 0.01$), and males exhibited elevated ACTH values at the same time points ($p < 0.01$). The differing contexts and intensities of the two exercise conditions appear to influence the HPA axis response directionally, reflecting both adaptive capacity to variable physical stress and preparatory adjustments to stimuli. In summary, training and racing elicited distinct HPA axis activations, wherein emotional arousal and physical maturity seemed to shape adaptive outcomes. Since ACTH and cortisol levels in mature horses exhibit wide variability, further research is necessary to clarify the factors modulating these endocrine patterns and their function as indicators of stress adaptation in equines.

Keywords: Standardbred, ACTH, Cortisol, Training, Racing

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Introduction

Exercise represents a physiological stressor that activates numerous endocrine mechanisms, allowing the body to re-establish a dynamic internal equilibrium and sustain homeostasis [1]. Among these, the hypothalamic-pituitary-adrenal (HPA) axis plays a central role by triggering adaptive hormonal responses that influence mechanical, metabolic, cardiovascular, and behavioral systems [2]. Studies in equine athletes and other species have clarified how HPA activity modulates both physiological and behavioral adjustments to stress. Cortisol, the main hormone produced by this axis, enhances hepatic gluconeogenesis and promotes lipolysis, thereby supplying energy substrates during prolonged or moderate workloads. Consequently, ACTH and cortisol together reflect the body's anabolic-catabolic balance, typically rising in response to physical strain or fatigue [3]. Physical exertion thus acts as a potent disruptor of equilibrium, inducing parallel secretion of ACTH and cortisol depending on the exercise's type, intensity, and duration in performance horses [4-7], as well as in dogs and humans [8-10].

Variables such as individual conditioning [11], age [12], novelty of the challenge [13], and prior competitive experience [14] may all alter the magnitude of ACTH and cortisol release. The quantification of HPA hormones has therefore become an important marker for assessing fitness, performance level [15-20], and overtraining in sport horses [12, 18-28], human athletes [9, 29], and particularly in Standardbred trotters [30-32]. It has been reported that prolonged exercise tends to elevate cortisol concentration, whereas brief, low-intensity activity produces minimal or no changes [33-35]. Over the last decade, attention has increasingly turned to assessing salivary cortisol as a non-invasive tool to evaluate equine stress during exercise and race training [36-38]. Numerous investigations have employed this method to examine ways of mitigating stress and quantifying cortisol through saliva sampling [36, 37, 39-42]. Strzelec *et al*. [36], for instance, found that cortisol decreased following dressage, with exercise intensity having no significant influence on its level, implying that moderate-duration exercise can reduce stress. Thus, variations in gait intensity—such as walking, trotting, or light cantering—do not appear to markedly alter stress responses. Nevertheless, further exploration is needed to elucidate the mechanisms underlying adaptive hormonal regulation across diverse athletic disciplines.

Based on these observations, it was proposed that hormonal patterns may differ between non-competitive training and competition, and that exercise intensity, age, and sex could affect the direction of the HPA response. Consequently, this research aimed to determine how ACTH and cortisol concentrations vary in 2- and 3-year-old male and female Standardbred horses during racing, and to compare circulating profiles between training and competitive conditions.

Materials and Methods

Animals

This study was conducted in compliance with Italian law (D.L. 04/3/2014 n. 26) and the EU Directive 2010/63/EU on the use of animals in research, and was approved by the Animal Ethics Committee of the University of Messina (Approval No. ME08/2023).

Ten Standardbred racehorses, consisting of three females and seven males, participated in the study. Four of the horses were 2 years old, while six were 3 years old, with an average body weight of 384 ± 42 kg (range: 331-485 kg). Horses were housed individually in stalls with wood shavings and allowed 5 hours of free exercise in a sand paddock each day. Their diet consisted of approximately 8-10 kg of grass per horse and 2 kg of commercial pelleted feed, split into two meals at 08:00 and 14:30. The feed was formulated according to the 1989 NRC requirements, with the concentrate providing 3.08 Mcal/kg digestible energy and 18% crude protein, and the hay containing 2 Mcal/kg digestible energy and 7% crude protein.

