ENDOCRINOLOGICAL AND BEHAVIOURAL ADAPTATIONS TO EXPERIMENTALLY INDUCED PHYSICAL STRESS IN HORSES

De Graaf-Roelfsema, Ellen.
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ENDOCRINOLOGICAL AND BEHAVIOURAL ADAPTATIONS TO EXPERIMENTALLY INDUCED PHYSICAL STRESS IN HORSES

ENDOCRINOLOGISCHE EN ETHOLOGISCHE AANPASSINGEN AAN EXPERIMENTEEL GEÏNDUCEERDE FYSIEKE STRESS IN PAARDEN

(met een samenvatting in het Nederlands)

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Ellen Roelfsema

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Promotor:	Prof. dr. A. Barneveld
Co-promotoren:	dr. H.A. Keizer dr. J.H. van der Kolk

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Voor mijn ouders,
Martin & Sanne

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Chapter 1

General Introduction

Introduction

In modern society, horses encounter a lot of different stressors due to intensive management, lack of social contact, and, probably the most important one, exercise. The horse is a natural born superb athlete and used extensively for this talent. The culmination of exercise stress can lead to the overtraining syndrome (OTS), a stress-related disorder seen in highly trained (race) horses. However, also horses involved in daily less intensive exercise can suffer from the OTS (37). Exercise stress is not only induced by too much physical exercise. It is a culmination of several different stressors, including physiological, environmental, social and psychological (179). Examples of such stressors are training monotony, high number of competitions, transportation etc. Therefore, it is very likely that a degree of overtraining occurs more frequently than is recognized in horses so far (20), simply because owners and veterinarians are not familiar with the syndrome or associate the syndrome with the highly trained sport horses. Well-known stress-related symptoms are the gastric ulcer syndrome and stereotype behaviours shown by horses that adapted to the stressor or a boring situation.

It is known that uncontrollable, unpredictable, and constant stress has far-reaching consequences on physical and mental health of our horses, and thus their welfare. This underlines the urge for finding parameters of early overtraining in order to detect OTS and other stress-related syndromes in horses in early stages. The following two sections will give a short overview of the phenomenon of stress and a description of the overtraining syndrome. This will be followed by sections on the three main topics studied in this thesis in relation to the OTS, namely a section on the regulation of the GH-IGF-1 axis as well as the influences of stress on this axis, a section on the regulation of glucose metabolism during exercise and at rest, and the influences of stress, and a section on stress induced behaviour in horses.

Stress

Stress-related syndromes are discovered more and more. In man, 70% to 80% of all illnesses seen in medical offices are either caused by stress or worsened by it. Burn out and posttraumatic stress disorder are stress-related diseases everyone has heard about.

Because of the overabundance of stress in our modern lives, we usually think of stress as a negative experience. But from a biological point of view, stress can be neutral, negative, or positive as is shown by the Yerkes-Dodson law (Figure 1) (364). It shows the relation between stress levels and performance. For optimal performance a certain level of stress is necessary.

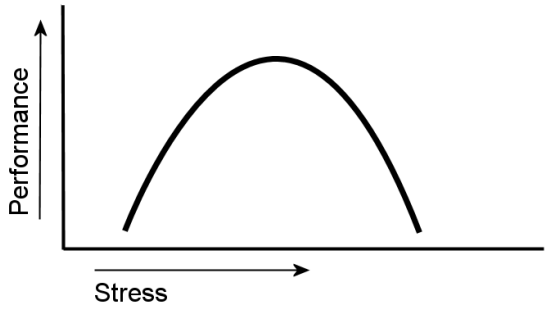

Figure 1. Yerkes-Dodson law

Definition of stress

In general, stress occurs when body homeostatic balance is disturbed (100, 287, 288, 291). When the body experiences environmental or internal stressors it responds with secreting a whole array of hormones to reestablish homeostatic balance. Regenerative processes continue after restoration of homeostatic balance such that, if the same stressor was imposed again, the homeostatic mechanisms would not be displaced to the same extent, resulting in overcompensation (289). The overcompensation is seen as positive stress.

Individuals can experience stress differently, because of their genetic make-up. Some people like challenges and enjoy the accompanying tension (stress), while others experience such challenges as threatening. When stress is not in someone's interest and is seen as a bad experience, it is called negative stress or distress.

The normal stress response

When the homeostatic balance is disturbed, the two main stress-related hormonal axes are activated: the sympathetic-adrenal medullary (SAM) axis and the hypothalamic-pituitary-adrenocortical (HPA) axis (9).

The brain handles the immediate response via activation of the SAM axis. This response signals the adrenal medulla to release epinephrine. The hypothalamus and the pituitary gland initiate the slower, maintenance response via the HPA axis. This response signals the adrenal cortex to release cortisol and other hormones. Many neural circuits are involved in the behavioural response. This response increases arousal, focuses attention, inhibits feeding and reproductive behaviour, reduces pain perception, and redirects behaviour. An anabolic phase follows with a higher adaptive capacity and enhanced performance capacity, in which both the GH-IGF-I axis as well as the gonadal-axis are activated (320). The massive activation of the sympathetic nervous system permits an individual to perform far more strenuous physical activity

than would otherwise be possible. The sympathetic nervous system operates by increasing the heart rate and blood pressure, redirecting blood flow to the heart, muscles, and brain and away from the gastro-intestinal tract, and releasing fuel (glucose and fatty acids) to respond immediately. It is also called the *fight or flight reaction* because a person or an animal in this state decides almost instantly whether to stand and fight or to run (16). In either event, the sympathetic alarm reaction makes the individual's subsequent activities vigorous.

The combined results of these three components of the stress response (SAM, HPA and neural circuits) maintain the internal balance (homeostasis), increase energy production and utilization, and alter electrolyte and fluid balance (124).

Prolonged/chronic stress

When homeostatic balance is not restored, the body experiences chronic stress which induces chronic activation of the endocrine system and possibly ending in a neuroendocrine disorder (stress-related disorder) like for instance burn out, chronic fatigue syndrome, post-traumatic stress disorder or overtraining syndrome. Stressors can be mental (e.g., emotional problems) or physical (e.g., overloading/overtraining) which is reflected in the variety of problems experienced by different individuals suffering from stress-related disorders like social workers, soldiers or athletes.

Selye is one of early researchers studying the stress phenomenon (100, 288). In his experiments, Selye induced stress in rats in a variety of ways. He found typical and constant psychological and physical responses to the adverse situations that were imposed on the rats. This implies that the response to chronic stress is the same for all stressors, physically as well as mentally in the rat. His research led to the development of a model, called the General Adaptation Syndrome (GAS) (289). The GAS is composed of three stages: the alarm reaction, the stage of resistance and the stage of exhaustion. The alarm reaction is equivalent to the fight or flight reaction as described above. The stage of resistance is a continued state of arousal. If the stressful situation is prolonged, the high level of hormones during the resistance phase may upset homeostasis and harm internal organs leaving the organism vulnerable to disease. The exhaustion stage occurs after prolonged resistance. During this stage, the body's energy reserves are finally exhausted and breakdown or catabolism occurs. Selye (286) has noted that, in humans, many of the diseases caused or deteriorated by stress occur in the resistance stage.

In rats, different kinds of stressors elicit the same behavioural responses. However, in humans, different stressors seem to induce various diseases or conditions. Heuther (138) discovered that the physiology of the stress response is much more complex in higher vertebrates and that this complex central stress response is triggered more frequently by social conflict, rather than by life threatening scenarios. The brain is the

key target of this stress response; there are complex feedback and feed-forward systems, which cause modification of the brain structure and function with repeated exposure to psychosocial stress.

The exhaustion stage is probably not so different for the multiple stressors known. However, recovery from the exhaustion stage is very difficult if possible. Therefore, it is important to define diagnostic parameters of early stage stress-related disorders (preferably in the resistance stage) to prevent the individual from the detrimental, irreversible effects of stress-related disorders in the exhaustion stage.

Training and the Overtraining Syndrome

The major objective in exercise training is to cause biologic adaptations to improve performance in specific tasks. The training response is highly specific and characterized by the so-called specificity principle. In aerobic or endurance training for example the mode of training must overload the appropriate muscles as well as the cardiovascular system. On the other hand in jumping events lasting around a few minutes, anaerobic power, strength of particular muscles and neuromuscular coordination determine performance. If the principle of specificity is not accomplished, performance enhancement cannot be anticipated. In addition, a specific exercise intensity or overload must be applied in order to force the body to adaptation. The latter type of training is called ‘overload’ training (179). If the exercise intensity is too low no gain in performance can be anticipated (179). The exercise intensity is the most important factor to increase performance and should be well above ‘normal’. Manipulating or varying the training frequency, intensity, and duration both within one training session as well as between training sessions can achieve an appropriate overload for each subject or animal. The product of these training modalities determines the endogenous load. If a training program is well constructed on an individual basis, and the endogenous training load is optimized, recovery and adaptation will occur within 3-7 days (179). If, however, training occurs before recovery is completed, the athlete comes in a situation of chronic overload, which results in a delayed adaptation process, (if any), and may lead to the overtraining syndrome.

At an early stage, overtraining is only reflected by increased fatigue and (sometimes) decreased performance, but with accumulation of fatigue other physical and psychological symptoms occur. The first stage is called overreaching, and 2-3 weeks of rest are enough to recover. The following or second stage is called overtraining syndrome and takes months or years of rest for recovery (if recovery is achieved at all).

The overtraining syndrome is clinically divided into two types, i.e. the sympathetic (Basedowian) and parasympathetic (Addisonoid) type (151). Sympathetic overtraining

is characterized by an increased sympathetic activity in the resting state. In parasympathetic overtraining there is a strong inhibition of the sympathetic system, and the parasympathetic activity in rest and during exercise predominates. It is hypothesized that the sympathetic type reflects a prolonged stress response whereas the parasympathetic type reflects an advanced state of overtraining with exhaustion of the neuroendocrine system (177, 179). Others associate the different types with different kinds of sports (102, 151). The parasympathetic type of OTS is more difficult to identify, since the symptoms are less alarming for the athlete and in the initial stages are more like the effects of training improvements (See Table 1) (102, 179).

Table 1. Summary of symptoms associated with different types of the overtraining syndrome in humans.

Sympathetic type	**Parasympathetic type**
Decreased performance	Decreased performance
Increased resting heart rate	Low resting pulse rate
Decreased appetite	Normal appetite
Weight loss	No weight loss
Retarded recovery HF after exercise	Quick recovery of heart rate after exercise
Increased irritability and emotional lability	Phlegmatic behaviour
Increased incidence of infection	Decreased plasma lactate during submaximal and maximal exercise
Increased incidence of injury	Hypoglycaemia during exercise
Disturbed sleep	Normal sleep
Increased resting blood pressure	

OTS is defined as a neuroendocrine disorder that results from an imbalance between the demand of the exercise and the functional capability (273). Alterations in the HPA as well as the GH axis have been described for overtrained athletes (16, 321).

Athletes of all performance levels may develop the syndrome. More than 200 symptoms have been associated with OTS (102). But despite such an extended list, up to this moment there are not yet quite established criteria for diagnosing OTS (273).

To date, in apparent clinically healthy human athletes showing a decrement in athletic performance, diagnosis of overreaching (first stage) and/or overtraining (end stage) is made by the use of the Profile of the Mood State (POMS) questionnaire (273). The tentative diagnosis of overreaching and/or overtraining in horses still remains a major challenge, because the use of questionnaires is not possible. However, a

translation from the POMS to a questionnaire for owners of the horses might be a valuable tool for predicting overtraining. At present, behavioural markers in concert with decrements in performance without any clinical adverse sign are the only options available (so far). More objective parameters would contribute positively to the diagnosis and adequate treatment of overreaching and prevent humans and horses from falling into the full-blown overtraining syndrome. Only a few researchers studied the OTS in horses and they mainly focused on the HPA-axis (33, 114, 127). The GH-axis has not been studied in horses yet in relation to training and early overtraining and will be discussed in this thesis.

Growth Hormone-IGF-I axis

Equine Growth Hormone

Equine Growth Hormone (eGH) (or Somatotropin (ST)) has a molecular weight of approximately 45,652 dalton and was isolated by Saxena and Hennema (282) and Hartree et al (132), who determined its amino acid composition and N- and C-terminal residues. In 1973, Conde et al (56) described a new procedure for obtaining eGH of high purity. In 1976, Zakin et al (370) described the primary structure of eGH molecule by its amino acid sequence. Stewart and Tuffnell (303) described the DNA sequence for eGH for the first time. Finally, recombinant DNA technology has led to the production of commercially available equine recombinant growth hormone in 1998 (Bresagen, Australia).

GH is released from the anterior pituitary gland. In the pituitary gland one cell population contains only GH (somatotropes) and an additional subset of cells contain GH and prolactin in the same secretory granules (mammosomatotropes, 6.5-16.5 % of all pituitary cells) (260).

Regulation of GH secretion

Pituitary GH secretion is pulsatile in horses like in other species (314, 304). GH release from the anterior pituitary gland is under the control of two hypothalamic peptides, Growth Hormone-Releasing Hormone (GHRH) and Somatostatin (SS). The mechanisms by which GHRH and SS interact to generate a pulse of GH are not clearly defined in humans and even less in horses. The idea is that SS and its withdrawal set the timing of each GH pulse, while GHRH determines the magnitude of the subsequent GH peak. Superimposed on this is the regulation of somatotroph responsivity by insulin-like growth factor I (IGF-I) (31). GHRH is important not only for release of GH but also in gene transcription and synthesis of GH. SS inhibits GH release without affecting GH synthesis from the pituitary gland.

The GH axis is one of the most dynamic of all endocrine systems. A large number of specific hormonal, metabolic, nutritional, and body-compositional factors as well as age, exercise, and sleep regulate GH secretion in humans and horses (43, 115, 172, 215, 230, 313, 314, 353, 355, 361). Pathologic conditions like stress, diabetes mellitus, chronic renal failure, starvation, hepatic disease, and acromegaly influence the GH-IGF-1 axis (83, 94, 147, 221). Moreover, multiple pharmacologic agents that alter the activity of various brain and neurotransmitter pathways (including acetylcholine, dopamine, serotonine, and noradrenergic and opiatergic pathways) can influence directly or indirectly the somatotropic axis (147). Relevant factors for this thesis will be discussed in this chapter, a detailed review for the interested reader has been written by Giustina and Veldhuis (111).

- Gonadal steroids and GH secretion

Sex-steroid hormones stimulate GH secretion. Both testosterone and oestradiol administration lead to increased GH secretion. The effect of testosterone is supposed to be mainly indirect, through aromatization of testosterone to estrogen (214). In humans, more and higher spontaneous GH pulses are found in women, probably due to higher estrogen levels (147).

GH release is also sex-dependent in horses. For horses, the gender difference in GH secretion is the opposite of the human gender difference. Stallions and geldings seem to have more pulses and greater average pulse amplitude than mares. No difference in baseline average for GH between the genders was found (304, 313, 314). A remarkable finding was that geldings were similar to stallions in their GH profile. Apparently, testicular products were not a factor in the difference between mares and stallions at the time of sampling (castration occurred at least 2 years earlier), but the presence of the testes throughout prenatal development or soon after birth may cause male-like differentiation of GH secretory characteristics that persists even when the testes are removed (313). Unfortunately, the researchers did not describe the cyclic state of the mares included in the study (313). In mares, seasonal changes appear to be related to the influence of ovarian steroids like in women. GH release is low outside the breeding season when mares are not cyclic, i.e. progesterone is absent and oestrogen concentrations are minimal. In pregnant mares, which are under a constant influence of gonadal and placental gestagens, plasma GH concentrations are elevated throughout the 11-month pregnancy. Furthermore, GH levels are constantly low in ovariectomized mares (12).

