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# Influence of repeated treadmill exercise on quality and freezability of stallion semen

F. Janett [a,*], C. Burkhardt [b], D. Burger [b], I. Imboden [b], M. Hässig [a], R. Thun [a]

[a] *Clinic of Reproduction, University of Zürich, Winterthurerstrasse 260, CH-8057 Zürich, Switzerland*
[b] *National Stud, Avenches, Switzerland*

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**Abstract**

The objective of this study was to investigate changes of quality and freezability of stallion semen in response to repeated acute treadmill exercise. Ejaculates from 11 stallions were collected, evaluated and frozen weekly during four periods of 4 weeks each defined as before (period 1), during (period 2) and after (periods 3 and 4) intense exercise. In fresh semen the gel-free volume, sperm concentration, motility, normal sperm and sperm with major defects (acrosome defects, nuclear vacuoles, abnormal heads, midpiece defects and proximal droplets) were evaluated. In frozen–thawed semen, motility as well as viability (SYBR-14/PI) were examined. In period 2, all stallions were exercised on an indoor high speed treadmill twice a week (total of eight sessions) using an incremental workload test. Heart rate was monitored telemetrically during exercise and blood samples were taken for determination of cortisol, testosterone and lactate. Results of our investigation demonstrate that heart rate and the plasma concentrations of cortisol, testosterone and lactate significantly ($P < 0.05$) increased during each exercise session. Furthermore, significantly more major sperm defects were present in periods 3 ($69.5 \pm 2.1\%$) and 4 ($66.8 \pm 2.1\%$) than in periods 1 ($62.2 \pm 2.4\%$) and 2 ($62.5 \pm 2.2\%$). Acrosome defects increased towards the end of exercise but improved 3 weeks later to values observed before exercise. In frozen–thawed semen, motility was significantly lower in period 2

* Corresponding author. Tel.: +41 44 635 8218; fax: +41 44 635 8942.
*E-mail address:* fjanett@vetclinics.unizh.ch (F. Janett).


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(45.4 ± 2.3%) compared to period 4 (51.6 ± 1.7%) and viability was significantly lower in period 2 (49.2 ± 2.0%) than in periods 1 (53.8 ± 2.1%) and 4 (53.7 ± 1.6%). Our results clearly demonstrate that in the stallion repeated strenuous treadmill exercise can negatively influence semen quality and freezability.


*Keywords:* Stallion; Semen; Stress; Exercise; Treadmill

## 1. Introduction

The use of artificial insemination in equine reproduction has been increasing worldwide. To maximize the efficiency of preserved semen it is imperative to obtain high quality ejaculates. Due to the great variation in semen quality between and within stallions, factors affecting quality should carefully be controlled. Performance in equestrian sport is considered one of the major selection criteria for breeding. However, due to a stallion's sporting career, semen may often be collected only during short periods of time between competitions or training, factors which may have a great influence on sperm quality and freezability.

Stress-induced suppression of the reproductive capacity in both humans and animals is a well-known phenomenon [1,2]. Hormones of the hypothalamic-pituitary-adrenal (HPA) axis secreted in response to stress may influence all three levels of the hypothalamic-pituitary-gonadal (HPG) axis. An important role is played by CRH, which in conjunction with β-endorphin can directly inhibit the secretion of GnRH in the hypothalamus [2,3]. Furthermore, the CRH-mediated increase in ACTH secretion leads to increased levels of circulating glucocorticoids. These have been proven to have an inhibitory effect on both the hypothalamus and the pituitary gland in many in vitro and in vivo studies in a variety of species including humans [4], rhesus monkeys [5], cattle [6], sheep [7,8] and rats [9]. A direct inhibitory effect of glucocorticoids on testosterone was described in an in vitro study of rats [10] and an in vivo study in humans [11]. In contrast to the influence of chronic stress, which decreases testosterone production, acute stressors have a much less predictable influence on testosterone concentrations, with authors reporting no changes [12–14], decreases [15] or even increases [16–22] in testosterone. The manner in which an organism reacts to stress is not only influenced by the type, duration and intensity of the stressor; it also depends on the presence of circulating sexual steroid hormones [23–26].