Measurements of height at the withers and body weight were performed for each horse. Body condition was scored on a 1-9 scale (1 = severely underweight, 9 = obese) as described by Henneke *et al*. [43]. Horses maintained stable body mass within 2-3% of baseline for at least two weeks prior to the study, with BCS ranging from 5 to 7. Water and salt blocks were available at all times, and all horses remained clinically healthy throughout the study.

Prior to the exercise sessions, horses underwent thorough physical and orthopedic evaluations. No signs of illness were observed. All animals were actively in training, and although the horse-driver pairs varied in competition experience, each had previously participated in national-level events.

The experiments were conducted in spring at the "La Favorita" racetrack in Palermo, Sicily (38°07′55″ N, 13°20′08″ E, 46 m above sea level), which features a 1000 m oval track. Environmental conditions were consistent across sessions, with a firm, dry track, cool sunny weather, and no wind. Air temperatures measured between 14:00 and 16:30 ranged from 17 to 21.5 °C, and relative humidity varied from 40.5% to 55.5%. Across study days, temperatures ranged from 18 to 20 °C, relative humidity was 40-60%, and wind speed ranged from 4 to 6 m/s.

Physiological parameters recorded included heart rate (HR), respiratory rate (RR), and rectal temperature (RT). Heart rate was continuously monitored using a Polar S710i™ pulsimeter (Polar Electro Oy, Kempele, Finland) and additionally recorded at rest, immediately post-exercise, and at 15 and 30 minutes of recovery using a stethoscope. Respiratory rate was measured at rest, within two minutes of completing a 500 m sprint, and at 15 and 30 minutes post-exercise. Rectal temperature was measured with a digital thermometer (Microlife AG, Widnau, Switzerland) at the same time points.

Horses were subjected to two exercise protocols: a non-competitive training session (exercise 1) and, three days later, a competitive race (exercise 2). Both sessions occurred between 14:00 and 17:30, and the same driver rode each horse for both conditions.

Table 1. Exercise Protocols

Training	Racing
Two rounds: strenuous training: velocity 8-10 m/s; duration 5 min	Two rounds: strenuous training: velocity 8-10 m/s; duration 5 min
1600 m: sprint training: velocity 10-12 m/s; duration 2.40 min	1600 m: sprint training: velocity 15-17 m/s; duration 1.58 min
Two rounds: basic training: velocity 5-8 m/s; duration 10 min	One round: basic training: velocity 5-8 m/s; duration 10 min
Cool down at the pass: duration 10 min	Cool down at the pass: duration 10 min

The training session consisted of two rounds of "strenuous training" on the racetrack at 8-10 m/s for 5 minutes, followed by a 1600 m "sprint training" at 10-12 m/s. The session concluded with two rounds of "basic training" at 5-8 m/s. Speed was monitored by the driver using trackside markers. After completion, horses returned to their stalls for a 10-minute walking cool-down. The total training session lasted 30 minutes, including the cool-down.

The racing session involved a 1600 m competitive effort. Horses began with a 5-minute warm-up at 8-10 m/s, including two sprint rounds. During the race, drivers encouraged maximum exertion, with peak speeds of 15-17 m/s at the start and finish phases. The session concluded with a "basic training" round at 5-8 m/s, followed by a 10-minute walking cool-down. Total duration of the race session, including cool-down, was 30 minutes.

Blood sampling and hormone analyses

Blood was collected from the jugular vein in 10 mL tubes before each exercise session at 14:00 (baseline), 5 minutes post-exercise (pre-cool-down, 17:00), and 30 minutes post-exercise (including cool-down, 17:30). Horses were familiar with handling, so no restraint was necessary, and informed consent was obtained from owners.

Plasma ACTH concentrations were measured in unextracted plasma using a commercial radioimmunoassay (ELSA-ACTH, CIS-BioInternational, Gif-sur-Yvette, France) in duplicate. The assay range was 0-440 pmol/L, with a sensitivity of 0.44 pmol/L. Intra- and interassay coefficients of variation were 6.0% and 15.0%, respectively.