In horses, opiodergic systems decrease GH release during but not outside the breeding season with a more pronounced effect in the mare compared to the stallion (12, 13).

- Influence of sleep on GH secretion.
In humans, there is a prominent association of increased GH release with episodes of deep sleep (stage III and IV). It is common believe that sleep is required for normal linear body growth. Sleep associated with rapid eye movements (REM) is accompanied by relative suppression of GH secretion, and the fully awake state is associated with further decreases (147). It is presumed that somatostatin withdrawal is involved during slow wave sleep. The strong physiologic coupling of deep sleep with GH secretion in healthy individuals raises the possibility that pathophysiologic conditions associated with disordered sleep (like chronic stress) may be accompanied by decreased GH release (147). As far as we know, no study in horses investigated the relationship between sleep and GH secretion. However, several studies describe a greater incidence of GH pulses occurring with the onset of or during the dark period of the day, which might indicate that the majority of GH pulses are formed at night during sleep in horses (40, 47).

- Aging and GH secretion
In healthy older individuals, decreased basal GH concentrations as well as a decreased response to secretagogues were found. Because adiposity tends to increase with age whereas sex steroid concentrations decrease with age, there are conjoint negative effects of age, body mass index, and steroid deficiency on endogenous GH production rates in older individuals (147). Obese horses produce and release less GH as well (43).

- Exercise and GH secretion
Exercise is a potent stimulator of GH secretion in humans as well as horses. Chapter 3 of this thesis addresses this subject extensively.

- Other factors influencing GH secretion described in horses
Ghrelin, an endogenous Growth Hormone-Releasing Peptide (GHRP)-like neuropeptides may be involved in GH regulation in horses (210). Synthetic GHRP-like compounds such as EP51389, are reported to work as potent GH secretagogues in horses (39).

The horse is unique among the farm animal species in that the only known effect of Thyrotropin Releasing Hormone (TRH) on GH secretion is inhibitory rather than stimulatory (175, 259). TRH inhibited the GH response to EP51389 (GH secretagogue), GHRH, exercise and aspartate infusion. This indicates that TRH action is not at the receptor level for either secretagogue, but probably acts directly via specific TRH receptors on the somatotropes, or indirectly through stimulation of somatostatin. It is not known whether this inhibition occurs in horses naturally. However, research from Pruett

et al (259) indicates that physiologic alteration of TRH secretion likely does not alter normal growth hormone secretion.

In addition, somatostatin-immunoreactive cells were found in equine thyroid and parathyroid glands in very small amounts compared to the amount of C-cells. Somatostatin may be involved in mechanisms for local regulation of calcitonin secretion, thyroid hormone secretion, or parathyroid hormone secretion (311).

In horses, knowing that some prolactin and GH are released from the same cells in the pituitary gland it is interesting that neither spontaneous or GHRH-induced surges in GH secretion are accompanied by changes in plasma prolactin concentrations (313). Moreover, increases in prolactin secretion after feeding or after administration of dopamine antagonists are not associated with changes in GH secretion (72, 314). The only physiologic event that consistently stimulates both hormones simultaneously is discharge of the sympathetic nervous system, such as during exercise (307, 313) or after epinephrine administration (55, 314).

Insulin-like Growth Factor-I (IGF-1)

The main site of GH action is the liver where GH stimulates the production of IGF I and II and Insulin-like Growth Factor Binding Proteins (IGF-BPs). On release of GH, the increases in IGF-I were shown to persist for longer periods of time than those for GH. This is mostly due to the binding of IGF-I with up to seven equine IGFBPs in circulation (37, 47, 60, 91, 193). The exact role of these IGFBPs is not clear, but it is known that they increase the effective lifetime of IGFs (18). Pharmacokinetic studies have indicated however, that a small fraction of IGF-I is not associated with IGFBPs (371). There is no consensus on the significance of so-called free IGF-I but, in theory, this could be the metabolically active form (122, 228). The secretion of IGF-I and IGFBPs is not pulsatile and their concentrations remain more or less constant under normal physiological conditions (193, 248). The physiologic role of IGF-I is mainly increasing the rate of protein synthesis (351).

Actions of endogenous GH

Growth hormone causes growth of almost all tissues in the body by promoting increased sizes of cells and increased mitosis. In addition, GH enhances almost all facets of amino acid uptake and protein synthesis by cells, while at the same time it reduces the breakdown of proteins. GH increases the release of free fatty acids from the adipose tissue and enhances fat utilization for energy. Furthermore, GH directly inhibits glucose uptake by the cells. This glucose-sparing action helps to maintain blood glucose at fairly high levels and contributes to one’s ability to sustain prolonged exercise. It is not completely understood how GH exerts this “anti-insulin” effect (123,

204). Nevertheless, increased levels of GH are associated with hyperinsulinaemia, hyperglycaemia and insulin resistance in man and horses (194).

Effects of exogenous GH

It is well known that growth hormone has anabolic effects, and its consequent abuse is a concern in many sports, including horse racing. The recent availability of reGH in Australia has attracted the attention of horse racing authorities worldwide and urged the development of methods for the detection of its abuse (65). The recent availability of reGH also attracted the attention of researchers to determine the responses to administration of reGH in horses.

The biological responses to reGH administration in adult horses are similar to those common in other species: hyperglycaemia, hyperinsulinaemia, insulin resistance, decreased plasma urea nitrogen concentrations, increased plasma IGF-I and IGFBP-3 concentrations (65, 166, 194, 248, 297, 312).

Beneficial effects of reGH administration reported to date in adult horses include increased nitrogen retention, musculation and granulocyte numbers in aged mares (194), increased ovarian follicle numbers in cyclic mares (52), and a potentiation of the ovarian sensitivity of seasonally anovulatory mares to sub-treshold GnRH treatment (53, 54).

No effects of reGH administration reported to date in adult horses were found in age-related declines in various immune parameters (121), on modulation of the in vitro biomechanical properties of superficial digital flexor tendon (SDFT) (77) and on second intention wound healing (61). Also no effects were found on aerobic capacity or exercise performance in geriatric mares (208) or on exercise capacity or indices of fitness in young Standard horses in training (110).

ReGH administration had a negative effect on the biomechanical properties of healing SDFT (78).

Long-term administration of reGH in foals did not influence body weight, long bone growth and other body sizes. Generally, basal glucose concentration and insulin response to glucose infusion were higher in reGH treated foals. Endogenous GH secretion in response to GH secretagogue (EP51389) was significantly reduced in treated foals. The prolactin and Thyroid-Stimulating Hormone (TSH) responses to TRH were not altered by reGH treatment. Mean serum IGF-1 concentrations were not significantly higher in reGH treated group. However, there was a significant increase of IGF-1 in the first five weeks of treatment in foals receiving reGH compared to controls. At necropsy, many internal organ weights were increased, but little effect on histopathologic characteristics was found in the same foals (42, 181).

Analysis of the function of the GH-IGF-1 axis

Because the secretion of GH is pulsatile, a single blood sample is not valid to evaluate GH axis functionality. Analysis of the hypothalamo-pituitary-GH axis requires measurements of multiple GH samples combined with determination of serum concentrations of IGF-I, and their circulating binding proteins, as well as stimulation tests with GHRH or other GH secretagogues (86). GH pulsatility characteristics can be determined from the GH data series by visual identification of presumptive pulses. However, this method is not very objective. Therefore, computerized algorithms have been developed in order to analyze GH hormone pulsatility and regularity. Three methods are used in this thesis, namely a pulse detection algorithm (Cluster 8), a deconvolution algorithm (AutoDecon) and Approximate Entropy (ApEn), which is able to calculate the regularity of the GH data series (159, 160). The three methods will be described in the following sections.

Several studies investigated GH pulsatility in horses (47, 62, 72, 304, 307, 313, 314). So far, only pulse detection algorithms were used to analyze GH pulsatility in equines. However, pulse detection analysis does not provide information about the secretion rate of the hormone into the blood stream and the elimination of the hormone from the serum. This information must be obtained from a deconvolution analysis. To make comparisons with the available literature, both methods are used in this thesis.

- Pulse detection algorithm: Cluster 8

Cluster (159) is a statistically based peak-detection algorithm, which is designed to locate all significant increases and decreases in a data series in relation to dose-dependent variance models. It starts with the first data point and compares it with the second data point and calculates whether there is a significant increase between the two points. Then, the second data point will be compared to the third for a significant increase. This search is continued throughout the entire experimental series, so that all individually significant increases are marked. After marking all the significant increases, the series is rescanned in consecutive order to search for significant decreases. A nadir is defined as a decrease followed by an increase. The occurrence of a peak is defined as a significant increase followed by a significant decrease. This formulation of a peak requires that a region of significant increase be associated with nadirs on both sides, which means that at the end of a data series partial peaks are not used for the calculations, unless the last peak is followed by a fully demarcated nadir. The user specifies the number of points to be used in testing nadirs against peaks (whether the comparison starts with one data point to another or a cluster of points (=nadir width) to a cluster of points (peak width).

Comparisons of nadir and peak clusters were made by pooled t-testing using the actual experimental replicates present in the test nadir and peak. The application of

pooled *t* testing assumes that variation within the experimental replicates is random and approximates a Gaussian distribution. The investigator must provide an acceptable estimate of measurement error either as a coefficient of variation or as a standard deviation (160).

In performing the analysis, the user specifies individual test clusters sizes for the nadir and peak (based on sample interval), a *t* statistic to identify a significant increase, and a *t* statistic to define a significant decrease, minimum peak size (which is the minimum hormone concentration to be defined as a possible peak), half-life and outlier T-score (to identify significant outliers). Veldhuis and Johnson (332) published a table from which the investigator can select test cluster sizes and identify an appropriate *t* statistic that will maintain a desired minimum false-positive rate associated with Gaussian-distributed measurement error. The algorithm calculates several parameters as shown in Table 2 (332).

Table 2. Definition of pulse detection parameters assessed by Cluster.

Parameters	*Definition*
Peak position	Location individual peak defined as sample number
Width of individual peak	Time elapsed between the first significant upstroke and first significant downstroke within the overall peak
Total number of peaks	
Mean interval between peaks	Time lapse between the maximum values within the consecutive peaks
Maximum peak height	Highest absolute value attained within the peak
Largest peak (%)	The percentage increase of the maximal peak amplitude above preceding nadir
Mean peak height (%)	Percent increase of the mean peak amplitude above prepeak nadir
Incremental peak amplitude	Algebraic difference between the maximal peak height and the prepeak nadir value
Area under the peak	Product of mean peak amplitude that is above the lower of the two flanking nadirs times the time interval (peak duration)
Valleys	Regions containing no significant increases or decreases.
Position valley	The location of the valley in relation to sample number
Width valley	Duration of the valley in minutes
Nadir	Mean concentration within the test nadir portion of the valley
Mean valley	Overall mean value within the entire value

Cluster does not provide information about the secretion of the hormone into the bloodstream and the elimination of the hormone from the serum. This information must be obtained from a deconvolution analysis like AutoDecon.

- Deconvolution analysis: AutoDecon

The concentration of a hormone in the blood is a balance between two processes: secretion into the blood stream and elimination from the blood. To obtain the concentration as a function of time the two processes are combined via a convolution integral. The secretion event is built up from infinitely small secretion events in a very short period. Once the secretion within each of these events occurs, the concentration increases by that amount, and then immediately begins to be eliminated according to its particular kinetics. The final concentration profile is the sum of all these individual eliminations. Analyzing hormone time series starts the other way around, namely with the concentration as a function of time and calculating both the secretion and the elimination that generated the concentration. This inverse process is known as deconvolution (160).

Table 3. Definition of hormonal secretion and elimination parameters assessed by Autodecon.

Parameters	*Definition*
Basal secretion	Non-burst-like secretion
Concentration at time zero	
Secretion SD	SD of random distribution of instantaneous secretory rates that constitutes a burst
Half-life	Metabolic clearance rate
Number of secretion peaks	
Mean secretion pulse height	Mean secretion rate
Mean secretion pulse mass	Mean area of the secretion burst
Mean secretion interpulse interval	Time between two pulses

The actual fitting is done using a damped, Gauss-Newton weighted nonlinear least-squares technique, which provides numerical values for all of the parameters (see Table 3). The user should provide estimates of several parameters before the calculation starts: elimination half-life, secretion SD (an initial good guess is one-half of the data interval spacing), basal secretion and the initial concentration at time zero. An

alternative is to use another deconvolution algorithm, which provides estimates of the above-mentioned parameters combined with estimates of the number and position of secretory bursts. These can then be utilized with the deconvolution analysis program, where iterative nonlinear least-squares parameter estimation at 95% statistical confidence intervals can be utilized to quantify all parameters of secretion (160).

Determination of the goodness-of-fit of the calculated concentration curve versus the original concentration curve can be done by visual inspection, autocorrelation plots and (or) residual(s) plots. Most goodness-of-fit tests are based upon the basic linear model least-squares assumptions. When an acceptable fit of the data has been achieved then the weighted differences between the data points and the estimated curve, will be Gaussian distributed about zero indicating that the residuals are normally distributed (160).

- Approximate Entropy (ApEn)

ApEn measures the logarithmic likelihood that runs of patterns in a time series that are close for *m* consecutive observations remain close when considered as *m+1* consecutive observations. Calculation of ApEn requires prior definition of three parameters, *m, r* and *N*. *m* is the length of run to be compared, *N* is the number of data points in the time series being considered and *r* defines close, which is simply a factor by which the standard deviation (SD) of the *N* points being considered is multiplied. *r* =0.2 means that “closeness” fits within 0.2 SDs of the data and is commonly used, because the value has shown from experience to be reasonable.

The first thing ApEn does is counting up all the individual values of the series (i.e., blocks of length m=1) that are close to each other (i.e., within *r* SDs of each other). Then, it counts how many vectors of length 2 (i.e., blocks of length *m+1=2*) are within r SDs of each other. The value of ApEn is then the difference of the logarithms of these two frequencies; specifically, that for *m=1* minus that for *m=2*.

Greater pattern regularity yields smaller ApEn values, whereas greater independence between consecutive values of an ordered time series yields larger ApEn values. ApEn is a relative metric that can range in value from zero to some upper, limiting value that depends on *N, m*, and *r*, as well as the particular values comprising the series. A reasonable estimate of the maximally irregular ApEn value for a given series can be provided by shuffling the data series in a Monte Carlo procedure and calculate ApEn thousands of times. The results provide an estimate for the distribution of ApEn values if the particular data series were actually random (160).

Adding Gaussian distributed random noise to the data time series and calculate ApEn thousands of time can provide a reasonable estimate of the precision of the ApEn value for a given series. The mean of the resulting distribution will be increased

because the sequence will be more irregular. The SD of the distribution can be used as a measure of the precision of the ApEn value.

Regulation of glucose metabolism

A horse needs energy sources to perform exercise and to allow muscle contraction. The main sources used to provide energy are carbohydrates (glucose and glycogen) and fat (fatty acids). Protein is only used to provide energy in cases of extreme exhaustion, starvation or disease (198). This thesis focused on the influences of exercise and training on carbohydrate metabolism.

Endocrine regulation of glucose metabolism
Insulin is the primary hormone controlling the metabolism and storage of body fuels, e.g. carbohydrates, fat and proteins. Its action influences three major tissues: liver, muscle and fat. Insulin is secreted by the ß-cells of the islets of Langerhans of the pancreas. Several factors influence directly the secretion of insulin by the pancreas. Glucose and glucagon stimulate the ß-cells, while somatostatin inhibits them. Both hormones are produced by the pancreas, namely α-cells secrete glucagon and δ-cells secrete somatostatin. Pancreatic function is also regulated by the sympathetic system, which inhibits insulin secretion and stimulates glucagon secretion. The parasympathetic system stimulates insulin secretion during food intake (263).