There are a few studies investigating the effect of stress on reproductive function in the stallion. The acute stress of teasing or mating leads to an increase in circulating cortisol concentrations [27,28] and to a short-term increase in GnRH, LH and FSH levels [29]. Baker et al. [30] found significantly higher values of cortisol but lower values of testosterone in stressed race horses performing below the expectations than in those performing at the expected level. Repeated semen analysis in 2-year-old stallions showed that regular exercise on the lunge in walk and trot during 16 weeks had no influence on semen quality [31]. Lange et al. [32] however, recorded significantly better sperm motility

in breeding stallions competing on a regular basis than in those used only for breeding. Similar results were obtained by Davies Morel and Gunarsson [33] who found significantly higher fertility in intensively trained Icelandic stallions, when compared to moderately trained or untrained stallions. The ability of stress to influence semen quality has also been known to humans [34–36]. The increasing training level of marathon runners has been shown to lower sperm motility and density and to increase the number of immature and abnormal sperm [35,37]. Because of well-designed controlled studies about the effect of intense physical stress on semen quality, are lacking, the aim of the present investigation is to evaluate the influence of standardized, repeated treadmill exercise on the quality and freezability of stallion semen.

## 2. Materials and methods

### 2.1. Animals

Experiments were performed using 11 healthy Franches–Montagnes stallions, aged between 7 and 19 years, from the National Stud Farm in Avenches (Switzerland). They were kept in box stalls bedded with straw and were fed hay, oats and pellets supplemented with minerals.

### 2.2. Treadmill exercise

Four months before the start of the experiment all stallions were introduced and adapted to exercise on an indoor high speed treadmill (Mustang 2200, Kagra AG, Fahrwangen, Switzerland) and a submaximal incremental workload test was established by monitoring heart rate and plasma lactate concentrations. During the exercise period of 4 weeks, each stallion was worked on the treadmill twice weekly (total of eight exercise sessions). The first two exercise sessions consisted of 5 min walk (1.5 m/s) at 0% inclination followed by two intervals of 7 min trot (3.5 m/s) at inclinations of 3% and 6%, respectively. Exercise sessions 3–8 consisted of intervals of 3 min trot (3.5 m/s) and 1 min walk (1.5 m/s) and sequentially increased inclination from 0% to 9%. At the maximal inclination of 9%, the trotting speed was further increased to 4 and 4.5 m/s. Exercise was stopped immediately when the stallion showed signs of exhaustion and could not maintain treadmill speed. The exercise tests were carried out at an ambient temperature of 12.0–18.5 °C and a relative humidity of 60–90%. A ventilator (Isler Bioengineering AG, Zürich, Switzerland) placed in front of the treadmill ensured that the horses received enough fresh air (4500 $m^3$/h) during exercise.

### 2.3. Heart rate

Heart rate was monitored during exercise sessions by telemetry electrocardiography (Polar Horse Trainer Advanced™, Polar Electro Oy, Kempele, Finnland). Values immediately before beginning and after completion of each exercise session were considered for analysis.

### 2.4. Hormone and lactate determination

Blood samples (EDTA) from all stallions were obtained by jugular venipuncture immediately before and after treadmill exercise. The samples were then centrifuged ($3000 \times g$, 10 min) and plasma frozen ($-18$ °C) until analysis. Cortisol and testosterone were determined by radioimmunoassay [38,39]. The sensitivity of the assays was 0.1 ng/mL for testosterone and 0.5 ng/mL for cortisol. Plasma lactate was measured by an enzymatic assay (EnzytecTM D/L-Lactic Acid Kit, Scil Diagnostics GmbH, Martinsried, Germany) with a detection limit of 0.3 mmol/L plasma.