For cortisol analysis, blood samples were centrifuged at 3000 × g for 15 minutes, and serum was stored at −20 °C until measurement. Total serum cortisol was determined in duplicate using a competitive enzyme immunoassay (Roche Diagnostics GmbH, Mannheim, Germany) on an automated analyzer (BRIO, SEAC, Rome, Italy). In the assay, the sample cortisol competed with horseradish peroxidase-conjugated cortisol for antibody binding. Unbound material was removed via washing, and the bound enzyme activity was inversely proportional to cortisol concentration. Signal was developed using tetramethylbenzidine substrate, and absorbance was read at 450 and 405 nm (Sirio S, SEAC, Florence, Italy). Assay sensitivity was 5 ng/mL, with intra- and interassay CVs of 4% and 6.9%, respectively. Samples were processed immediately on-site and stored at −20 °C, with analysis performed within one week.

Statistical analysis

All numerical results are reported as mean ± standard deviation (S.D.) based on duplicate measurements and expressed with their corresponding international units (UI). Data normality was verified using the Kolmogorov-Smirnov test.

To investigate the influence of age, sex, and exercise type on hormone levels relative to baseline, a one-way ANOVA was performed. For physiological variables measured repeatedly over time, a two-way repeated measures ANOVA (Two-way RM ANOVA) was applied to assess the effects of the training and racing sessions, as well as their interaction. Where significant differences were detected, Tukey's post-hoc test was used for pairwise comparisons. A p-value of less than 0.05 was considered statistically significant. All analyses were conducted using GraphPad PRISM software (version 10.3.0, GraphPad Software Inc., San Diego, CA, USA).

Additionally, percentage changes (Δ%) were calculated to compare post-exercise values with resting baselines, and racing session values with training session values.

Results and Discussion

Average ACTH and cortisol concentrations (mean ± S.D.) are shown in **Tables 2-4** and **Figures 1-3**.

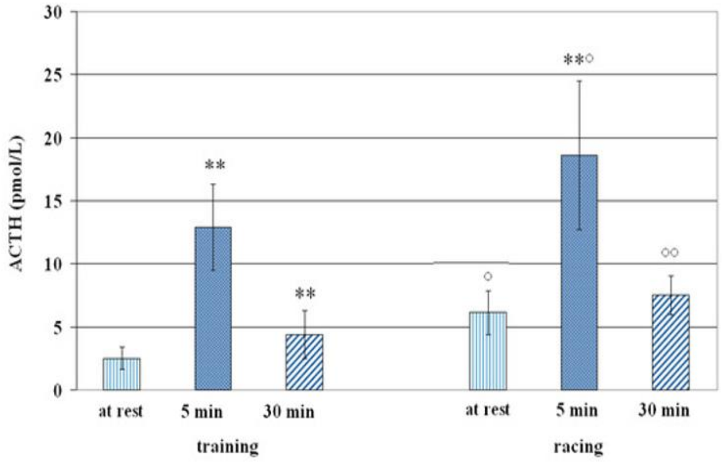
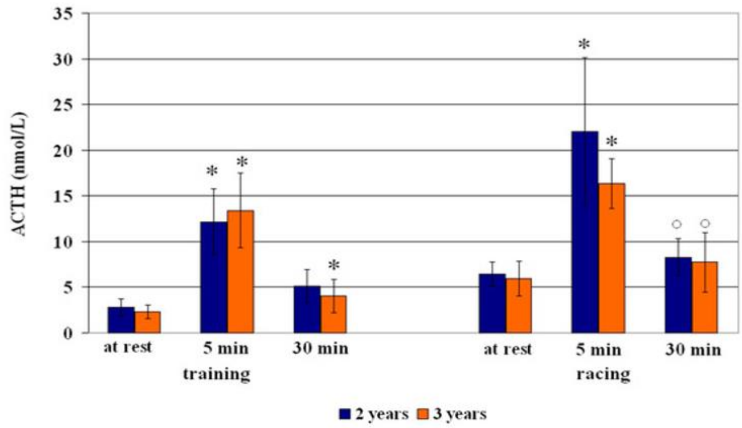


Figure 1. Circulating ACTH (a) and cortisol (b) in Standardbreds before and after training and racing. Symbols indicate statistical significance versus resting (*) or training sessions (°).