Effect of insulin on carbohydrate metabolism
Insulin is secreted in great quantity when there is an excess amount of carbohydrates in the blood, for instance after a meal. The role of insulin is to store this excess of energy. The carbohydrates are mainly stored as glycogen in the liver and muscles. All the excess carbohydrates that cannot be stored as glycogen are converted into fat and stored in the adipose tissue. In case of excess of proteins, insulin has a direct effect in promoting amino acid uptake by cells and conversion of these amino acids into protein. Several other hormones influence insulin actions on carbohydrate metabolism, like glucagon, catecholamines, GH and glucocorticoids.

Glucagon is secreted when blood glucose decreases, such as between meals and during exercise. Glucagon and insulin have opposing effects on the hepatic glucose metabolism. The effect of glucagon is to make the liver release the glucose it has stored in its cells into the blood stream, with the net effect of increasing blood glucose. Glucagon also induces the liver (and some other cells such as muscle) to take up amino acids for conversion to glucose (263).

Catecholamines inhibit glucose-stimulated insulin secretion. In addition, catecholamines raise glucose concentrations by stimulating glycogenolysis and

gluconeogenesis, inhibiting insulin-mediated glucose uptake, increasing lipolysis, and stimulating glucagon secretion (263)

Growth hormone counteracts insulin action on lipid and glucose metabolism. Growth hormone decreases glucose use and storage and promotes protein synthesis and lipolysis. High growth hormone levels (as seen in acromegaly) are associated with insulin resistance (123, 263)

Glucocorticoids have insulin-antagonistic effects like decreasing the skeletal muscle and adipocyte responses to insulin-stimulated glucose uptake. Further, glucocorticoids stimulate proteolysis, increase glycogen formation, and stimulate gluconeogenesis. Increased glucocorticoid concentrations are associated with insulin resistance (123, 263).

Regulation of cellular glucose metabolism

Muscle cellular glucose transport can be activated by at least two separate and distinct pathways (Fig 2): one stimulated by insulin and insulin-like growth factors and the other activated by contraction/exercise and/or hypoxia (133).

Insulin stimulates the cellular uptake of glucose via a signaling pathway shown in Figure 2A. The effects of insulin on glucose uptake are mediated via the insulin receptor and finally result in the translocation of intracellular glucose transporter (GLUT) 4 to the cell surface. It is the increased amount of GLUT4 on the cell plasma membrane that results in an increased rate of glucose transport into the cell. When insulin is no longer available, these vesicles separate from the cell membrane within 3 to 5 minutes and move back to the cell interior to be used again as needed (124).

Contractile activity in the muscle directly increases muscle glucose transport via a separate signaling pathway shown in Figure 2B. The exact cellular mechanisms responsible for activating the glucose transport system in response to contractile activity are not completely understood. Traditional theories concerning the role of intracellular calcium (released from the sarcoplasmic reticulum to induce the contractile process) have been supplemented by recent studies suggesting a role for 5'AMP-activated protein kinase (AMPK), nitric oxide (NO) or mitogen activated protein kinase (MAPK) p38 as mediators of the exercise effect (133).

Beyond the transient, insulin-independent stimulation of glucose transport that results from contractile activity, *acute exercise* can also enhance insulin sensitivity in the post-exercise period. This effect lasts for several hours for up to 48 hours in humans (38, 152, 178, 218, 224). One factor that appears to regulate the duration of the post-exercise effect is the replenishment of muscle glycogen stores. However, experimental manipulation of glycogen repletion has demonstrated that this is certainly not the only factor regulating the time course of post-exercise insulin sensitivity, as

these variables are not always tightly coupled. The cellular mechanisms underlying the post-exercise enhancement of insulin action are unknown.

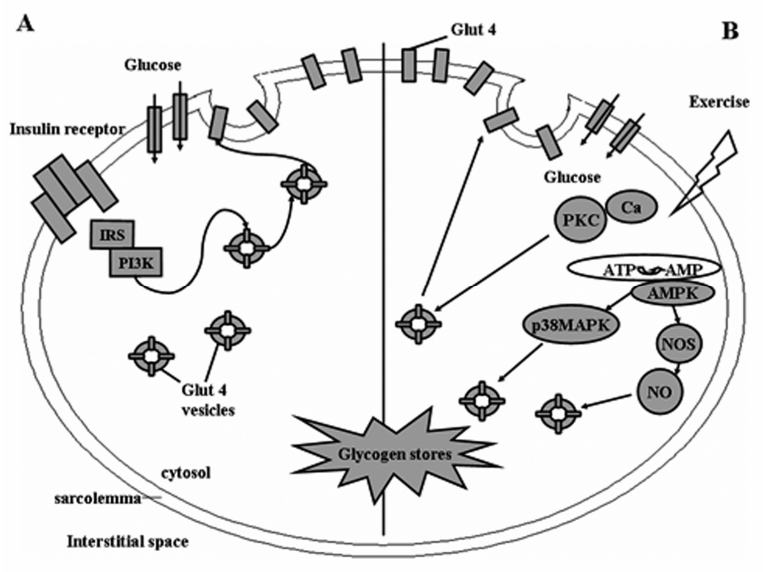

Figure 2. Insulin (A) and acute exercise (B) induced signaling pathways involved in stimulating muscle glucose transport
A. Insulin mediates its action via the insulin receptor which, following insulin binding, undergoes autophosphorylation on tyrosine residues activating its tyrosine kinase activity. The activated IR then phosphorylates IRS-1 and other substrates. Tyrosine phosphorylated IRS-1 then serves as a docking protein for PI 3-kinase, which is activated by this interaction and finally results in the translocation of intracellular GLUT4 to the cell surface.
B. Muscle contractile activity induces a recruitment of a separate pool of intracellular GLUT4 to the plasma membrane and a subsequent increase in glucose transport. This effect does not involve the components of the insulin signaling pathway. Muscle contraction is initiated by a necessary release of calcium to permit cross bridge formation. Intracellular calcium activates PKC serine kinases which have been hypothesized to stimulate GLUT4 recruitment by unknown mechanisms. Contractile activity alters the AMP/ATP ratio leading to the stimulation of AMPK. AMPK activation leads to an increase in glucose transport, possibly through several mechanisms. AMPK can phosphorylate and activate eNOS, and NO production by this enzyme may contribute to exercise stimulated glucose transport. AMPK can also lead to the phosphorylation of p38 MAPK, which may be involved in the GLUT4 translocation response. Adapted from Piercy and Riviero (240).

In addition, exercise induced improvements in glucose metabolism after training is described as well. However, it is not fully understood how chronic exercise training improves insulin effectiveness in muscle. It has been shown that muscle GLUT 4 content increases after training in human and horses (133, 198). However, this is not

the only factor improving muscle glucose uptake after exercise training. The improvements may also be related to upregulation of the expression and /or activity of proteins involved in insulin signal transduction in skeletal muscle (133).

Insulin sensitivity

Insulin resistance can be defined as the metabolic state in which normal concentrations of insulin stimulate a lower-than-anticipated biological response (cellular glucose uptake) (170). In horses, a decrease in insulin sensitivity has been described with aging (192), during pregnancy and associated with several disease states, including obesity (97, 155), hyperadrenocorticism (105), equine metabolic syndrome or peripheral hyperadrenocorticism (158), laminitis (155, 236), hyperlipemia (155, 236), and excessive amounts of growth hormone (262). An increase in insulin sensitivity is associated with exercise as described above (253).

Analysis of insulin sensitivity

Insulin resistance can be quantified by examining glucose tolerance, a variable that can be measured most accurately by use of two clamp techniques (7), i.e., the hyperglycemic clamp and the euglycemic-hyperinsulinemic clamp technique, both of which have been validated for use in horses (87, 268). The hyperglycemic clamp technique is primarily a method for quantifying the sensitivity of the pancreas to exogenous glucose, whereas the euglycemic-hyperinsulinemic clamp technique is a method for quantifying tissue sensitivity to insulin.

In this thesis, the euglycaemic hyperinsulinaemic clamp (EHC) technique is used for determination of the effects of training/overtraining on peripheral tissue sensitivity to insulin. During the EHC insulin is infused to increase glucose disappearance and the glucose is maintained at euglycaemic levels by exogenous glucose infusion. Assuming zero endogenous glucose production, the rate of glucose infusion equals the rate of glucose disposal. The rates of glucose disposal calculated during insulin infusion can be directly compared under differing physiologic conditions if the concentration of glucose perfusing and glucose-utilizing by the tissues is the same. Considering the data in this thesis we have to take into account that venous blood sampling can give a systematic error in comparing individuals at equivalent venous glucose level and matched plasma insulin if their sensitivities to insulin are not the same. In the more insulin sensitive individual, arterio-venous glucose difference across a peripheral bed will be greater than in the resistant individual. If venous glucose is identical, arterial glucose must be higher in the sensitive individual, and disposal will be overestimated.

Behaviour and stress

Animal welfare and stress-related disorders are increasingly being recognized by horse owners and breeders. The lack of knowledge on the psychology of the horse led to deficiencies in the way the horses are trained and housed, resulting in stress-related disorders. The most common stressors for horses are housing, management, training, transport, veterinary and farriery procedures. Stress-related syndromes associated with chronic stress in horses are, for instance, stereotypic behaviours, hyper- or hyporeactivity and learned helplessness (207, 269).

Currently, the assessment of stress responses in horses is difficult (267). Considerable inter-individual animal variation occurs in the observed endocrine response subsequent to exposure to stressors, and indicates considerable variation in individual animals' psychological responses to stress (95). The specificity of a single parameter representing a certain response to stress is poor and therefore different parameters need to be recorded simultaneously. Therefore, new and easy applicable methods are necessary to obtain more knowledge on behavioural stress responses in the horse.

Analysis of stress behaviour

Several tools are used for studying influences of stress on behaviour in equines and are based on the assumption that stressed horses respond differently towards arousal than unstressed horses. Since horses are primarily flight animals, stress must give recognizable responses among different situations. Horses can be observed at rest and/or during normal routine events like training. Behaviours can be scored by the observer directly or afterwards from videotapes, allowing calculations of exact durations. Additional tests can be used to observe the horse’s response to stressors like new objects or changing situations. To get an impression of the functioning of the two main hormonal axes associated with the stress response, blood cortisol can be sampled as a representative for the HPA axis and heart rate and heart rate variability (HRV) are used as representatives of the SAM axis during the observations.

- Observational tests

Several symptoms are described in literature to indicate stress in a horse, namely tail sweeping, defecation, eye expression and ear position (207, 352). Vigorous tail sweeping, especially when there are no insects present, can express uncertainty, discontent or increasing motivation to erupt into fear or aggressive behaviour (352). Increased defecation frequencies (often combined with less dehydrated faeces) are typically observed together with tension and fear (207, 267). In humans, the Profile of the Mood State (POMS) questionnaire has proven itself to be a reliable measure of

mood states and their fluctuations in psychiatric patients and overreached/overtrained athletes (212). The original POMS assessment measured six identified mood factors by 65 items: tension-anxiety, depression-dejection, anger-hostility, vigor-activity, fatigue-inertia and confusion-bewilderment. Definitions of these mood states are shown in Table 4.

Table 4. Definition of mood states used in POMS

Mood states	*Definition*
Depression-dejection	Depression accompanied by a tense of personal inadequacy
Anger-hostility	Anger and antipathy towards others
Vigor-activity	Vigorousness, ebullience, and high energy
Fatigue-inertia	Weariness, inertia, low energy level
Confusion-bewilderment	Muddleheadedness, appearing to be an organized-disorganized dimension of emotion

In this thesis, a start with the translation from the POMS for humans to a POMS for horses expressed in measurable biologically meaningful expected behaviours during certain “moods” is made. This equine POMS (ePOMS) is used for the observational studies in order to find behavioural parameters for diagnosing mental aspects of early overtraining in horses.

Two standardized observational tests were used in this thesis to assess changes in mood state of the horses: the High Intensity Training (HIT) observation and the Time Budget (TB). The HIT observation assesses the motivation (and performance) during the treadmill training by measuring items related to equine acceptance, resistance, or stress during preparation before training, during the training and post training. Parameters are shown in Table 5a. The TB observation assesses behavioural patterns related to maintenance as well as reactions to the environment in the home stable measuring items related to depression, vigor and fatigue for instance (parameters shown in Table 5b).

Table 5a. Summary of parameters used to assess mood state in horses in their home stable

Category	*Parameter**
Resting/sleeping	Lateral recumbency, sternal recumbency, standing
Eating/drinking	Eating hay, eating concentrate, eating uneatable bedding, eating faeces, foraging, drinking water, licking manger, licking lickstone, anticipation behaviour towards food provision
Vegetative behaviour	Defaecating, urinating, autogrooming
Other Activities	Standing alert, walking in stable, rolling, scraping, buck/jumping, rearing, inspection of object
Non-relaxed behaviour	Biting bars, biting/licking stable, cribbing

*Parameters were scored either as frequencies or as time spent on this behaviour in relation to the total observation time. In addition, parameters like people coming in/feeding/cleaning etc. and horses passing by were scored to measure responses towards known stimuli (feeding) and unknown stimuli.

Table 5b. Parameters used to assess mood state in horses during treadmill training

Part of training	*Parameter**
Preparation for training	Entering the training hall, cooperation, body tension, alertness
Entering/leaving treadmill	Walking, jumping on/off, refusing
Training on treadmill	Amount and direction of earplay, body tension, jaw tension, playing with bit, looking backward to trainer, alertness, jumping/rearing, stumbling, kicking, scraping, neighing, snorting, defaecating, position on treadmill, stopping on treadmill, cantering, pulling assistance rope, sniffing, tail sweeping

*Parameters were scored either as frequencies or as time spent on this behaviour in relation to the total observation time

- *Stress tests*

The third assessment used in this thesis is the Novel Horse (NH) test. The Novel Horse test was adapted from the more commonly used Novel Object test, originally developed for rodents (9), and is commonly used to test emotional reactivity especially anxiety. In a Novel Object test, the individual is suddenly exposed to a new, unknown object in its otherwise familiar environment. The reaction to sudden novelty involves behavioural as

well as physiological responses. Behavioural changes that are often observed in Novel Object tests include changes in speed of locomotion, changes in postural tonus (head and tail), specific movements of the eyes and ears, and an increase in specific alarm-related vocalizations (9). Physiological changes often measured are changes in corticosteroid concentrations or by heart rate variables (350).

It is commonly accepted that a repetition of a Novel Object test can only be performed with at least a 4-week interval. Since the processes measured in these thesis were at a shorter interval, it was chosen to replace the Novel Object by a stimulus which should always be interesting for a horse: another unknown (Novel) Horse (NH).

The NH test assessed the mental attitude towards social contact with another horse measuring items related to anger-anxiety (but also depression, vigor and inertia) (parameters shown in Table 5c).

Heart rate variability

HRV refers to the variation in time between two beats (R-R intervals). Variations in inter-beat intervals are used as an index of autonomic responsiveness (27, 191). Two methods of analysing HRV can be used, namely time domain analysis and the more recently developed frequency domain analysis.