### 2.5. Semen collection, processing and examination

One week before the start of the study, extragonadal sperm reserves of all stallions were minimized by five daily semen collections. From September to December, ejaculates were collected, evaluated, and cryopreserved weekly and the experimental time divided in period 1 (4 weeks before exercise), period 2 (4 weeks during exercise) and periods 3 and 4 (4 weeks each after exercise). After removal of the gel fraction (in-line gel filter), the volume (graduated cylinder) and the concentration (spectrophotometer) were estimated. Progressive motility was assessed with a Mika Motion Analyzer (Cell Motion Analyzer SM-CMA-1074, MTM, Switzerland) in fresh diluted semen using standardized threshold values for stallion semen [40]. For morphological examination five drops of fresh semen were fixed in 2 mL Hancock solution and smears prepared [41]. At least 200 spermatozoa were evaluated by phase contrast microscopy (Olympus BX50, UplanFl 100x/1.30) and abnormal spermatozoa classified in major and minor defects [42,43]. The gel-free semen was diluted with centrifugation medium (INRA 82-Hepes + 2% (v/v) egg yolk) to a concentration of $100 \times 10^6$ spermatozoa/mL and centrifuged at $1000 \times g$ for 2 min. After centrifugation, the supernatant was removed and the sperm pellet resuspended in freezing medium (75 mL lactose solution 11% (w/v), 20 mL egg yolk, 5 mL glycerol) to a final concentration of $300 \times 10^6$ spermatozoa/mL. Thereafter, semen was cooled to 4 °C during at least 30 min, packaged into 0.5 mL straws and frozen in an automatic freezer (Minidigitcool 700 ZB 290, IMV, France) at a cooling rate of 60 °C/min from + 4 C° to $-100$ C° and 30 °C/min from $-100$ C° to $-140$ °C. The straws were then stored in liquid nitrogen until examination. From each ejaculate three straws were thawed (37 °C, 30 s), pooled and diluted with 3 mL centrifugation medium to determine the motility and viability. Viability was performed by dual DNA staining (LIVE/DEAD® Sperm Viability Kit, Molecular Probes Europe, Leiden, NL) using SYBR-14 in combination with propidium iodide (PI). SYBR-14 (component A) was diluted with anhydrous dimethyl sulfoxide (DMSO) 1:10 (SYBR-14 working solution), while PI (component B) was used in original concentration. Two microliter of the SYBR-14 working solution was added to 1 mL of diluted thawed semen. After incubation for 10 min at 37 °C, 5 μL of component B was added. Five minutes later, 5 μL of stained semen was placed on a slide, covered with a coverslip (24 mm × 24 mm) and examined using fluorescence microscopy (Olympus BX50, UPlanApo 40x/0.85, FITC filter U-MWIB, high pressure Hg-lamp). Different sequences of fluorescence stained spermatozoa were monitored by connection of a video camera

(SANYO VCC-2972) to the microscope. At least 500 sperm cells were counted on the screen and the percentage of green fluorescing sperm was defined as viability [44].

### 2.6. Statistical analysis

Data were analyzed using StatView 5.0 software program (SAS Institute, Switzerland). The effect of each exercise session (before and after) and of the session number (1–8) on heart rate and plasma concentrations of cortisol, testosterone and lactate were analyzed by ANOVA with repeated measures. Values determined before and after exercise were compared by paired *t*-test. To determine changes in semen quality parameters during periods 1–4 a multivariate analysis of variance (ANOVA) with repeated measures was used. Period means of semen quality parameters were compared with Fisher's PLSD post hoc test. Data are presented as mean ± S.E.M. Statistical significance was based on $P < 0.05$.

## 3. Results

### 3.1. Heart rate

Mean heart rate of all stallions before and after each exercise session is shown in Fig. 1a. The average values before exercise ranged from 70.4 ± 5.2 to 97.4 ± 5.5 beats per minute (bpm) and increased to values between 166.8 ± 2.3 and 194.0 ± 2.8 bpm just after