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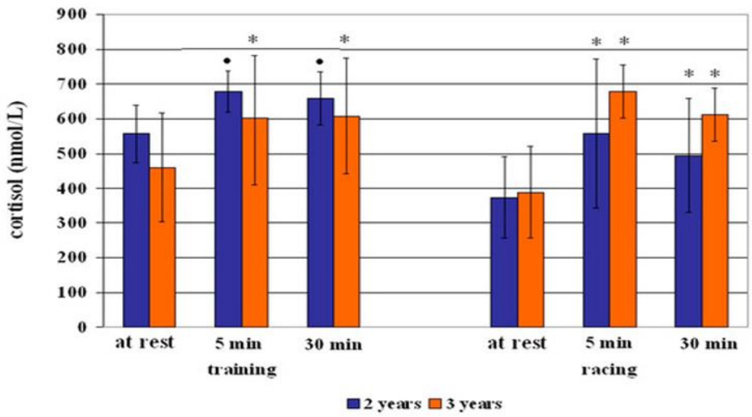

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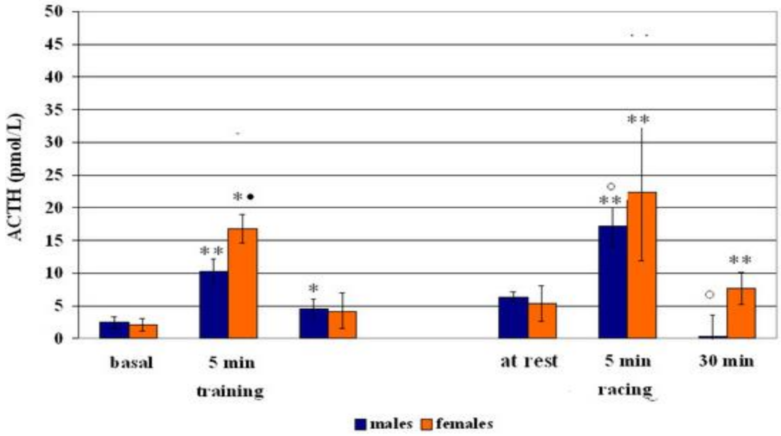
Figure 2. ACTH (a) and cortisol (b) in 2- and 3-year-old horses across exercise sessions. Symbols show significant differences versus baseline (*), training (°), or age group (•).



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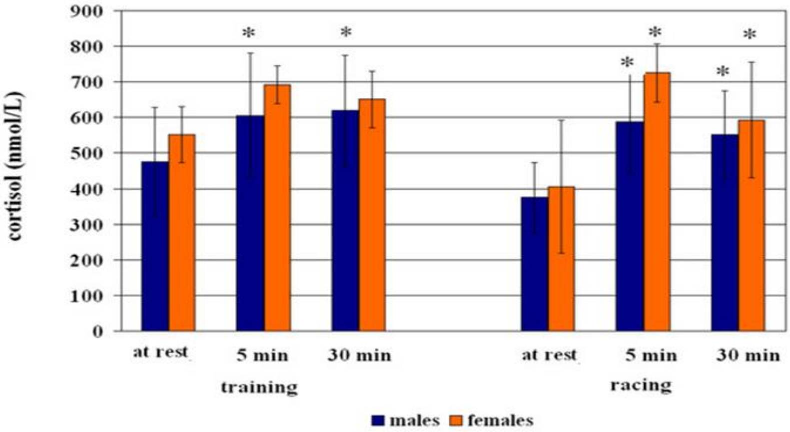

b)

Figure 3. ACTH (a) and cortisol (b) in male and female horses. Symbols denote significant differences versus rest (*), racing session (°), or sex (•).

Table 2. ACTH and cortisol values for training sessions.

	ACTH (pmol/L)				
	at Rest	5 min	Δ%	30 min	Δ%
Total	2.50 ± 0.89	12.91 ± 3.43 b	+416	4.40 ± 8.66 b	+76
2-year-old	2.78 ± 0.95	12.13 ± 3.66 a	+336	5.10 ± 1.80	+83
3-year-old	2.31 ± 0.78	13.40 ± 4.06 a	+480	4.04 ± 1.83 a	+75
males	2.48 ± 0.89	10.28 ± 1.89 b	+314	4.51 ± 1.51 a	+82
females	2.11 ± 0.94	16.81 ± 2.21 aB	+697	4.19 ± 2.72	+98
	cortisol (nmol/L)				
Total	498 ± 136	632 ± 151 b	+27	629 ± 133 b	+26
2-year-old	555 ± 82	678 ± 59 A	+22	659 ± 76 A	+19
3-year-old	460 ± 158	601 ± 190 a	+31	608 ± 165 a	+32
males	475 ± 153	606 ± 175 a	+27	619 ± 156 a	+30
females	552 ± 79	692 ± 54	+25	651 ± 79	+18

Letters mark differences versus baseline (a: $p < 0.01$; b: $p < 0.001$), age (A: $p < 0.01$), and sex (B: $p < 0.01$).