Table 5c. Parameters used to assess mood state in horses meeting other horse (NH)

Phase	*Parameter**
Pre- and post-exposure phase	Exploration while standing or walking, foraging, standing still, standing alert, startle reactions, friskiness, autogrooming, snorting, neighing, urinating, defaecating, flehming, anticipation after announcement of presentation Novel Horse
Exposure to NH	Walking to NH, standing at NH, nose contact, initiating play subject, playing, scraping, rearing, walking away, relaxing penis, latency for first contact, total time in contact/ total time within 1 horse length / total time outside 1 horse length

*Parameters were scored either as frequencies or as time spent on this behaviour in relation to the observation time of each phase

- Time domain analysis

During time domain analysis R-R intervals in milliseconds are plotted against time. Parameters calculated to express HRV in the time-domain are: the standard deviation of all normal intervals (SDNN), which reflects the overall variability of the heart. Differences between successive R-R intervals provide an index of cardiac vagal control. This can be quantified by calculating the root mean squared of successive difference (r-MSSD) of all R-R intervals and the number of adjacent R-R intervals differing more than 50ms expressed as a percentage of all intervals over the collection period (pNN50) (1). The 50 ms interval is an arbitrary interval used for standard HRV calculations in humans (27), however, this cannot be used in equines due to their lower basal HR.

- Frequency domain analysis

In frequency domain analysis HRV is plotted as the frequency at which the length of the R-R interval changes. The main parameters on the frequency domain are: very low frequency power (VLFP), low frequency power (LFP), high frequency power (HFP), ratio between LFP and HFP (LFP/HFP) and total power (TP). The measurements at different frequencies are usually expressed in absolute values of power (milliseconds squared). The peaks at different frequencies reflect the different influences of the parasympathetic and sympathetic nervous system. Part of the HRV is caused by respiratory sinus arrhythmia mainly mediated by parasympathetic activity to the heart, which is high during expiration and absent or attenuated during inspiration. It has been shown that the high frequency (HF) peak is mediated only via the vagal nerve (and thus ventilation) and the low frequency (LF) peak mediated by the vagal and sympathetic nerves (27). The ratio of LFP to HFP is considered to reflect the sympatho-vagal balance and high values suggest a sympathetic predominance. It has been shown that pNN50 and r-MSSD provide the same information as the HFP component. (1, 27)

Several factors can influence the interpretation of R-R interval data. Firstly, the accurate detection of R waves is essential, as misinterpretation of normal waveforms will lead to large errors in heart rate variability results, especially when frequency domain analysis is used (27). Secondly, part of the HRV is determined by respiratory sinus arrhythmia and therefore any change in breathing pattern will have an influence on the power spectrum. Thirdly, body position influences HRV significantly comparing data from supine and upright positions in humans. Fourthly, HRV is influenced by age and sex. HRV decreases with increasing age and women have lower HRV than men.

Overtraining and HRV

HR and HRV are closely related to parasympathetic and sympathetic activity, and changes in the autonomic nervous system due to overtraining may be reflected in changes in HR and HRV (191). Uusitalo et al (322) showed an increase in LFP in the

intensified trained group versus control group (indicating increased sympathetic activity), while in a case study describing a young overtrained cross country skier an increased HFP and TP were found (indicating increased parasympathetic activity) (134). So far, the effects of intensified training/early overtraining on HRV appear to be unclear at present, mainly due to the small number of studies addressing this problem (1).

Aim and scope of the thesis

To date, diagnosing overreaching or overtraining remains still a major challenge. In athletes, a decrease in performance despite the maintenance of or an increase in training effort is indicative for overreaching or overtraining. In man, the diagnosis is made by the use of the Profile of the Mood (POMS) questionnaire, a valuable but subjective tool. However, the use of questionnaires is not possible in horses, therefore behavioral markers combined with loss of performance without any adverse clinical signs are the only options available yet. More objective parameters would contribute positively to an early diagnosis of OTS in humans and horses. Some evidence exist, that a neuroendocrine disorder is associated with the origin of the OTS. Several hormonal axes have been studied and found disturbed in overtrained individuals among them the HPA axis and the GH-IGF-1 axis. The HPA-axis has also been studied in overtrained equines. However, the results were not conclusive. Therefore, the major objective of this thesis is to determine the influences of training and early overtraining on the GH-IGF-1 axis and to find possible markers for the detection of early overtraining. This will be combined with a behavioural study and physical performance study as to date these seems to be the most reliable predictors of the OTS. Since stressful situations are described to disturb carbohydrate metabolism, i.e. decrease insulin sensitivity, the second part of this thesis aimed to determine the influences of training and early overtraining on glucose metabolism and peripheral insulin sensitivity in order to find possible markers for the detection of overtraining. Glucocorticoids, GH as well as training itself influences glucose metabolism and insulin sensitivity in different ways, which makes it difficult to hypothesize which effect might be expected after early overtraining and therefore research is needed to determine these separate effects to understand the possible effect of combinations.

Chapter 2 describes and discusses the original research design of the whole training study. The study consisted of four periods, namely acclimatisation, training, intensified training and recovery period with at the end of each period (endocrinological) tests. Standardized exercise tests were used to monitor performance and behavioural studies were used to monitor mood changes. A summary of the already published

results will be given to offer the possibility to provide a broader overview of possible parameters of early overtraining in the general discussion at the end of this thesis.

Chapter 3 gives an overview of the literature concerning the influence of exercise, training and overtraining on the HPA axis as well as the GH-IGF-1 axis in humans and horses. Since most hormones, including GH, are secreted in a pulsatile fashion, we hypothesize that normal pulsatility might be disrupted in a period of very demanding training or overtraining. At the moment, however, the knowledge on basal GH pulsatility in equines is scarce. In order to study influences of training/early overtraining on GH pulsatility, normal values for basal GH pulsatility needed to be determined first. Hormone concentrations in peripheral blood are at any time determined by the secretion rate of the pituitary and the simultaneously occurring clearance rate. Therefore the objective of **Chapter 4** was to determine first the half-life of equine Growth Hormone (eGH) (endogenous and exogenous) in order to provide the information needed to use the relative new techniques of deconvolution analysis and approximate entropy on basal GH data series for the first time in horses. These techniques provide more detailed information on GH data series. In the same chapter, the validation of the used GH assay will be described. The next step was to use the same techniques on the GH data series collected after the training period, the intensified training period and the recovery period in order to determine the influences of training/overtraining on the GH-IGF-1 axis. The hypothesis was that the nocturnal pulsatile secretion would increase during the intensified training period followed by a possible decrease as described in severely overtrained athletes (**Chapter 5**).

The second part of this thesis focuses on the influences of GH, cortisol and training/early overtraining on glucose metabolism and peripheral insulin sensitivity. Since increased GH concentrations are associated with hyperinsulinaemia, hyperglycaemia and insulin resistance, alterations in functioning of the GH-IGF-1 axis were expected to influence glucose metabolism at rest and during exercise. Also hypercortisolaemia is associated with decreased insulin sensitivity. In contrast, acute exercise as well as training improves insulin sensitivity. The alterations in functioning of the GH-IGF-1 axis and the HPA axis combined with the effects of exercise/training might determine the actual effect of overtraining on insulin sensitivity and to our opinion the outcome was not predictable on the forehand. Therefore the influence of exercise as well as the influence of increased plasma concentrations of eGH and cortisol on glucose metabolism and insulin sensitivity were studied in separate studies before studying the influence of overtraining itself. In **Chapter 6,** the effects of a single dose of exogenous recombinant eGH and cortisol as well as the long-term administration of recombinant eGH on glucose metabolism and insulin sensitivity were determined by use of the euglycaemic hyperinsulinaemic clamp technique. In **Chapter 7,** the results of

the euglycaemic hyperinsulinaemic clamp performed at the end of the acclimatisation period and the training period were compared to measure the influence of long-term training on glucose metabolism and insulin sensitivity. The clamp was performed 24 or 72 hours after the last exercise bout to exclude the influence of acute exercise. **Chapter 8** describes influences of the intensified training period on glucose metabolism and insulin sensitivity at rest measured by the euglycaemic hyperinsulinaemic clamp technique. Influences of training and overtraining on changes in plasma glucose and insulin concentrations during acute exercise are described in **Chapter 9.**

Last but not least, instead of Profile of the Mood questionnaires, the behaviour of the horses was observed and objectively scored regularly during treadmill training and at rest in their home stables during the period of training, intensified training and recovery. Thereby, the horses performed a newly developed behaviour test, the Novel Horse Test, several times during the same periods. Results will be shown and discussed in **Chapter 10**. These behavioural results combined with the outcome of performance during the standardized exercise test should reveal whether early overtraining was induced in the horses during this study.

Finally, in **Chapter 11** the results of the thesis are integrated and discussed.

Chapter 2

General Research Design

Introduction

This thesis focuses on the influence of training and intensified training on endocrinological variables and behaviour. However, during the experiment multiple parameters were studied. An overview of these other studies will be given and the results (that are already published elsewhere) will be summarized at the end of this chapter in order to provide some extra information for the general discussion (Chapter 11).

Experimental Set up

Animals
12 healthy Standardbred geldings, aged 20 ± 2 (mean ± SD) months, were included in the study. The horses had no known history of health and exercise problems and had not been involved in any kind of organized exercise or training regimen previously. The choice for geldings was based on the knowledge that gonadal hormones have important influences on the GH-IGF-1 axis, which we would exclude on the forehand.

Quarantine period
On arrival, a clinical as well as a routine blood examination was performed. The first week, the horses were dewormed, vaccinated for tetanus and influenza, and castrated. The horses were individually housed in boxes on wooden shavings. Their diet consisted of grass silage supplemented with concentrate feed according to the daily estimated energy requirements of 58 MJ NE (range 54-66). During the intensified training period, a supplement with vit E, Se, electrolytes and other vitamins was added to the diet of all horses. The horses were fed at fixed times: concentrate feed at 0600 h, grass silage at 0730 h, little bit of straw at 1400 h, concentrate feed with a little bit of straw at 1800 h, and finally grass silage at 1930 h. Salt blocks and water were available ad libitum. The horses were weighed on a weekly basis minimally.

The study started after a two-months quarantine period at the research centre during which the horses walked daily for 45 mins in the walking machine. At the start of the experiment, horses had a mean body weight of 368 ± 45 kg. For organizational purposes, the study was performed in two different groups of six horses in two successive years. At the start of each year, the horses were divided in three pairs based on age. Of every pair, one horse was randomly selected for the intensified training program in period three.

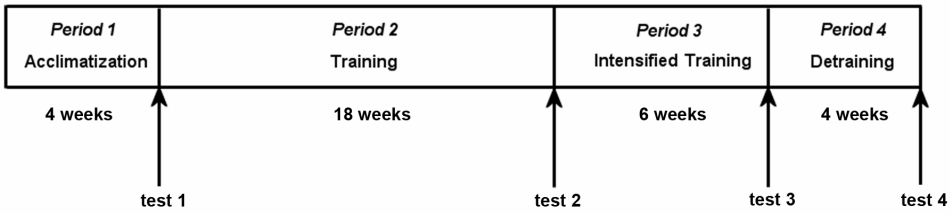

Figure 1. Schematic view of experimental set up divided into four periods. At the end of each period a test week was scheduled.

Table 1. Schedule of tests performed in test week at the end of each period.

Day of week	Morning	Afternoon	Evening/Night
Monday	Training	Behaviour	
Tuesday	Incremental Exercise Test (1-2)*		
Wednesday	Rest or training**		
Thursday	Training	EMG (1-4) Behaviour	
Friday	Standardized exercise test (1-4) Muscle biopsies (1-4) Routine blood examination (1-4)		GH-profile (1-4) followed by GHRH stim test*** (1-4)
Saturday	Rest		
Sunday	Rest or Training**		
Monday	Endoscopie stomach (1-4)	EHC (1-4)	
Tuesday	GHRH-stimulation test (1,3)	Training	
Wednesday	CRH-stimulation test (1,3)		

(periods in which test is performed), *only performed in first year
** Rest during period 1,2, and 4, training for the IT group period 3.
***only performed directly after GH profile in second year.
EHC = Euglycaemic hyperinsulinaemic clamp; EMG = Electromyography;
GHRH-stimulation test = Growth Hormone Releasing Hormone-stimulation test;
CRH = Corticotrophin Releasing Hormone stimulation test; GH-profile = Growth hormone profile.

Training protocol

Prior to the start of the experiment, the horses were acquainted with the Equine Exercise Laboratory and were acclimatized to running on the high-speed treadmill (Mustang 2000, Kagra, Graber AG, Fahrwangen, Switserland). The training period consisted of 32 weeks in total divided into four periods. A schematic representation of

these periods is provided in Figure 1. At the end of each period the horses underwent several assessments in one week, always in the same order (see Table 1)

The training period was divided into four periods. Since the horses had never been trained before, a low intensity basic training period of 4 weeks was applied first (period 1). The goal of this basic training period was to improve the aerobic capacity/general fitness and limb strength of the horses as well as to educate the horses. It has been suggested that the incidence of injuries is lower in horses that started with a basic training period before strenuous or interval exercise was started (199). Baseline values of the studied parameters were collected after this period.

During period 2, an alternating high intensity (HIT) and endurance training (ET) was performed. It has been shown that high intensity interval exercise is necessary to improve performance capacity to the maximum level (199). This type of exercise, however, increases the risks of injuries when performed on a daily basis. Alternating days of high intensity exercise and light/endurance exercise prevents the occurrence of such injuries, and provides sufficient recovery to enable the physiological systems to adapt. During the HIT exercise the number of repetitions should be kept as low as possible to prevent injuries as well. Three repetitions were proven to be sufficient for an increase in oxygen transport capacity and performance with the best result at an intensity of 85% HF_{max} (34). The differences in the number and duration of the repetitions during the HIT exercise were developed for the horses, to keep them alert and not anticipating too much to the training. The difference in endurance training was based on light exercise (60% HF_{max}) or moderate endurance exercise (75% HF_{max}).

The ultimate goal of the training protocol was to induce early overtraining during period 3 of the study. The training protocol was based on a previously validated protocol of early overtraining as described by Bruin et al (33), who showed that elucidating resting days from the training protocol was the step needed to decrease the performance of the already intensified trained horses. Therefore, the rest days were decreased to one in the first three weeks of period 3 and skipped during the last three weeks of period 3.

Period 4 is the detraining period or recovery period. The horses performed a low intensity endurance training to give them the opportunity to recover. Because evidence of overtraining can manifest after a period of rest, all the variables studied were also evaluated at the end of period 4 (Keizer, personal communication).

Trainingload

During period 1, the horses were acclimatised to the high-speed treadmill for four weeks. The horses of year 1 trained longer in period 1 which appeared to be too much for the young horses, they showed fatigue in the beginning of period two, which resulted in a different scheme in year 2 (number between brackets). Each training

session was preceded by 30 mins warm-up at the horse walker followed by 8 mins warm-up at the treadmill, which consisted of 4 mins at 1.6 m/s and 4 mins at 3.0-4.0 m/s, no incline. The training program during period 1 consisted of endurance training: week 1, 30% $HF_{est\text{-}max}$ for (20)-30 min 3 x week; week 2, 30% $HF_{est\text{-}max}$ for (25)-45 min 4 x week; week 3, 40% $HF_{est\text{-}max}$ for (30)-45 min 4 x week; and week 4, 50% $HF_{est\text{-}max}$ for (35)-45 min 4 x week. Each training session ended with a cooling down consisting of a 5 min walk at the treadmill followed by 30 mins walk at the walking machine.