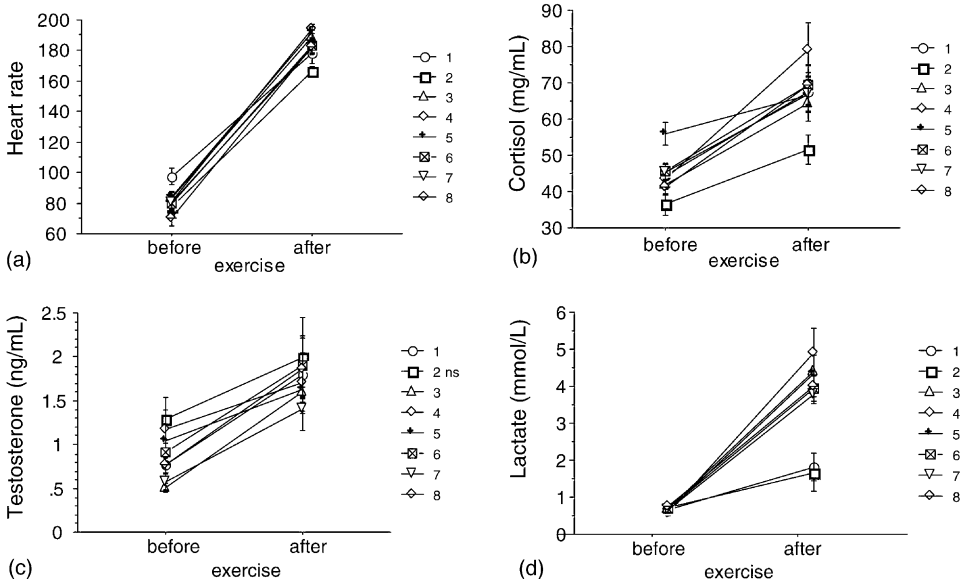


Fig. 1. Mean (±S.E.M.) of heart rate (a) and plasma concentrations of cortisol (b); testosterone (c); and lactate (d) before and after exercise sessions 1–8 in 11 stallions; ns: not significant, $P > 0.05$.

Table 1
Effect of exercise (before and after) and exercise session (1–8) on heart rate and plasma concentrations of cortisol, testosterone and lactate in 11 stallions

| | Exercise, *P*-value | Session, *P*-value |
|---|---|---|
| Heart rate | <0.0001* | 0.0232* |
| Cortisol | <0.0001* | 0.0364* |
| Testosterone | <0.0001* | 0.2870 |
| Lactate | <0.0001* | <0.0001* |

*Significant at $P < 0.05$.

completion of the session. The increase in heart rate during exercise was significant in each of the eight sessions ($P < 0.05$) and a significant influence of the session number was present (Table 1).

### 3.2. Cortisol

Mean plasma cortisol concentrations of the 11 stallions before and after the eight exercise sessions are shown in Fig. 1b. The values ranged from 36.5 ± 2.9 ng/mL to 56.0 ± 3.2 ng/mL before and from 51.6 ± 4.0 ng/mL to 79.1 ± 7.5 ng/mL after exercise. Both, sampling time (before and after) and session number (1–8) significantly influenced cortisol values (Table 1) and a significant increase in cortisol was present in all eight exercise sessions.

### 3.3. Testosterone

Fig. 1c shows mean plasma testosterone concentrations of all stallions before and after exercise. Average values at rest ranged between 0.5 ± 0.1 ng/mL and 1.3 ± 0.2 ng/mL and increased to concentrations between 1.4 ± 0.2 ng/mL and 2.0 ± 0.5 ng/mL after each exercise session. Plasma testosterone was significantly influenced by sampling time (before and after exercise) but not by session number (Table 1). In all sessions except number 2, testosterone was significantly increased after exercise.

### 3.4. Lactate

Mean plasma lactate concentrations are shown in Fig. 1d. All values before exercise were lower than 0.8 mmol/L. After exercise, values of session 1 (1.8 ± 0.4 mmol/L) and 2 (1.7 ± 0.5 mmol/L) were lower than those in the remaining 6 sessions in which concentrations ranged between 3.8 ± 0.3 mmol/L and 4.9 ± 0.6 mmol/L. Both, sampling time and session number significantly influenced lactate concentrations (Table 1) and a significant increase was observed in all eight exercise sessions.