Table 3. ACTH and cortisol during racing sessions.

		|ACTH (pmol/L)			
Category	at Rest	Δ%	5 min	Δ%	30 min

Total	6.13 ± 7.79 C	+203	18.61 ± 26.82 Cb	+23	7.52 ± 1.54 CD
2-year-old	6.43 ± 6.13	+243	22.05 ± 8.10 Ca	+29	8.29 ± 2.0 C
3-year-old	5.92 ± 8.70	+176	16.32 ± 2.74 C	+31	7.74 ± 3.28 C
males	6.30 ± 3.52	+172	17.15 ± 3.05 bC	+32	8.35 ± 3.25 C
females	5.34 ± 12.35	+68	22.29 ± 10.41 b	+44	7.67 ± 2.41 b
cortisol (nmol/L)					
Category	at Rest	Δ%	5 min	Δ%	30 min
Total	383 ± 121 C	+64	629 ± 141 b	+47	565 ± 126 a
2-year-old	375 ± 118	+48	57 ± 215 a	+32	494 ± 165 a
3-year-old	389 ± 132	+74	678 ± 77	+57	612 ± 77
males	375 ± 99	+56	587 ± 143 a	+47	552 ± 123 a
females	405 ± 187	+79	725 ± 82 a	+46	593 ± 162 a

Letters indicate differences versus baseline (a, b) and training session (C, D).

Table 4. HR, RR, and RT (mean ± S.D.) at rest, post-exercise, and during recovery (15 and 30 min).

Parameter	At Rest	End	15 min	30 min
Training				
HR (beats/min)	38 ± 10	180 ± 20 a	80 ± 15 a	65 ± 5 a
RR (breaths/min)	18 ± 8	80 ± 5 a	75 ± 10 a	40 ± 10 a
RT °C	37.5 ± 0.3	38.6 ± 0.3 a	38.5 ± 0.2 a	37.8 ± 0.4
Racing				
HR (beats/min)	39 ± 11	189 ± 30 a	84 ± 10 a	55 ± 5 a
RR (breaths/min)	18 ± 3	80 ± 5 a	78 ± 10 a	45 ± 8 a
RT °C	37.6 ± 0.3	39.2 ± 0.4 a	38.6 ± 0.3 a	37.8 ± 0.4

Letters indicate significance versus resting values ($p < 0.05$).

Hormonal response to training

ACTH and cortisol responses observed in this study are in line with previously reported equine data, with minor deviations likely due to methodological differences in hormone assays [4, 5, 6, 26-28, 44-46].

During training, ACTH increased sharply relative to baseline, with a +414% rise at 5 minutes ($p < 0.001$) and a +76% rise at 30 minutes ($p < 0.001$). Cortisol also rose significantly, with +27% at 5 minutes ($p < 0.001$) and +66% at 30 minutes ($p < 0.001$) **(Table 2, Figure 1)**. Statistical analysis confirmed a significant effect of training on ACTH ($F = 55.70$; $p < 0.01$) and cortisol ($F = 27.70$; $p < 0.01$).

Age-related differences: 2-year-old horses showed ACTH increases of +336% at 5 min ($p < 0.01$) and +83% at 30 min ($p < 0.01$). For 3-year-olds, ACTH rose by +480% at 5 min ($p < 0.01$) and +75% at 30 min ($p < 0.01$). Training significantly influenced ACTH in both age groups (2-year-olds: $F = 28.73$; $p < 0.01$; 3-year-olds: $F = 29.80$; $p < 0.01$).