In period 2, the horses received an 18-week training program of mixed endurance training (ET) and high intensity training (HIT). Days of ET were alternated with HIT. Each training session was preceded by 30 mins warm-up at the walking machine followed by 8 mins warm-up (4 mins at 1.6 m/s and 4 mins at 4.5 m/s) at the treadmill. The endurance running included 20-24 min of continuous level running at 60% $HF_{est\text{-}max}$ or 16-18 min at 75% $HF_{est\text{-}max}$. The interval training included three 3-min bouts at 80-85% $HF_{est\text{-}max}$ or four 2-min bouts at 80-85% $HF_{est\text{-}max}$ and interspersed with 3-min or 2-min periods at 60% $HF_{est\text{-}max}$. Each training session ended with a cooling down consisting of a 5 min walk at the treadmill followed by 30 mins walk at the walking machine. The horses exercised 4 days/wk throughout period 2.

In period 3, the horses were divided in a control group (C) and an intensified trained group (IT). The control group continued training at the volume and intensity they received in the second period for six weeks adapted from a previously validated protocol for overtraining as published by Bruin et al (33). For the IT, the intensified training regimen consisted of alternating days of HIT and ET for 6 days/week during the first 3 weeks followed by 7 days/week for the last 3 weeks. Each training session was preceded by 30 mins warm-up at the walking machine followed by 8 mins warm-up (4 mins at 1.6 m/s and 4 mins at 4.5 m/s) at the treadmill. Exercise intensity and volume during the ET was gradually increased to 24-35 mins 60-75% $HF_{est\text{-}max}$. High intensity exercise gradually increased to five 3-min bouts at 80-85% $HF_{est\text{-}max}$ interspersed with 2-min periods at 60% $HF_{est\text{-}max}$ or six 2-min bouts at 80-85% $HF_{est\text{-}max}$ interspersed with 1-min or 2-min periods at 60% $HF_{est\text{-}max}$.

In period 4, the horses received a 4-week training program of light endurance exercise for detraining. The horses performed endurance training for 20 min at 60% $HF_{est\text{-}max}$ for 3 days and 70% $HF_{est\text{-}max}$ for 1 day a week.

On the resting days the horses walked for 60 minutes at the walking machine throughout all periods.

Monitoring training

In order to standardize training to the individual exercise capacity of the horses, training and exercise intensity were adapted to the maximal individual heart frequency. For this purpose, an incremental exercise test was performed at the end of period 1. After thirty

minutes walking in a horse-walker, horses performed an incremental exercise test on a high-speed treadmill. The incremental exercise test started with a warm-up and thereafter horses trotted for 2 mins of 5 m/s, followed by 2-min at 6 m/s. Intensity was increased by 1 m/s every 2 minutes until the horses reached fatigue, which was defined as the speed where the horse could not keep up with the treadmill despite humane encouragement. Heart frequency was monitored with an on-line ECG recording (Cardio Perfect Stress 4.0, Cardio Perfect Inc, Atlanta, GA, USA) and an external heart rate meter (Polar S610, Kempele, Finland). In the first year, all six horses performed this test at the end of period one and period two, and reached a mean maximal heart frequency of 221±17 beats per minute (bpm). A plateau in heart frequency at maximal exercise intensity was, however, not observed in all animals. Therefore the measured maximal heart frequency can only be considered as average peak heart frequency. Based on the maximal heart frequency of horses that did reach a plateau in maximal heart frequency during the test (n = 3), and on measurements in a previous study with 2-year old Standarbred stallions (34), an estimated maximal heart frequency ($HF_{est\text{-}max}$) of 240 bpm was predicted. This $HF_{est\text{-}max}$ was used to guide training intensity (speed and inclination) on the treadmill, and was adjusted on a weekly basis to the measured peak heart frequencies during training. The incremental exercise test was because of coordination problems difficult to perform for the young, relatively untrained horses. Therefore, and also to minimize the risk of injuries during the incremental exercise test, the test was not performed in the second year group.

During each training session, temperature and humidity inside the training centre as well as the speed and incline of the treadmill, total distance covered during the exercise session and the heart frequencies were recorded for each horse.

Tests performed in test week

Routine measurements

Horses were weighed every week during period 1 and 2 and twice a week during period 3 and 4. Body temperature was measured as a daily morning routine.

Endoscopy of the stomach

One of the hypotheses was that the diminished appetite observed in other equine overtraining studies might have been caused by gastric ulceration due to the stress of the overtraining. Therefore, endoscopic examination of the stomach was performed in conscious horses after feed was withheld for 12 hours and water for 6 hours with a 3.5 meter Video gastro-endoscope. During the endoscopic procedure, the stomach was

distended by insufflation of air through the endoscope biopsy channel until the squamous and glandular regions of the gastric mucosa were visible.

Lesions scores were assigned to areas of the stomach (squamous mucosa, glandular body, antrum/pylorus, and duodenum) and scored as described by Murray et al (226). Grade 0 = intact epithelium with no apparent mucosal changes, grade 1 = mucosal reddening or squamous hyperkeratosis, grade 2 = small single or multifocal lesions, grade 3 = large single or multifocal lesions or extensive superficial lesions, and grade 4 = extensive lesions with apparent deep ulceration.

Standardized Exercise Test (SET)

SETs were performed at the end of each period in the test week (SET 1, 2, 3, 4). Those were combined with muscle biopsies before and after the SET. During period 2 & 3 the horses performed also SETs every other 3 or 4 weeks to measure performance improvement (SET 1a, 1b, 1c, 1d, 2a performed in week 9, 13, 17, 21, and 25, respectively). Those were not combined with muscle biopsies and blood was only sampled for lactate, pH, electrolytes, glucose and insulin during the SET at 0, 9, 14, 19, 24, 29, 34 and 39 minutes.

Before the SET started, horses walked for 30 minutes in the horse walker followed by a warming up at the treadmill that consisted of 4 mins walking (1.6 m/s) and four minutes of slow trotting (4.5 m/s) and one minute walking (1.6 m/s). This was immediately followed by the time trial procedure where the horses trotted for 20 minutes at a speed and inclination that elicited a heart frequency of approximately 80% of the HF_{estmax}. The cooling down consisted of walking on the treadmill for 5 minutes (1.6 m/s) followed by 30 minutes of walking in the horse walker. Body temperature was measured before the SET started and immediately after the cooling down on the treadmill. Heart frequency was monitored constantly with a Polar S610 and on-line ECG measurement. Speed and inclination of the treadmill could be adjusted to achieve the desired heart frequency. In period 3 & 4, the speed and inclination were not further increased. This made comparison between the tests possible. Venous blood was drawn from the jugular vein before the test (t=0 mins), after the warming up (t=9 mins), every 5 minutes during the SET (t=14, 19, 24, 29 mins), after the cooling down (t=34 mins) and 1,3,6 and 24 hours after the SET. Samples were kept on ice until heparinized whole blood lactate, glucose, electrolytes and pH had been analyzed (ABL-605 Radiometer Copenhagen, Westlake, Ohio, USA). Subsequently, the samples were centrifuged and plasma and serum were stored at –20°C for analysis of insulin.

Muscle biopsies

It was hypothesized that changes in muscle glycogen storage, activities of key metabolic skeletal muscle enzymes and muscle substrate transport proteins as well as

muscle protein expression and/or activation upon intensified training might be suggestive of early markers of overtraining. Therefore, the enzyme activities of 3-hydroxy-acyl dehydrogenase (HAD), citrate synthase (CS), hexokinase and the expression of monocarboxylate transporters (MCTs) were investigated after (intensified) training. CS is mainly used as a marker for mitochondrial density in muscles. HAD does reflect mitochondrial potential for fatty acid oxidation. Hexokinase is involved in muscle glucose transport. MCT's are involved in lactate transport (325). In addition, the localization and activation of the signaling proteins protein kinase C (PKC), extracellular signal-regulated kinase (ERK), p38 mitogen activated protein kinase (MAPK), Jun N terminal kinase (JNK) and heat shock protein (HSP)27 were investigated after a period of (intensified) training. Proteomic technology was used to elucidate (intensified) training-induced muscle adaptation at the protein level (329).
In order to study the influence of training and intensified training on muscle metabolism, before and immediately after the SET muscle biopsies were taken under local anesthesia (lidocain hydrochloride 2% without adrenalin) using a modified (for increased tissue yield) Bergström biopsy needle (Maastricht instruments, Maastricht, The Netherlands) with a diameter of 7 mm. Approximately 60 minutes before the SET, a 5 cm deep biopsy of the M. vastus lateralis (VL) was taken at a point 15 cm ventral to the centre of the tuber coxae and 7 cm caudal to the cranial border of the VL muscle. Also a 4 cm deep biopsy was taken at a point 20 cm caudal to a line extending through the shoulder joints in the middle of the muscle from the M. pectoralis descendens (PD). These muscles were selected for their easy accessibility for biopsy procedures and EMG analysis. Furthermore, based on previous studies in different horse breeds the muscles were expected to differ in muscle fiber type distribution, the VL has more type II and the PD has more type I fibres (8, 188). Both muscles play a role in propulsion, which made them good candidates to measure influences of training on muscle metabolism. The M. gluteus medius appeared to give a bad EMG signal and was therefore not used in this study (325). Within thirty minutes after cessation of the SET a second muscle biopsy was obtained from a new incision 3 cm caudal to the point of the first biopsy. Blood and fat tissue were carefully removed from the biopsy and the biopsy was thereafter split in two. One part was frozen in isopentane cooled to its melting point in liquid nitrogen for immuno-histochemical procedures and another part of the biopsy was immediately frozen in liquid nitrogen for biochemical procedures. Frozen muscle tissue was stored at –80°C (325).

Routine blood examination
In the test week, on the day of the SETs 1, 2, 3, and 4, a routine blood examination was performed to check the horses health and performance. Blood was sampled at t=0 mins for determination of WBC and differentiation, activity of liver enzymes gamma-glutamyl

transferase (GGT) and alkaline fosfatase (AF), activity of muscle enzymes creatine kinase (CK), lactate dehydrogenase (LDH) and aspartate aminotransferase (ASAT), ammonia, NEFAs, ß-hydroxybutyrate, vitamin E, Selenium, glutatione peroxidase (GSH-PX), and IGF-1. Ammonia was also sampled at the end of the SET before the cooling down at t=29 mins. Blood was collected for determination of NEFAs, ß-hydroxybutyrate and activity of muscle enzymes at t=29 mins, and 1, 3, 6, and 24 hours after the SET. Subsequently, the samples were centrifuged and plasma and serum were stored at –20°C for later analyses.

Outside the test week, on the day of the SETs 1a, 1b, 1c, 1d, and 2a activity of muscle enzymes (CK, LDH and ASAT) were determined in blood samples collected at 0 and 180 minutes after the SETs.

Electromyography (EMG)

In needle electromyography examination the electrical activity generated by the motor unit is recorded and analyzed. As neurological adaptations and motor unit firing patterns are among the first adaptations after training (103), needle EMG can provide valuable information on neuromuscular adaptations to training. Furthermore, needle EMG provides one means of discriminating between neurogenic and myogenic adaptation that might occur with an intensified training regimen.

At the end of each training period, quantitative needle electromyographic (EMG) analysis was performed of the vastus lateralis (VL), pectoralis descendens (PD) and subclavian (SCL) muscle. After applying a small amount of local anaesthetic (lidocain hydrochloride 2% without adrenalin) a probe connected to a thermometer was inserted through a 16 gauge, 60 mm needle into a muscle to record intramuscular temperature. EMG recordings were made using a disposable concentric needle (50-100 mm) (Nicolet Biomedical Inc., Madison, WI, USA) with a sampling area of 0.68 mm^2. A portable EMG apparatus (Nicolet Meridian, Nicolet Biomedical Inc., Madison, WI, USA) was used for the recording of EMG. Amplifier gain was 50-100 µV for spontaneous activity and 10-500 µV for MUP recording. At least 3 insertions and three directions per insertion were made per investigated muscle. Insertional activity, pathological spontaneous activity, motor unit action potentials and satellite potentials were recorded. Spontaneous activity was assessed outside the endplate region in the same regions in which motor unit action potentials (MUPs) were obtained. Per muscle 20-30 MUPs were analysed. To be analysed, a MUP had to have a rise time of maximally 0.80 ms and had to occur repeatedly at least four times. For each semi automatically analysed MUP, reproducibility was checked by superimposition of at least four waveforms. Analysis of waveform was checked manually. EMG examination in conscious horses took place in stocks.

Endocrinological and behavioural tests
Alterations in the sensitivity of the hypothalamic-pituitary-adrenal (HPA)-axis to human corticotropin releasing hormone (CRH) after intensified training was studied, however results will be presented elsewhere (not in this thesis). The influence of training and intensified training on the GH-IGF-1 axis was studied. Results are described in Chapter 5. The influence of training and intensified training on *in vivo* insulin sensitivity was examined by use of the euglycaemic-hyperinsulinaemic clamp technique. Results are described in Chapter 8 and 9. Three tests to assess the influence of training and intensified training on behaviour were used. Results are described in Chapter 10.

Results

One couple out of six was unable to finish the experiment due to injuries. They were removed from the study after period 2 in the second year.

Routine measurements
All horses gained weight during the experiment. No significant differences in weight between control and intensified trained horses were found during period 3.

Endoscopy of the stomach
The fasting period needed for the clamp was combined with the fasting period needed for the stomach endoscopy to minimize the risk of inducing gastric ulceration by fasting. Evaluation of the stomach revealed only minor changes (grade 1 or 2) in a couple of horses, most horses showed no changes at all (see Table 2). No deterioration of the lesions was observed during the experiment. In addition, diminished feed intake was not observed in any of the horses during this study.

Table 2. Results of endoscopy of the stomach showed as the number of horses with different lesion scores at the end of each period.

Lesion scores ***Period***	***0***	***1***	***2***	***3***	***4***
1	6	1	3	0	0
2	7	3	0	0	0
3	7	2 (1 C, 1 IT)	1 (C)	0	0
4	10	0	0	0	0

C=control group, IT = intensified trained group

Standardized Exercise Test

The SETs were performed throughout the experiment to monitor performance. The performance of the horses increased upon training, as observed from the later time trial at higher speeds and inclination at the end of period 2 (SET 2) compared to period 1 (SET 1).

In the intensified trained group, the mean duration of SET 3 was decreased compared to SET 2 with 12%. One IT horse actually stopped during SET 3. Three IT horses changed their gate at the planned speeds during training at period 3 and SET 3; they were unable to continue trotting and started cantering. The IT horses maintained trotting at high speeds during SET 3 for 15.8±2.1 minutes only as compared with 19.2±1.6 minutes (P=0.036) in control horses. IT horses started galloping frequently at the beginning of SET 3 despite human encouragement to trot or stopped. As a consequence, the mean duration of trotting during SET 3 was decreased significantly by 18% in the overtrained group as compared with control horses. One IT horse only wanted to trot at the end of treadmill, we were unable to keep him in front of the treadmill during the training at the end of period 3 and SET 3.

A right shift of the lactate curve during SET 3 compared to SET 2 was observed in both groups (see Figure 2) possibly indicating an adaptation to longer training. However, the right shift of the lactate curve was still observed during SET 4 for the intensified trained group and not for the control group (Figure 3) which might indicate that the IT group adapted better to training than the control group due to the intensified training program during period 3. Another explanation could be that the right shift shows the lactate paradox seen in the parasympathetic form of overtraining in the IT group and that the recovery period was not long enough to compensate for the effects of overtraining.

Muscle biopsies

No effects of training were found on MCT, HAD and CS activities. Hexokinase activity decreased significantly after period 2. The intensified training significantly increased CS activity in the IT group versus control group. In contrast, HAD and hexokinase activity increased significantly as well during period 3, however, in both control and IT group. No effect of training or intensified training was observed in muscle glycogen concentrations (325).