### 3.5. Semen characteristics

The effects of the periods 1–4 on semen characteristics assessed by ANOVA are presented in Table 2. Significant changes were noticed for the parameters major defects, acrosome

Table 2
Effect of period (1–4) on quality parameters in fresh and frozen–thawed semen in 11 stallions

| Parameter | Period, *P*-value |
|---|---|
| Volume | 0.9381 |
| Concentration | 0.2576 |
| Total sperm | 0.5369 |
| Motility fresh semen | 0.1614 |
| Normal sperm | 0.6586 |
| Major sperm defects | 0.0007* |
| Acrosome defects | 0.0380* |
| Nuclear vacuoles | 0.0360* |
| Motility frozen–thawed semen | 0.0399* |
| Viability frozen–thawed semen | 0.0392* |

* Significant at $P < 0.05$.

defects, nuclear vacuoles in fresh semen as well as motility and viability in frozen–thawed semen. Comparison of mean values between the four periods are summarized in Table 3. In fresh semen motility was significantly higher in period 2 (78.1 ± 2.1) than in period 4 (73.9 ± 2.2) and the percentage of major defects in periods 1 (62.2 ± 2.4) and 2 (62.5 ± 2.2) was significantly lower than in periods 3 (69.5 ± 2.1) and 4 (66.8 ± 2.1). Acrosome defects showed a significant increase from period 2 (6.3 ± 0.9) to 3 (8.1 ± 1.1) and dropped again in period 4 (6.1 ± 0.8). Acrosome defects continuously increased towards the end of exercise, peaked (10.2 ± 2.6) 2 weeks later and thereafter steadily declined to initial values (Fig. 2). The percentage of sperm with nuclear vacuoles significantly increased between period 1 (16.8 ± 1.6) and periods 3 (22.0 ± 1.5) and 4 (21.7 ± 2.2). In frozen–thawed semen, the motility was significantly higher in period 4 (51.6 ± 1.7) than in period 2 (45.4 ± 2.3) and the viability was significant lower in period 2 (49.2 ± 2.0) than in periods 1 (53.8 ± 2.1) and 4 (53.7 ± 1.6). The lowest mean viability (43.6 ± 3.4) was seen 1 week after the end of exercise but values recovered within 2 weeks (Fig. 3).

Table 3
Mean (±S.E.M.) of semen characteristics during periods 1–4 in 11 stallions

| | Period 1 (mean ± S.E.M.) | Period 2 (mean ± S.E.M.) | Period 3 (mean ± S.E.M.) | Period 4 (mean ± S.E.M.) |
|---|---|---|---|---|
| Volume (mL) | 26.4 ± 2.6 | 24.6 ± 3.0 | 24.3 ± 2.2 | 24.7 ± 2.5 |
| Concentration ($\times 10^6$/mL) | 312.7 ± 17.5 | 328.5 ± 17.4 | 307.8 ± 18.2 | 328.9 ± 20.1 |
| Total sperm ($\times 10^9$) | 7.0 ± 0.5 | 6.8 ± 0.6 | 6.1 ± 0.4 | 6.6 ± 0.5 |
| Motility fresh semen (%) | 77.9 ± 2.3$^{ab}$ | 78.1 ± 2.1$^{a}$ | 76.9 ± 1.8$^{ab}$ | 73.9 ± 2.2$^{b}$ |
| Normal sperm (%) | 23.7 ± 2.0 | 24.0 ± 2.0 | 22.1 ± 1.9 | 23.3 ± 1.6 |
| Major sperm defects (%) | 62.2 ± 2.4$^{a}$ | 62.5 ± 2.2$^{a}$ | 69.5 ± 2.1$^{b}$ | 66.8 ± 2.1$^{b}$ |
| Acrosome defects (%) | 7.2 ± 1.0$^{ab}$ | 6.3 ± 0.9$^{a}$ | 8.1 ± 1.1$^{b}$ | 6.1 ± 0.8$^{a}$ |
| Nuclear vacuoles (%) | 16.8 ± 1.6$^{a}$ | 19.9 ± 1.6$^{ab}$ | 22.0 ± 1.5$^{b}$ | 21.7 ± 2.2$^{b}$ |
| Motility frozen–thawed semen (%) | 48.5 ± 2.3$^{ab}$ | 45.4 ± 2.3$^{a}$ | 48.7 ± 2.3$^{ab}$ | 51.6 ± 1.7$^{b}$ |
| Viability frozen–thawed semen (%) | 53.8 ± 2.1$^{a}$ | 49.2 ± 2.0$^{b}$ | 50.4 ± 2.0$^{ab}$ | 53.7 ± 1.6$^{a}$ |

Means with different letters (a, b) indicate significant difference ($P < 0.05$).