Cortisol changes were significant in 3-year-olds, increasing by +31% at 5 min and +32% at 30 min ($p < 0.01$). The effect of training on cortisol was significant only in 3-year-olds ($F = 39.57$; $p < 0.01$). Interestingly, 2-year-olds had higher cortisol values than 3-year-olds at both 5 and 30 minutes ($p < 0.01$).

Sex-related differences: Males exhibited ACTH rises of +314% at 5 min ($p < 0.001$) and +82% at 30 min ($p < 0.01$). Females showed even higher ACTH elevations, +697% at 5 min ($p < 0.01$) and +98% at 30 min ($p < 0.01$). Training significantly affected ACTH in both sexes (males: $F = 47.30$, $p < 0.01$; females: $F = 10.66$, $p < 0.01$), with females showing the highest ACTH at 5 minutes ($p < 0.01$).

Cortisol concentrations increased in males by +27% at 5 min and +30% at 30 min ($p < 0.01$), with training exerting a significant effect on male cortisol ($F = 19.02$; $p < 0.01$) **(Table 2, Figure 1)**.

Hormonal response during racing

After completing the racing session, ACTH concentrations showed a sharp rise from baseline, increasing by +203% at 5 minutes ($p < 0.001$) and +23% at 30 minutes ($p < 0.01$). Cortisol levels followed a similar trend, with +64% at 5 minutes ($p < 0.001$) and +47% at 30 minutes ($p < 0.01$) compared to resting values **(Table 3, Figure**

2). Statistical evaluation indicated that the racing session had a significant impact on both ACTH ($F = 42.17$; $p < 0.01$) and cortisol ($F = 16.45$; $p < 0.01$) concentrations.
When considering age, the younger horses (2-year-olds) exhibited a +243% increase in ACTH at 5 minutes ($p < 0.01$), while the older horses (3-year-olds) showed a +176% rise ($p < 0.001$), both compared to baseline. The effect of the racing session was statistically significant in 2-year-olds ($F = 21.47$; $p < 0.01$) and in 3-year-olds ($F = 31.41$; $p < 0.01$) **(Table 3, Figure 2)**.
Cortisol also increased in both age categories. In 2-year-olds, levels rose by +48% at 5 minutes and +32% at 30 minutes ($p < 0.01$), whereas in 3-year-olds, rises of +74% and +57% were observed at the same time points ($p < 0.01$). The influence of racing on cortisol was significant in both younger ($F = 14.35$; $p < 0.01$) and older horses ($F = 7.75$; $p < 0.01$).
Sex-related patterns were apparent as well. Male horses had an ACTH increase of +172% at 5 minutes ($p < 0.001$), while females showed +68% at 5 minutes and +44% at 30 minutes ($p < 0.001$) relative to resting values **(Table 3, Figure 2)**. These changes were statistically significant for males ($F = 49.95$; $p < 0.01$) and females ($F = 10.66$; $p < 0.01$).
Cortisol followed a comparable pattern. In males, concentrations increased +56% at 5 minutes and +47% at 30 minutes ($p < 0.01$), whereas females experienced rises of +79% at 5 minutes and +46% at 30 minutes ($p < 0.01$) **(Table 3, Figure 2)**. Significant effects of racing were detected in both sexes (males: $F = 9.24$; $p < 0.001$; females: $F = 27.87$; $p < 0.01$).

Comparison between racing and training
Relative to training sessions, ACTH levels were higher in racing at rest ($p < 0.001$), at 5 minutes ($p < 0.01$), and at 30 minutes ($p < 0.001$). Cortisol was lower after racing only at rest ($p < 0.01$). Both age groups showed higher ACTH at 5 and 30 minutes post-race ($p < 0.01$), and males specifically had greater ACTH levels at the same time points following racing ($p < 0.01$) **(Table 3, Figure 2)**.