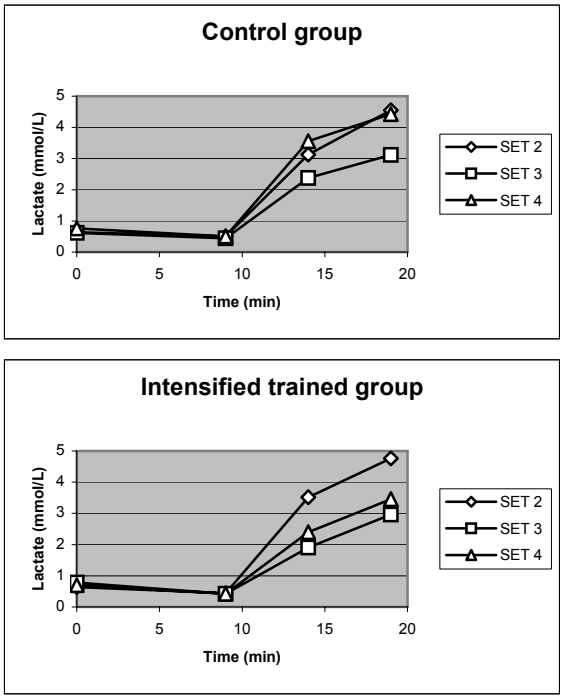

Figure 2. Lactate curves of SET 2, 3 and 4 presented as mean concentrations at the first 10 minutes of trot (t = 9, 14 and 19 mins) in the control and intensified trained group.

The most remarkable findings were the changes in the skeletal muscle proteome upon intensified training. Analysis of the differential expression of muscle proteins in horses before and after normal or intensified training yielded a group of 7 proteins that showed an increase in expression after intensified training. Their exact identity and biological relevance has to be established in future studies. Exercise training did not result in a significant change in MHC distribution. A period of normal or intensified training had no effect on p38 MAPK, JNK or HSP 27 protein expression or exercise-induced HSP27 phosphorylation. Basal p38 phosphorylation is decreased upon training. The latter finding might be indicative for the beneficial effects of training on basal stress levels in equine skeletal muscle (329).

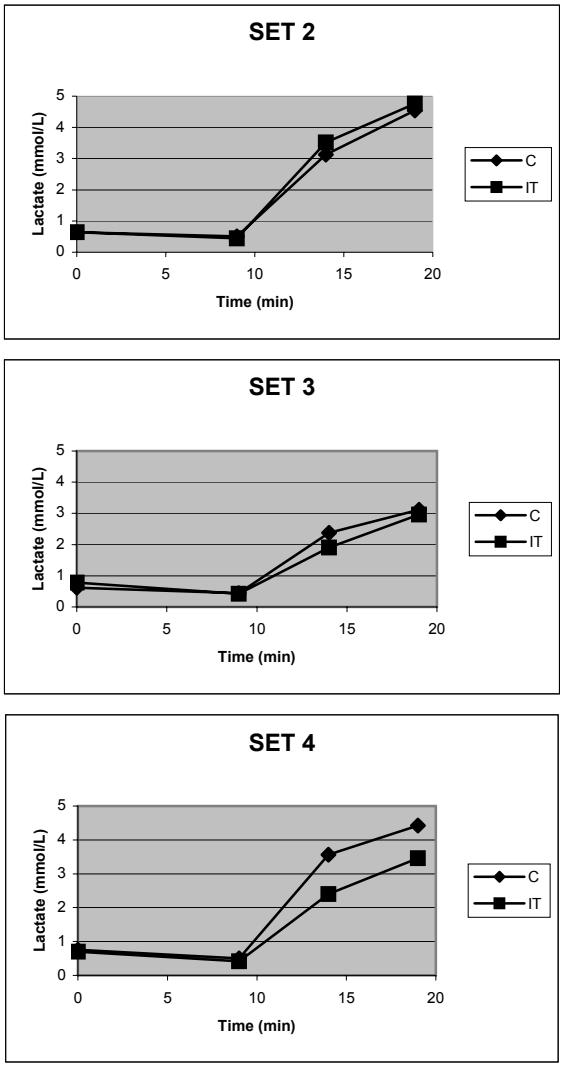

Figure 3. Lactate curves of the control and the intensified trained group presented as mean concentrations at the first 10 minutes of trot during the different SETs for control group (C) and intensified trained group (IT).

These results from the muscle biopsies led to the conclusion that most of the adaptations found were related to normal physiological training adaptations even in the intensified training period. Moreover, horses that were trained according to our

intensified training schedule followed physiological adaptation more closely than the horses in the control group. It is therefore possible that the equine skeletal muscle is resistant or responds later to the amount of stress induced by the intensified training protocol used in this study.

Routine blood examination
All horses remained within the reference ranges for WBC/differentiation and the activities of liver enzymes at all periods. Activity of muscle enzymes did not indicate muscle damage at any point measured.

EMG
Needle EMG was performed for two reasons. Firstly, to examine the horses for possible myopathies due to mechanical overloading, (a potential complication of intensified training program) and secondly, to elucidate adaptations of the motor unit system to training and intensified training.

Needle EMG can measure the electrical stimulus of the motor unit, which results in muscle contraction and subsequent force generation of the muscle fibre. In the recorded signal the motor unit action potential (MUP) amplitude is positively influenced by both hypertrophy and increased neuromuscular excitability, whereas MUP variables as duration, period and turns are influenced by synchronization of the MU. EMG can distinguish myopathic and neurogenic changes in the muscle. In the current study, no signs of myopathic changes were found at any time in any of the horses. However, in one couple, both horses showed neurophatic changes on their EMG recordings at all SETs indicating the presence of a neuropathy, which might have influenced some results. For all horses, MUP variables showed a significant training adaptation after period 2. Intensified training induced a stronger training adaptation in MUP variables, however no differences between control and intensified trained group were observed. There were no signs of overtraining apparent in the selected EMG parameters (325).

Final remarks

So far, no overtraining at the neuromuscular level could be demonstrated in this study. However, since the OTS is described as a neuroendocrine disorder the expectation is that central changes might precede peripheral changes. This thesis will focus on the neuro-endocrinological and behavioural adaptations to training and intensified training.

Chapter 3

Hormonal responses to acute exercise, training and overtraining: a review with emphasis on the horse

Ellen de Graaf-Roelfsema[1]
Hans A. Keizer[2]
Eric van Breda[3]
Inge D. Wijnberg[1]
Johannes H. van der Kolk[1]

[1]Department of Equine Sciences, Medicine Section, Faculty of Veterinary Medicine, Utrecht University, The Netherlands. [2]Department of Human Physiology and Sportsmedicine, Free University of Brussels, Brussel, Belgium. [3]Department of Movement Sciences, Faculty of Health Sciences, University of Maastricht, The Netherlands.

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Abstract

Overtraining is an imbalance between training and recovery leading to symptoms associated with a neuroendocrine dysbalance called the overtraining syndrome, a disease characterized by behavioral, emotional and physical symptoms similar with depression. Although the prevalence of overtraining is high in human and equine athletes, at present no sensitive and specific test is available to prevent or diagnose overtraining. Nowadays, it is believed that combination of different (hormonal) parameters appear to be the best indicators of overtraining. Therefore, this review provides a summary of previous literature examining the response of the HPA axis and the GH-IGF-I axis to acute and chronic exercise as well as overtraining in humans and horses. The exercise induced hormonal responses seem to be equal for the equine as well as the human athlete, which makes comparisons possible. Repeated bouts of exercise are suggested to provide a way to detect subtle changes in hormonal responses in the individual athlete, which may make them an important tool in detecting early overtraining. This should be combined with CRH stimulation tests and basal ACTH and GH pulsatility determination. Further research is needed to establish the correct training intensity and rest period for the exercise test in equines.

Introduction

In general, stress occurs when body homeostatic balance is disturbed (279, 287, 288, 291). When the body experiences environmental or internal stressors it responds with secreting a whole array of hormones to reestablish homeostatic balance. Regenerative processes continue after restoration of homeostatic balance such that, if the same stressor were imposed again, the homeostatic mechanisms would not be displaced to the same extent, resulting in overcompensation (289). The overcompensation is seen as positive stress.

One example of an acute stressor is exercise. Each exercise bout induces an acute disturbance of homeostasis in cells and organs, which may result in decreased mechanical output and fatigue (179). In the recovery phase, homeostasis is reestablished and supercompensation occurs. Ideally, a subsequent overload exercise bout should not take place until supercompensation has occurred, which implicates that sufficient time for recovery must be incorporated in the training programme (179). If training starts before recovery and adaptation has occurred, the athlete is in danger for overtraining. At an early stage, overtraining is only reflected by increased fatigue and decreased performance. Recovery and supercompensation will usually occur within 1-2 weeks with decreased training; this stage is called overreaching (OR). When the imbalance between training and recovery exists for a longer period, the homeostatic disturbance at the cellular level will be expressed at the somatic (whole body) level, and be recognized as a disease (232). This state is called the overtraining syndrome (OTS) associated with behavioral, emotional and physical symptoms as shown in Table 1 (102, 179, 320).

Table 1. Clinical findings associated with overreaching and/or the overtraining syndrome (102, 179, 320).

Symptoms associated with overtraining
Decreased performance
Lethargy, depression
Poor appetite
Weight loss
Mental instability and irritability, anxiety
Loss of competitive drive
Menstrual irregularities
Sleep disturbances
Increased susceptibility to and severity of illness/colds/allergies
Changes in heart rate at rest, exercise and recovery
Changes in blood pressure
Low plasma lactates during submaximal and maximal exericse (lactate paradox)

The major objective in exercise training is to cause biologic adaptations to improve performance in specific tasks. The training response is highly specific and characterized by the so-called specificity principle. In aerobic or endurance training for example the mode of training must overload the appropriate muscles as well as the cardiovascular system. On the other hand in jumping events lasting around a minute, anaerobic power, strength of particular muscles and neuromuscular coordination determine performance. If the principle of specificity is not accomplished, performance enhancement cannot be anticipated. In addition, a specific exercise intensity or overload must be applied in order to force the body to adaptation. The latter type of training is called ‘overload’ training (179). If the exercise intensity is too low no gain in performance can be anticipated (179, 203). The exercise intensity is the most important factor to increase performance and should be well above ‘normal’. An appropriate overload for each subject or animal can be achieved by manipulating or varying the training frequency, intensity, and duration both within one training session as well as between training sessions. The product of these training modalities determines the endogenous load. If a training program is well constructed on an individual basis, and as a consequence the endogenous training load is optimized, recovery and adaptation will occur within 3-7 days (179). If however, the alternation of training load and rest is shifted towards the former, the athlete comes in a situation of chronic overload, which results in a delayed adaptation process if any.

Very short exercise bouts for a few seconds usually rely on ATP as an energy source, whereas maximal exercise bouts of about 10 seconds mainly rely on ATP and creatine phosphate breakdown besides the anaerobic breakdown of muscle glycogen. During maximal exercise bouts of longer duration (for example 20-60 seconds) the energy for muscle contraction is derived predominantly from anaerobic breakdown of muscle glycogen. It is of importance to realize that only the relative contribution of each energy source varies according to the exercise intensity and duration rather than one or more of these being totally switched off (203).

The hormonal events during reestablishment of homeostasis due to (exercise) stress can be divided into two phases. Initially, a catabolic phase can be distinguished, with decreased tolerance of effort, characterized by reversible biochemical, hormonal and immunological changes. The two main hormonal axes activated in this phase are the sympathetic-adrenal medullary (SAM) axis and the hypothalamic-pituitary-adrenocortical (HPA) axis (10). An anabolic phase follows with a higher adaptive capacity and enhanced performance capacity, in which both the GH-IGF-I axis as well as the gonadal-axis are activated (320). When homeostatic balance is not restored, the body experiences chronic stress which induces chronic activation of the endocrine system and possibly ending in a neuroendocrine disorder like for instance burn out, chronic fatigue syndrome or OTS.

A neuroendocrine disorder at the hypothalamic-pituitary level has been suggested as the underlying cause for the overtraining syndrome (10, 16, 185). Many researchers have attempted to describe the mechanism involved in OTS by observing the hormonal changes during overreaching and overtraining (11, 86, 112, 118, 185, 186, 190, 357). So far, two types of OTS are described, i.e. the sympathetic and the parasympathetic type (179, 185, 323). It is yet to be established whether these two types are different phases of the same syndrome or two different syndromes (179, 323).

In athletic training, the workloads of exercise sessions are gradually increased (called overload training) to provide an optimal stimulus for adaptation and supercompensation, which gradually improves performance (179). The optimal workload is difficult to determine for athletes and coaches, which may explain the high incidence of OTS in athletes. Signs and symptoms of the OTS appeared in over 60% of distance runners during their athletic careers (223), more than 50% of professional soccer players during a 5-month competition season (186), and 33% of basketball players participating in a 6-week training camp (10, 342).

Overtraining, overreaching and OTS was also observed in (race)horses (20, 33, 179, 237, 298). Nowadays, equine athletes experience a lot of exercise stress, due to an increased number of competitions and therefore more and longer transportations, environmental changes, etc. Because horses cannot complain, finding the optimum training load and preventing OR/OTS is even more difficult in horses than it is for humans. Since exercise stress is a culmination of several different stressors, including physiological, environmental, social and psychological stressors (179), not only highly trained (race)horses, but also horses involved in daily intensive or monotone exercise and competition might suffer from overreaching or overtraining (96). It is very likely that some degree of overtraining occurs more frequently than is recognized in horses, and is responsible for a minor (but financially significant in racehorses for instance) reduction in performance (20). The occurrence of OR/OTS might be severely underestimated in equine practice and every horse referred for complaints of loss of performance without obvious other diseases should be considered as possibly suffering from OR/OTS.

So far, no single diagnostic parameter has been identified for OR and OTS in humans or equines and so far a combination of different (hormonal) parameters appear to be the best indicators of OR and OTS (145, 265). Until recently most studies of overtraining in horses focused on a phenomenon referred to as red cell hypervolaemia, characterized by an excess of red blood cells that limited performance (209, 237). However, red blood cell volume did not increase in horses that were overtrained (33, 114, 127) and attention is now drawn to investigation of hypothalamic-pituitary dysfunction in overtrained horses (33, 114, 127).

The equine studies generally focused on the cortisol response to (over)training. Cortisol plays an important role during the catabolic phase. However, hormonal changes during the catabolic phase influence the hormonal response during the anabolic phase (233). It is thus important to study both the catabolic and anabolic hormonal responses to exercise and during recovery. A good hormonal representative for the anabolic phase is Growth hormone (GH). The GH-IGF-I response to exercise is well described in humans, but not in horses (172, 215, 230, 353, 361). However, recently, GH drew the attention of equine researchers as well, albeit not for its role in overtraining, but for its role in the anti-aging process and its possible performance improvement or therapeutical potential (61, 77, 110, 121, 194, 208, 275). Nevertheless, some studies provide information about the acute changes in GH-IGF-I axis due to a bout of exercise (43, 115, 313, 314).

So far, little information is available as to how the hypothalamic-pituitary-axis behaves in equines during exercise/training/OTS. Further research is needed to describe these hormonal changes and provide reference values useful for diagnosing overreaching and the overtraining syndrome in horses. Therefore, this review will focus on the knowledge around the hormonal disturbances during overreaching and OTS in humans and compare it to the data found in horses. Recommendations for tests to optimize diagnosing early overtraining in horses based on the current knowledge in humans will be given. This review will focus on the effects of endurance type of training and not on a resistance type of training.