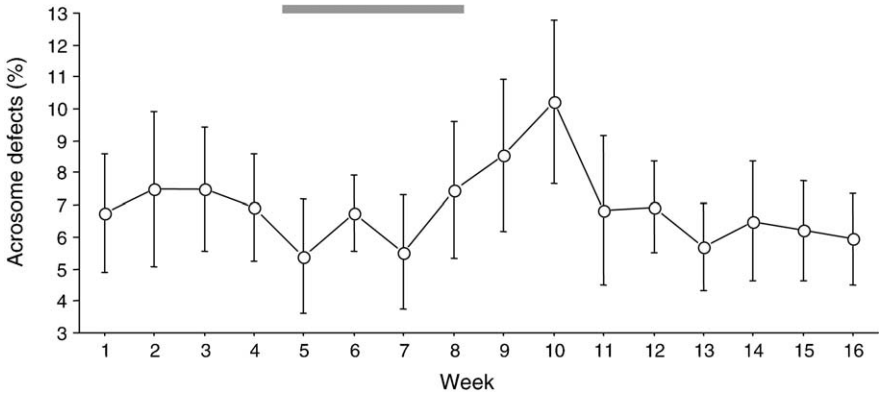


Fig. 2. Mean (±S.E.M.) of acrosome defects before, during (▬) and after treadmill exercise in 11 stallions.

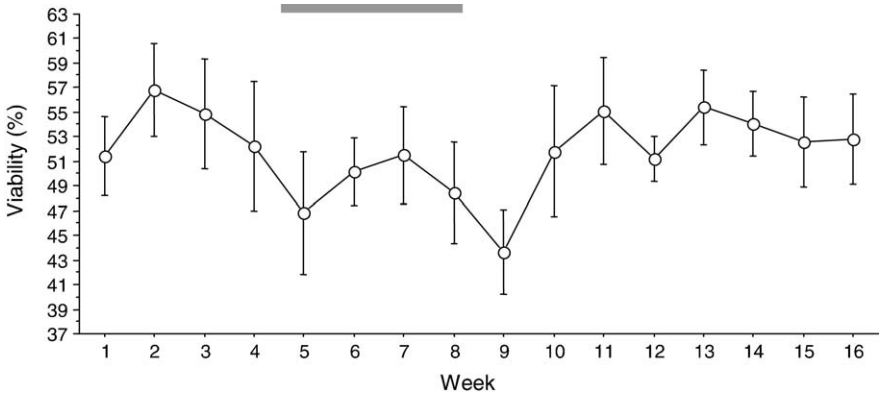


Fig. 3. Mean (±S.E.M.) of sperm viability in frozen–thawed semen before, during (▬) and after treadmill exercise in 11 stallions.

## 4. Discussion

Results of this study demonstrate that strenuous treadmill exercise caused a significant increase in heart rate and plasma cortisol, testosterone and lactate concentrations. Furthermore, the quality of fresh and frozen–thawed semen was negatively influenced by repeated exercise sessions over a period of 4 weeks.

The use of an equine high speed treadmill enables a standardized evaluation of physical stress on various body functions. The exercise protocol used in this study proved to be an effective means of examining the effects of stress on reproductive function. Most importantly, the manipulation of treadmill inclination allowed us to exercise the stallions to a level of anaerobic metabolism avoiding excessive speeds [45]. Numerous studies in horses [46–52] have shown that heart rates above 160 bpm are accompanied by an exponential rise in blood lactate concentrations, indicating the transition from aerobic to

anaerobic metabolism. The anaerobic threshold of plasma lactate concentrations occurs at 3–4 mmol/L and is known as onset of blood lactate accumulation (OBLA). This lactate level was achieved in all stallions during exercise sessions 3–8 but was lower in sessions 1 and 2 when stallions where acclimatized to treadmill exercise.

Before exercise, the heart rates showed high variation but significantly increased to mean values between 166.8 and 194.0 after exercise, indicating that exercise sessions represented a considerable stress for the untrained stallions. The big variation in heart rates before exercise and the gradual decrease towards the end of the 4-week exercise period demonstrate that heart rates before exercise were mainly influenced by psychic stress and environmental factors [53,54] suggesting that the stallions grew accustomed to the treadmill.