Functional parameters
Both heart rate (HR) and respiratory rate (RR) were elevated immediately following training and racing exercises and remained above baseline values at 15 and 30 minutes post-exercise ($p < 0.05$). Rectal temperature (RT) increased at the end of exercise and at 15 minutes, returning to resting levels by 30 minutes of recovery ($p < 0.05$) **(Table 3)**.
The current findings demonstrate that endocrine responses to exercise vary depending on the type of activity, age, and sex of the horses. ACTH and cortisol levels increased following both training and racing sessions, supporting previous reports in athletic horses where these hormones peaked between 5 and 30 minutes after exercise cessation in Thoroughbreds [23, 47], Warmbloods [16], and show-jumping horses [4, 25].
Baseline measurements taken before racing indicated ACTH and cortisol were +145% and +38% higher, respectively, than before the training session. This anticipatory elevation likely reflects psychological arousal related to the imminent race, highlighting the role of emotional and temperament-related processes in influencing performance outcomes [48].
Post-race ACTH concentrations at 5 and 30 minutes were 44% and 70% higher than corresponding values after training, whereas cortisol displayed smaller increases of 7% and 19% at the same time points. This pattern aligns with observations by Nagata *et al*. [23], who reported peak ACTH at 5 and 30 minutes following incremental treadmill exercise in Thoroughbreds, demonstrating greater sensitivity of ACTH to exercise intensity than duration.
Our results also correspond with studies showing anticipatory responses in Standardbred racehorses, with pre-training cortisol of 97.3 ± 16.4 nmol/L versus pre-race cortisol of 171.8 ± 18.7 nmol/L [49]. Participation in competitions activates the hypothalamus-adrenocortical axis and sympathetic nervous system, reflected in transient cortisol elevation and increased heart rate [26].
In this study, training speeds were approximately 10-12 m/s, while racing reached 15-17 m/s. Kurosawa *et al*. [22] reported that the timing and magnitude of ACTH increases during treadmill exercise are closely linked to exercise intensity, supporting its role as a stress and performance biomarker. Our data confirmed that the HPA axis response in Standardbreds scaled with exercise intensity, as racing elicited higher ACTH levels at baseline, 5, and 30 minutes post-exercise compared to training.

Previous research also indicated that ACTH responses correlate with blood lactate levels [23] and circulating arginine vasopressin during incremental exercise [13]. In Thoroughbreds, increases in ACTH at 5 and 30 minutes after racing are consistent with results from standardized treadmill exercise tests (SET) [16], whereas submaximal treadmill exercises in young horses did not modify ACTH concentrations [20].

In young Friesian horses, dressage training elicited significant increases in circulating ACTH, but cortisol levels remained unchanged post-training [28]. Physical activity clearly stimulated ACTH release, yet increases in cortisol did not always parallel ACTH changes, suggesting differential regulation of the two hormones in response to mental stress during exercise [25].

The pronounced ACTH rise observed after the first training session indicates that novel stimuli provoke an early stress response in horses, resulting in heightened arousal, particularly on the initial training day. These findings corroborate earlier studies demonstrating that exposure to unfamiliar exercise stimuli increases plasma ACTH in Thoroughbreds [13].

Participation in both competitive and non-competitive race activities induced activation of the hypothalamic-pituitary-adrenal (HPA) axis, reflected by a temporary elevation in cortisol secretion. Typically, cortisol release is triggered endogenously following stimulation by ACTH, and during riding, this response is at least partially driven by the physical exertion imposed on the horse. Consistent with prior findings, cortisol levels increased significantly following both training and racing sessions, indicating that moderate exercise, such as the training protocol used here, as well as high-intensity exercise like racing, can stimulate cortisol release [49].

Previous studies have reported elevated cortisol in response to incremental physical exertion [19], as well as after a variety of equestrian events, including cross-country competitions [49], endurance training, events for Arabian horses [50], and standardized exercise tests (SET) on tracks [51]. In endurance races, post-exercise cortisol levels have been shown to rise by 100-200% relative to baseline values [25] Ferlazzo, 2012. In our study, cortisol levels measured at 5 and 30 minutes post-racing were consistent with prior observations of cortisol increases in trotters following physical activity [52] Lindner, 2002. Although the type of exercise varied, cortisol trends 5 minutes after racing were similar to those after training, paralleling findings in both horses and riders during dressage sessions [53].