Hypothalamic-Pituitary-Adrenal axis: CRH, ACTH and Cortisol

Regulation of the HPA axis

Stimulation of the HPA axis starts with the excitation of the hypothalamus by different types of stress. Stress stimuli activate the entire system to cause rapid release of cortisol, and cortisol in turn initiates a series of metabolic effects directed toward relieving the damaging nature of the stressful state (124). The limbic system controls stress and influences the HPA axis either by stimulating or inhibiting ACTH and cortisol release (137). Corticotropin Releasing Hormone (CRH) released from the hypothalamus controls the secretion of ACTH by the anterior pituitary gland. Vasopressin (Arginine vasopressin, AVP) seems to be another controller of ACTH secretion in humans and horses (5, 174). Pituitary venous blood samples from conscious horses revealed that AVP is the primary signal driving short-term fluctuations in ACTH, as CRH plays a permissive role in regulating ACTH secretion; low CRH concentrations suffice to potentiate the ACTH response to AVP. Also, exercise induced ACTH levels are mainly regulated by AVP in horses (3, 5).

ACTH controls the secretion of cortisol by the adrenal cortex. Cortisol has a direct negative-feedback effect on the hypothalamus to decrease formation of CRH and on the anterior pituitary gland to decrease the formation of ACTH. The intracellular transduction pathways used by AVP are less susceptible to glucocorticoid negative feedback (5). Another controler of the HPA-axis is the limbic system, whereby the hippocampus and prefrontal cortex are largely inhibitory to HPA axis secretion, whereas the amygdala is implicated in acitivation of glucocorticoid secretion in humans. Limbic dysfunction is highly associated with stress-related disorders

Determination of HPA axis activity at rest offers several possibilities. Peripheral blood samples can be collected for measuring basal ACTH and cortisol concentrations. Pituitary venous blood samples can be easily collected by means of catheterization of the V. Facialis in the horse for determining basal pituitary CRH, AVP and ACTH concentration, which provide more detailed information on the activity of the hypothalamus and the pituitary gland (5, 148). Stimulation of the HPA axis can be performed by for instance administration of exogenous CRH or ACTH or acute exercise. However, before interpreting the blood results, several factors influencing the blood levels of HPA hormones should be taken into account, e.g. hormonal secretion rhythms, the amount of hormone bound with plasma proteins (278), varying densities of receptors (i.e. corticosteroid receptor type I and II) in target tissues (28) and prereceptor metabolism of cortisol by tissue-specific enzymes, i.e. 11-B-hydroxysteroid dehydrogenase (11-ß-HSD) (5, 11, 279).

Basal plasma ACTH secretion in the horse is characterized by three distinct rhythms: a circadian rhythm, an ultradian rhythm and a high frequency micropulsatility secretion. The latter is only measurable in venous pituitary blood (5).

Basal plasma cortisol secretion in humans and horses is characterized by two rhythms, firstly a circadian rhythm with peak levels in the early morning and a nadir in the late afternoon/evening and secondly an ultradian rhythm with mean cortisol peak frequency in the horse of 0.56 +/- 0.03 per hour (5, 171). The circadian rhythm can be easily disrupted by physical and psychological stress which results in elevated cortisol levels during the normal trough and a consequent damping of the daily rhythm (149).

Social stress is reported to decrease the binding capacity of Cortisol Binding Globulin (CBG) in horses, which resulted in increased free cortisol concentrations associated with normal total cortisol (3).

Two isoenzymes of 11-ß-HSD interconvert hormonally active cortisol and inactive cortisone within target cells. They have been shown to modulate the action of the hormone at an autocrine level in several peripheral tissues 11-ß-HSD-2 inactivates cortisol to cortisone, and is mainly expressed in the kidney in which it protects the mineralocorticoid receptor from cortisol excess. 11-ß-HSD-1 is expressed in numerous tissues where it converts the inactive cortisone to the active cortisol (11, 279).

Thus, plasma concentrations of total cortisol may not accurately reflect changes in the HPA axis status in humans and horses. Stimulation of the HPA axis with for instance CRF and ACTH might provide more accurate information.

Actions of CRH, ACTH, Cortisol

CRH is more than a releasing factor alone. CRH is considered an integrator of the (neuro)endocrine, autonomic, and behavioral responses to stress (174). CRH has also profound effect on mood-state and it stimulates cytokine production in peripheral tissues.

ACTH increases cortisol synthesis and the amount of low-density lipoprotein (LDL) receptors in the adrenal cortex. Extra-adrenal effects of ACTH involve increases in glucose and amino acid uptake by skeletal muscle and stimulation of lipolysis in the adipocyte (174).

Cortisol has a wide spectrum of functions in metabolic control. Activation of catabolic processes, anti-anabolic and anabolic processes are included and they are geared towards sparing glycogen and increasing protein synthesis. Cortisol stimulates substrate mobilization by enhancing gluconeogenesis and the mobilization of free fatty acids (anabolic effect). At same time, cortisol release will decrease glucose utilization by some tissues, sparing it for use by the CNS. The purpose of the catabolic changes is to create an increased pool of free amino acids (24). In part, this is caused by the competition of glucocorticoids with androgen receptors in skeletal muscle (174). During exercise, the branched chain amino acids can be used as an additional source of substrate oxidation when glucose level begins to drop. After exercise, in the late recovery phase, the increased pool of free amino acids is available for protein synthesis. RNA synthesis and thus protein synthesis has been shown to influence the work capacity in rats directly (343). Thus, the catabolic processes have to be initiated during exercise in order to provide a stimulus for adaptation after exercise, i.e. protein synthesis (202, 295, 344).

Another important cortisol effect during exercise is the increase in arousal (280), and the induction of enzymes of catecholamine synthesis (201, 247). In addition, cortisol also modulates immune function, acts as an anti-inflammatory agent and suppresses immune reactions (124, 210). In conclusion, the view to see cortisol as a mere stress hormone is only a simplification.

Acute exercise and response of HPA axis

Most exercise studies conclude that both high intensity and endurance exercises cause an increase in plasma ACTH and cortisol in humans (57, 84). However, the exercise-induced plasma cortisol increase may not be directly related to the exercise-induced ACTH increase, because both hormones show a different exercise related response.

ACTH increases curvilinear with exercise intensity, while cortisol increases more with increasing exercise duration. In horses, the relationship between relative work intensity and plasma cortisol concentrations was studied during an incremental exercise test (157). A significant increase in plasma cortisol concentration was found following exercise. However there was no relationship with relative work intensity or blood lactate concentration. The suggestion was made that the exercise test was too short (total time test 270 s) to induce a consequent increase in cortisol concentration. However, another study in horses confirmed that there was no detectable relationship between cortisol and relative work intensity and in addition showed that there was no significant relationship between plasma ACTH and cortisol concentrations during exercise (227). The hormonal responses to an incremental exercise test until exhaustion and two relative workload treadmill exercise tests (105% and 80% VO_2 max till exhaustion) were evaluated in this study. All exercises produced significant increases in plasma ACTH and cortisol concentrations. ACTH continued to increase with exercise intensity and duration, but cortisol remained constant after the initial increase and showed a relationship to the exercise duration (e.g. peak cortisol was highest at 80% VO_2 max with total run time of 12 minutes compared to 105% VO_2 max with total run time of 4 minutes).

In general, for humans and horses, high intensity exercise results in a 2-3 fold increase in cortisol, peaking usually 15-30 minutes after exercise, and returning to pre-exercise levels within the hour (198, 210). Endurance exercise usually requires duration of exercise of more than 20 minutes with an intensity of at least 60% of the VO_2 max to obtain a significant increase in cortisol (176, 210, 211, 320). High intensity exercise results in an increase in ACTH, usually peaking the end of exercise and returning to baseline within 60-120 minutes. Prolonged submaximal exercise or endurance exercise shows a later peak in plasma cortisol and a slower return to pre-exercise levels than high intensity exercise (198).

Several factors influence the exercise-induced cortisol response, e.g. circadian rhythm, multiple exercise bouts, high intensity versus prolonged submaximal exercise, nutrition, and training. The existence of a circadian modulation of cortisol to exercise is demonstrated in several studies (171, 283). Kanaley (171) found in men that peak cortisol concentrations were highest after moderate to intense exercise (30 min 85% VO_2 max treadmill test) at 0700 versus 1900 and 2400 h. In contrast, maximal increases in cortisol concentrations over time in comparison to control day conditions occurred at 2400 h. Scheen et al (283) found a significant stimulatory effect of exercise on cortisol levels only in the afternoon and not in the early morning or during the night after performing low intensity exercise (3h at 40-60% VO_2 max). However, the intensity of exercise might have been too low to induce a good response in this study.

Multiple bouts of acute exercise and response of HPA axis
Repeated bouts of high-intensity endurance exercise (2 bouts of 65 min 70% VO_2 max, 3h rest in between) resulted in increased plasma concentrations of ACTH and cortisol during exercise and early recovery (274). Two bouts of high intensive exercise with a 4h rest period in between showed an exaggerated response for ACTH and cortisol after the second bout. In addition, the first bout of exercise was not able to induce an increase in cortisol concentration (213). Two bouts of moderate intensity exercise (30 min 50% VO_2 max, 45 min rest in between) did not change the cortisol response to the second bout of exercise, four bouts of moderate intensity (30 min, 50% VO_2 max, 30 min rest) progressively increased concentrations of cortisol throughout the series of exercise bouts. However, cortisol concentrations were still augmented during the rests in the last study, which may partly account for the progressive increase (29,169). Galassetti et al (104) found a blunted cortisol response after the second bout of exercise (50% VO_2 max, 90-min, 3-h rest) in men and an increased cortisol response in women. Comparison to other experiments is difficult, because the athlete's plasma glucose was maintained at euglycaemia during the experiment by glucose infusions, which may have altered the results.

Recovery phase and response of HPA axis
Some studies investigated the HPA axis during long-term recovery after exercise. Kern et al (176) investigated the influence of daytime exercise on the secretory pattern of sleep-related cortisol release in men and found that after long-duration moderate intensity exercise (120-150 km biking at 60% HFmax) cortisol levels were increased, followed by significant decreases compared to control conditions 6-10 hours post exercise. They explained the decrease by a prolonged negative feedback of the high cortisol levels after exercise, which disrupted the normal secretion pattern. Golland et al (116) studied the cortisol response after an incremental exercise test till exhaustion and during a recovery period of 32 hours in 4 geldings. A significant increase of mean plasma cortisol concentration after exercise was found, followed by a significant decrease 4-8 hours post exercise and a significant increase 20-24 hours post exercise compared to basal levels, the same alterations as Kern et al (176) found in men.

Summarized, a similar exercise-induced response of the HPA-axis is found in humans and horses. Combined results found in both species lead to the following conclusion. To study the response of the HPA axis to an acute bout of exercise, a submaximal exercise bout of about 30 min of 70-85% VO_2 max can be used to induce a reliable increase in cortisol and ACTH. Repeated bouts of sufficient exercise should exaggerate this response, which is easy to measure. And therefore seems to be more useful in the individual athlete, because testing circumstances will vary, which hampers determining

reference values for the initial response in the individual. A fixed time of the day should be used to compare individuals to prevent biases due to the circadian rhythm of the HPA axis and there should be at least 24 hours between the last training and the planned exercise test.

Chronic exercise and response of HPA axis

Most studies find elevated ACTH levels at rest in trained subjects versus untrained subjects (135, 189, 363). Mostly, basal cortisol levels are not elevated (81, 167), which might indicate that the adrenal gland is less sensitive to ACTH due to training. This is supported by the findings of Rietjens et al (265), who found a decreased cortisol response to a CRH administration after two weeks of intensified training (no resting days combined with doubled training volume and 15% increase in intensity) in well-trained athletes.

Duclos et al (81) describe a decreased pituitary sensitivity to glucocorticoids in endurance-trained men (50-70 km running per wk, completed marathon in less than 3h30min) compared to sedentary men (<1h physical activity per wk). At rest, 3 out of 9 endurance trained men responded with an increase in ACTH and cortisol after administration of oCRH while at the mean time dexamethason was given to suppress endogenous cortisol secretion. The sedentary group as well as 5 out of 9 endurance trained athletes did not respond to an injection with oCRH. Basal cortisol and ACTH levels were not significantly different between both groups. It was suggested that the decreased pituitary sensitivity represents an intermediary stage that has the potential to progress towards a decreased sensitivity of the adrenal gland eventually or it might be an endpoint of successful acclimation to exercise training.

Studies reporting on the effect of training on the response of the HPA axis to acute exercise differ in their results, showing increased responses as well as blunted responses of ACTH and cortisol after exercise which might reflect different stages of adaptation of the HPA axis to chronic stress induced by training (57, 167, 211). Elevated plasma ACTH responses to exercise in trained athletes were reported by Farrel et al (91) and Duclos et al (82). A blunted ACTH response to an incremental treadmill running test till exhaustion was reported by Heitkamp et al (135) in 23 untrained women after a training period of 8 weeks combined with significantly increased basal concentrations of ACTH after the training period. Luger et al (189) also found a blunted response of ACTH and cortisol to exercise in highly as well as moderate trained runners compared to sedentary subjects. Only the highly trained runners showed basal elevated levels for ACTH and cortisol and a blunted response of ACTH and cortisol to administration of oCRH.

Studies looking at overreaching show similar results of HPA axis adaptation as studies looking at overtraining, which makes it even more complicated to distinguish between normal physiologic adaptation to training and overreaching and overtraining.

The same problem occurs in data from studies in equine athletes. Researchers reporting on HPA axis adaptation to training show the same results as researchers reporting on HPA axis adaptation to short-term overtraining.

Marc et al (197) found a blunted cortisol response following ACTH administration and a significant blunted cortisol response following a standardized exercise treadmill test in trained horses compared to untrained horses, which is indicative of decreased adrenal sensitivity. Golland et al (115) studied the effects of training and short-term overtraining (10 days of higher intensities and longer duration training with only 1 day of rest) on plasma cortisol concentrations at rest, after a standardized exercise test and after stimulation with ACTH in horses. In the overtrained group, a blunted cortisol response to a standardized treadmill exercise test was found compared to the trained group. However, when the peak cortisol concentration after exercise was corrected for the pre-exercise cortisol concentration, the increment was significant for all horse over time, however there was a trend for the overtrained group. In the trained group, a blunted cortisol response to stimulation with ACTH was found. Both results are indicative of decreased adrenal sensitivity. Two other studies reporting on the effects of overtraining on the HPA axis show decreased basal plasma cortisol concentrations, decreased adrenal response to ACTH (237) and blunted post-exercise cortisol responses (127).

These results might indicate that the increase in chronic stress from normal trained to highly intensified trained induces the same response of the HPA axis as an increase from untrained to trained.

In contrast with the studies above, Bruin et al (33) reported an increased cortisol response to administration of ACTH in overtrained horses, suggesting an increased sensitivity. Resting cortisol levels showed no significant change during short-term overtraining. They induced short-term overtraining in horses by intensifying the training for 10 days and although their horses showed no sustained performance decrement during an exercise test, they were not able to fulfill their complete training suggesting overreaching/overtraining.

The conflicting outcomes in data dealing with cortisol responses to (exercise) stress might be explained by studies of socially stressed teachers (258) and horses (5) suggesting that although chronic stress traditionally has been thought to produce hypercortisolaemia, there is growing realization that hypocortisolaemia can also ensue. The effects of burnout and perceived stress on early morning free cortisol saliva levels after awakening were investigated in a group of teachers. Normally, cortisol levels show a significant increase after awakening. Teachers with high levels of burnout showed

blunted cortisol levels after awakening and an increased suppression of cortisol levels after dexadreson. Teachers with high levels of perceived stress showed stronger increases of cortisol levels after a low-dose dexamethasone pretreatment suggesting decreased pituitary feedback sensitivity. In teachers with both high levels of perceived stress and high levels of burnout, a lower overall cortisol secretion was observed, with stronger cortisol increases after dexamethason suppression (258).