Mean plasma cortisol concentrations also showed a significant rise during exercise. Values ranged between 36.5 and 56.0 ng/mL before and increased to levels between 51.6 and 79.1 ng/mL plasma after stress. In a previous study [55] plasma cortisol measured in warmblood horses increased by at least 50 ng after a treadmill exercise of 20–30 min duration and continuous treadmill exercise for 2.5 h resulted in high cortisol concentrations between 110.9 and 195.5 ng/mL plasma [56]. When compared to distinct cortisol elevations after physical stress, much smaller levels were observed after transport for several hours [57,58]. These differing results lead us to conclude that a stress induced rise in plasma cortisol is not only dependent on the intensity, but also on the type and the duration of the stressor.

With the exception of only one exercise session (session 2) mean testosterone levels also rose significantly during exercise. Similar to cortisol, the rise in plasma testosterone is probably also caused by the stress-induced release of ACTH, stimulating testosterone secretion from the adrenal cortex. An increase of testosterone after treadmill exercise has also been observed in dogs [22] and in men [12]. In humans, it is presumed that the rise of testosterone is not primarily due to higher secretion but to an overall decrease in plasma volume [12] accompanied by lower metabolization rate of testosterone in the liver [59]. A rise in testosterone secretion after application of exogenous ACTH has been observed in mares [60] and in boars [18].

Besides the stress-mediated activation of the HPA-axis, an activation of the HPG-axis must also be considered. In the stallion [29] but not in the bull [14] sexual stimulation has been shown to temporarily increase plasma LH and testosterone. However, in cyclic cows a 2 h immobilization stress increased plasma LH [25] and in rodents subjected to various types of stressors also responded with an elevation of LH and sexual steroid hormones [16–19,61].

Most importantly, the results of our study demonstrate that repeated strenuous treadmill exercise had a negative effect on the quality of fresh and frozen–thawed stallion semen. The evaluation of fresh semen revealed that the percentage of major defects, particularly acrosome defects and nuclear vacuoles, started to rise 3 weeks after the beginning of the exercise period (period 2). Towards the end of the study (period 4), the acrosome defects had normalized to values observed before stress (period 1). In contrast, the percentage of nuclear vacuoles did not improve until the end of the study what might be explained by seasonal effects. In a previous study we have shown that nuclear vacuoles in Franches–Montagnes stallions were highest during winter months [62]. Motility in fresh semen was highest during the exercise period but surprisingly viability and motility in

frozen–thawed semen were at their lowest during exercise. The impairment of semen quality commencing 3 weeks after treadmill exercise was started and the beginning of improvement 3 weeks after the end of exercise suggest that physical stress most likely had a negative influence on spermiogenesis, where differentiation of spherical spermatids to spermatozoa takes place. The adverse effect of physical stress might be the result of elevated glucocorticoid levels or increased body temperature compromising testicular thermoregulation during strenuous exercise. Direct or indirect action of glucocorticoids on spermatogenesis has been observed in bulls [63] and damage to spermatogenic cells is also known to occur by increased testicular temperature in the stallion [64] and other species [65–67]. In contrast to our findings, Dinger et al. [31] found that regular exercise on the lunge in walk and trot did not influence semen quality in warmblood stallions. Lange et al. [32] even found improved sperm motility in warmblood stallions when competing on a regular basis compared to stallions only engaged in breeding activities. Icelandic stallions have also been shown to have significantly better fertility when intensively trained rather than moderately trained or untrained [33]. These contradictory results are difficult to compare as intensity of exercise was either minimal [31] or not standardized [32,33] and semen quality [33] or sperm morphology [32] were not evaluated and freezability was never tested.

In conclusion, we have found that repeated strenuous exercise on a high speed treadmill leads to a distinct stress reaction in stallions with significant increases in heart rate as well as in plasma cortisol, testosterone and lactate concentrations. Furthermore, repeated physical stress during 4 weeks can compromise the quality of fresh and frozen–thawed semen during and up to 1 month after exercise.

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