Cortisol recovery was most efficient during the training session. After racing, cortisol concentrations remained elevated at 30 minutes, resembling the 5-minute post-exercise values, likely due to the combined emotional and physical demands of competitive racing. Previous research in trotters reported cortisol elevations persisting for 40-60 minutes post-race [54], and in Standardbreds, cortisol often decreases 4-8 hours after exercise, returning to baseline roughly 24 hours later [11]. In show-jumping horses, animals competing over higher fences exhibited slower cortisol recovery, with 30-minute post-exercise levels exceeding those measured at 5 minutes [25]. Furthermore, significant cortisol increases before and after show-jumping events were observed in horses previously transported, with post-competition rises correlating more with exercise than transport stress [6]. Rivera *et al*. [55] recorded peak cortisol at 15 minutes post-endurance training, which declined by 75 minutes.

In our study, circadian effects on ACTH and cortisol fluctuations can be ruled out, as all blood samples were collected consistently in the afternoon (15:00-17:30). It is known that exercise can modify normal circadian cortisol rhythms, which peak around 10:50 a.m. in horses [56]. Similarly, in humans, the cortisol response is influenced by time of day, and failing to account for circadian patterns may lead to erroneous conclusions about exercise-induced hormonal responses [57].

Considering age, 2-year-old horses exhibited lower ACTH and cortisol at 5 minutes post-training, and lower cortisol at 5 and 30 minutes post-training, consistent with previous SET studies in Standardbreds on dirt tracks [51]. Interestingly, although not statistically significant, younger horses tended to have higher cortisol at all post-race time points compared to post-training, possibly reflecting cumulative fatigue from both sessions. Younger animals may expend more energy than older horses, which likely possess greater exercise adaptation and recovery capacity due to prolonged training experience.

Comparable trends have been observed in Arabian racehorses, where cortisol gradually increased across successive training sessions in the racing season, and peak performance correlated with lower cortisol levels [7]. Similar adaptation of the HPA axis is reported in human runners: trained athletes exhibited smaller cortisol increases under incremental exercise, whereas untrained controls showed higher cortisol at each intensity level, with significant elevation only occurring after exhaustive exercise [20].

Age and the level of training were shown to positively affect locomotion parameters in Thoroughbreds, as well as the metabolic demands associated with competitive exercise [58]. Moreover, existing literature indicates that 2-

year-old Thoroughbred racehorses exhibit higher cortisol concentrations than their older counterparts [17]. This suggests that younger horses may have a more responsive HPA axis due to limited experience in competitive racing, with the novelty of the event producing a greater emotional stress response [13].

Even though all horses in this study were adults, it is well established that factors such as age, genetics, and environmental conditions can reduce the HPA axis's responsiveness. Aging can alter hormonal reactions typically activated to manage physical exertion, affecting cardiovascular regulation, metabolism, and substrate utilization [59]. In fact, studies on Standardbred mares demonstrated that the functional efficiency of the HPA axis declines with age, although the underlying mechanisms remain unclear. Nevertheless, structured exercise training may mitigate these age-related deficits [30].

Regarding sex differences, no significant variations in ACTH concentrations were observed between males and females during either training or racing sessions. Cortisol levels were generally lower in males than in females at all measured time points and following both exercise sessions, although these differences were not statistically significant. The mechanisms behind sex-related differences in HPA axis modulation in response to exercise remain uncertain, warranting further study.

Limitations of the study

A notable limitation of this investigation is the small number of horses completing the protocol, primarily due to unforeseen events during the experiment. Ten potential participants were excluded due to retirement from racing, while another ten were omitted because trainers declined to allow their horses to perform the study exercises. Another constraint was the absence of continuous heart rate monitoring to assess speed, trot parameters, acceleration, and total distance covered, which are critical for evaluating fitness and performance. Additional limitations include the reliance on plasma cortisol measurements instead of a non-invasive salivary method and the lack of other stress indicators such as lactate, PCV, and hematocrit. These methodological choices were dictated by restrictions imposed by race authorities and owners during a national competition, which allowed only a single plasma sample per horse to measure both hormonal parameters.

Conclusion

Exercise intensity, differing between training and competitive contexts, influenced the direction of HPA axis responses, which may impact horses' adaptability to physical stress. Literature indicates that multiple factors can modify ACTH and cortisol concentrations. Basal ACTH levels are particularly sensitive to pre-analytical variables, and even low-intensity exercise can elicit significant hormonal increases within 30 minutes. To optimize athletic performance in horses, further research is necessary to evaluate how variables such as emotional experience, age, sex, and sample handling influence the ability to cope with exercise-induced stress.

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