The same response is described for horses. Horses confined with new and hostile equine companions respond either by withdrawing from confrontation or by fighting for a place in the hierarchy. In passive horses, plasma cortisol concentrations decline progressively during daily sessions of social stress to reach a nadir of less than half of initial values within one week. In aggressive horses cortisol concentrations rise. In addition, in socially stressed, passive, hypocortisolaemic horses at rest, no ACTH secretion was detectable, however acute perturbations could induce ACTH and cortisol responses, in addition elevated concentrations of pituitary venous CRH and no response to exogenous CRH administration were found. An increased secretion of an ACTH inhibitory factor was suggested to counteract the action of CRH raised by chronic social stress in the passive horses.

It is possible that the teachers with high burnout levels, like the passive socially stressed horses, like to withdraw from insults they cannot control and in doing so increase the secretion of the ACTH inhibitory factor (5). From this perspective, the increased cortisol response to ACTH administration in overtrained horses reported by Bruin et al (33) might also be explained by the theory of decreased hypothalamic-pituitary feedback sensitivity instead of increased adrenal sensitivity. The other equine studies (115, 127, 197) describe more or less a decreased adrenal sensitivity, which might also have been combined with an increased secretion of an ACTH inhibitory factor. The physiological reason for decreasing adrenal sensitivity is a protection of the body to hypercortisolaemia during prolonged stress. This is useful, because prolonged hypercortisolaemia has a lot of negative side-effects, like, for instance, the competition between progesterone/testosterone and cortisol for receptors in the muscle resulting in prolonged anti-anabolic action of cortisol, reducing the induction of muscle protein synthesis by testosterone (346). On the contrary, the decreased feedback sensitivity allows a prolonged or evoked stress response, which is only useful when the body tries to adapt to the increased stress. This might implicate that this is a short-term adaptation. When the body succeeds to adapt, the stress response disappears and when the body is not able to adapt, it is more useful to prevent the body against the harmful hypercortisolaemia and thus reduce adrenal sensitivity. Based on this hypothesis, the results of Bruin et al (33) might indicate an earlier stage of overreaching than the other equine studies.

Some researchers tried to evaluate prolonged periods of training by determination of the ratio of catabolic to anabolic hormones using cortisol as catabolic hormone and sex steroids or IGF-I as anabolic hormone. However, based on the current literature it is difficult to conclude whether any androgen to cortisol ratio is a useful indicator of training status in endurance athletes (57, 320).

It has been shown that the peripheral metabolism of cortisol can be assessed accurately from the urinary cortisol/cortisone (C/Cn) ratio. A decreased 24h C/Cn ratio was associated with an increase in performance in elite swimmers during intensified training and detraining. A decreased 24h urinary C/Cn ratio was also found in highly trained triathletes. In both studies increased cortisone concentrations were responsible for the decreased ratio, suggesting a greater inactivation of cortisol to cortisone as a response to training (11, 119).

In conclusion, two problems occur when comparing the data reporting on the HPA axis responses to training. Firstly, studies show conflicting results, which might be explained by the initial different response to chronic stress leading to decreased sensitivity of the adrenal cortex or decreased hypothalamic-pituitary sensitivity to negative feedback. Secondly, similar HPA-axis responses are found in studies comparing untrained versus trained athletes and moderately trained versus highly trained athletes, highly trained versus overreached athletes. Probably the responses of ACTH and cortisol reflect the stress impact of the current trainingload on the body, but it does not necessarily indicate overtraining.

Hypothalamic-Pituitary-Growth hormone axis

Regulation of the GH-IGF-1 axis

GH is released from the anterior pituitary gland in a pulsatile fashion in humans and horses. Hypothalamic Growth Hormone-Releasing Hormone (GHRH) stimulates GH release and synthesis from the pituitary gland, while hypothalamic somatostatin inhibits GH release without affecting GH synthesis from the pituitary gland (210, 361). In addition, endogenous Growth Hormone-Releasing Peptide (GHRP)-like neuropeptides (e.g. ghrelin) may be involved in GH regulation as well (210, 361). In plasma, the majority of GH is bound to a carrier protein known as GH-binding protein (GHBP); this is however a weak binding and therefore GH has a short half-life in the blood of less than 20 minutes (6, 112, 124). The physiological role of GHBP at rest is thought to act as a damper on GH oscillation, as a reservoir for later release and potentially to augment GH's biological activity and GH receptor expression (351). The main site of GH action is the liver, where the production of insulin like growth-factor (IGF)-I is

stimulated. IGF-I attaches strongly to its carrier protein and remains therefore longer in the blood with a half-life of about 20 hours (124). Seven IGF-binding proteins (IGFBP) have been reported to be present in equine serum (47; 37, 60, 193, 257). IGF-I is also produced locally by many tissues in which it exerts paracrine and autocrine effects (112). Recently, different IGF-I isoforms were identified in humans and divided into two main groups: class-I isoforms, which are produced locally in muscle and tendon tissue and presumably act in an autocrine-paracrine manner; class-2 isoforms, produced in hepatocytes with systemic actions on myocytes and fibroblasts (74, 126, 129). The peripheral actions of IGF-I will be regulated via coupling of IGF-I, IGFBP-3 and acid-labile subunit (ALS) into a ternary complex (19, 74). IGF-I can inhibit release of human GH directly by affecting somatotrope cells in anterior pituitary or indirectly by affecting release of GHRH and somatostatin.

GH secretion is influenced by several factors, e.g. sleep, age, gender, nutrition, body composition, fitness, reproductive state (124, 171, 355, 361). For instance, in humans, the majority of GH pulses are formed at night during the onset of slow wave sleep characterized by an increase in GH secretion. As far as we know, no study in horses investigated the relationship between sleep and GH secretion yet. However, several studies describe a greater incidence of GH pulses occurring with the onset of or during the dark period of the day, which might indicate that the majority of GH pulses are formed at night during sleep in horses (40, 49). This implies that GH measurements are best obtained at night in order to detect GH pulses.

Older people and aged horses as well as obese people produce and release less GH. For the elderly, approximately 14% less GH per decade is secreted after the age of 40 years (43, 211, 355, 361).

A clear gender difference in GH profiles exists in humans and other species. In particular, women secrete twice the amount of GH per burst, maintain the same mean GH pulse frequency and generate quantitatively more irregular GH secretory patterns, compared to men (111, 336, 341, 361). This sexual dimorphism of the human somatotropic axis is based on a difference in negative-feedback control of endogenously driven pulsatile GH secretion. For horses, the gender difference in GH secretion is the opposite of the human gender difference. Stallions and geldings seem to have more pulses and greater average pulse amplitude than mares. No difference in baseline average for GH between the genders was found (304, 313, 314). A remarkable finding was that geldings were similar to stallions in their GH profile. Apparently, testicular products were not a factor in the difference between mares and stallions at the time of sampling (castration occurred at least 2 years earlier), but the presence of the testes throughout prenatal development or soon after birth may cause male-like differentiation of GH secretory characteristics that persists even when the

testes are removed (313). In addition to the gender difference, in cyclic mares during the breeding season and in pregnant mares endogenous GH release is increased (12).

The influence of exercise stress on the GH-axis can be analysised by measurements of circulating GH, IGF-I, and their circulating binding proteins as well as measurements of GH pulsatility (86). The selection of an appropriate assay (method) for the detection of GH is not that simple for several reasons. Recent work evaluating the impact of exercise on *in vivo* bioassayable versus immunoassayable hGH has shown a disparity between the two assays for hGH concentrations. In addition, a variety of immunoassays are commercially available as well. Because these immunoassays employ different monoclonal and polyclonal antibodies directed at specific epitopes on the GH molecule and because various molecular isoforms exist, the results obtained for GH concentrations from the same sample can vary (67, 230, 361). Therefore, caution must be used when comparing data from studies using different assay techniques or different assays.

The sensitivity of the assay is a concern when measuring growth hormone. Levels of this hormone at rest and in the elderly can reach as low as 0.10 µg/L, which is below the detection limit of most assays (57).

When evaluating the measurement of IGF-I, one must consider the methodological problems associated with the binding proteins in the assays. There is no apparent universal procedure in which IGF-I is separated from its binding protein (57). Therefore, research is needed to distinguish between the bound and free levels of this hormone, before it can be applied in studies leading to reference values for trained and overtrained individuals.

Actions of GH

Growth hormone causes growth of almost all tissues in the body by promoting increased sizes of cells and increased mitosis. In addition, GH enhances almost all facets of amino acid uptake and protein synthesis by cells while at the same time it reduces the breakdown of proteins. GH increases the release of free fatty acids from the adipose tissue and enhances fat utilization for energy. Furthermore, GH decreases carbohydrate utilization.

The physiologic role of the exercise induced GH pulse is not fully understood. It is hypothesized that the GH increase after exercise may provide an energy source during recovery from exercise by inducing lipolysis and promote synthesis of new proteins, while at the same time the proteins already present in the cells are conserved (124, 353). The study of Pritzlaff et al (255) promotes this view. They found that the increase in fat expenditure during recovery after acute intensive exercise was directly related to GH release.

The physiologic role of IGF-I is mainly increasing the rate of protein synthesis (351).

Acute exercise and response of GH axis

The GH response to acute exercise has been studied extensively in men (172, 215, 230, 353, 361) and to a lesser extend in horses (43, 116, 313, 314), with most investigators reporting that acute bouts of exercise increase the plasma concentration of GH. Several candidates have been indicated as potential stimuli for the exercised induced GH release (111), e.g. neural input, either from muscle afferents and/or centrally from the motor cortex (68, 190, 362), feedback from release of IGF's, direct stimulation by catecholamines (355), B-endorphines, lactate, NO and changes in acid-base balance (57, 112).

Most studies indicate that the GH increase in response to exercise is dependent on the relative workload and that a threshold of exercise intensity may be necessary before a significant rise in GH levels is detected (50, 171, 357). Pritzlaff et al (256) concluded that the GH secretory response to exercise is related to exercise intensity in a linear dose-response pattern in young men. The variability in results of the studies may be due to the fact that a certain exercise intensity or duration might be needed to overcome GH autonegative-feedback, as will be discussed below.

In general, to induce a significant plasma GH concentration elevation, exercise of at least 10 min with intensity above lactate threshold (50-70% VO_2 max) should be performed (112). The GH peak will appear within the exercise bout or immediately after exercise, and baseline values will be reached approximately 1-2 hours after exercise (172, 361).

The human studies correlate well with studies in equine athletes, where a certain level of activity seems to be needed as well to overcome GH autonegative-feedback. Thompson et al (313) studied differences in the GH response to 5-minute lunge exercise of moderate to fast trot with some cantering. In this study, the variation among horses was large and 2 out of 24 horses did not respond with a GH increase after exercise at all. The variation might be caused by previous spontaneous episodes in GH secretion in which case the exercise stimulus was not enough to overcome autonegative-feedback of GH (93, 111, 172, 308, 313).

In the same study, they looked at gender differences in the GH response to the 5 min lunge exercise. Mean concentrations of GH in geldings immediately after exercise were greater than those in mares or stallions, but the areas under the GH curves did not vary significantly, because the variation among horses was large (313).

Stokes et al (309) studied the reproducibility of the individual GH response to exercise in men after a 30s cycle ergometer sprint. The athletes performed the sprint twice with a 7-day period in between. 7 out of 11 athletes had a significantly greater GH

peak concentration after the second sprint. The authors concluded that the GH AUC is a more reproducible measure for reporting the GH response to sprint exercise than peak GH concentrations, because the GH AUC demonstrated a significant correlation between the two tests, while a large variability between subjects in time to reach peak serum GH concentrations (some athletes did not respond with a GH peak at all) and individual serum GH concentrations after exercise (10-60 min post exercise) were found. Given the large variability in peak GH values and time to reach peak GH in this study, the duration of the exercise (30 seconds) might not be enough to evoke a GH response in all athletes and that results might be influenced by this (309).

Several other factors modulate the GH response to exercise, including nutrition, age, body composition (51) and fitness level of the subject. For instance, in the elderly the GH response to exercise is reduced (369). GH release at rest and during exercise is also reduced in individuals with increased body fat (211). A fat meal just before exercise blunts the GH response to exercise (172).

On the contrary, some factors have been studied and reported not to influence the GH-response to exercise, like aerobic versus resistance exercise (361) and time of the day (171, 283, 361), which is interesting because the majority of GH pulses is secreted at night. Apparently, the exercise stimulus is powerful enough to overcome the decreased GH pulsatility during the day.

GH and IGF-I activity depends, in part, on their circulating binding proteins, which may alter biological activity, and alterations may present an interesting pathway of adaptation to stress. Several researchers studied the effect of exercise on IGF-I concentrations as well as the concentrations of binding proteins of GH and IGF in humans and/or equines (21, 57, 351). Increases in IGF-I are reported to be transient and only observed during the onset of exercise. The concurrent increase in serum GH is unlikely to be related to the increase IGF-I secretion, because it normally takes a couple of hours to increase serum IGF-I after GH release (21, 57).

In horses, there is no indication that exercise modifies IGF-I concentrations in plasma. Popot et al (248) reported that 64 post-race (intensity and duration were not described) blood samples collected less than 1.5 hour after the race for determination of serum IGF-I did not show significant differences compared to the concentrations of a control group of 194 horses. In addition, blood samples of trained horses collected after an incremental treadmill exercise test at 2, 5 or between 5-20 minutes post exercise revealed no difference in IGF-I plasma concentrations as well (248).

Wallace et al (351) reported that a single bout of exercise induced fast and marked increases in total IGF-I, IGF-BP3 and ALS in humans, which together form the ternary complex. Levels increase 5-10 min after short-term exercise (30-min cycling), returning to baseline within 45 (IGF-BP3, ALS) – 60 (IGF-I) min post exercise. It is opposed that the ternary complex is stored in tissue, from where it enters and leaves the central

circulation in response to exercise. The skeletal muscle itself may be the tissue source, because during exercise a marked increased perfusion of the skeletal muscle occurs, another possible tissue source may be the liver (21, 351). The biological effect of an exercise-induced change in IGF ternary complexes may be to enhance post-exercise reparative processes, such as protein anabolism (351).

IGFBP-1 may also be involved in regulation of biological action of IGF-I during short-term and prolonged exercise. A study involving prolonged endurance exercise (7.5 hours of a 75 km cross-country ski race or cycle ergometer exercise) reported that IGF-I levels were decreased after the ski-race and IGFBP-1 were significantly increased (310). In the same study, significant increased IGFBP-1 concentrations were reported, while the IGF-I concentration did not change after cycle ergometer exercise of 3-h at 45-50% VO_2 max. Wallace et al (351) found the same increase in IGFBP-1 after acute short-term exercise. In their study IGFBP-1 increased prominently 30 min after acute exercise, with a peak at 60 min post-exercise.

IGFBP-2 is a potential alternative carrier for the IGFs when IGFBP-3 levels are insufficient. Wallace et al (351) found no significant effect of acute exercise on IGF-BP2.

A post-exercise increment in GHBP concentrations may prolong the GH signal for post-exercise protein synthesis, tissue repair and muscle glycogen replenishment, or assisting in metabolic adaptations during prolonged exercise to provide fuel via lipolysis. Serum GHBP increased in response to acute exercise, with a peak concentration at the end of exercise and declined to basal values within 2 hours (351).

Repeated bouts of acute exercise and response of GH axis

Kanaley et al (172) reported that repeated bouts of exercise (three 30-min aerobic exercise sessions at 70% peak VO_2 max with 90 minutes in between or three 30-min sessions with 4 hours in between) on the same day were able to consistently stimulate GH secretion. The GH response significantly exaggerated with repeated bouts of exercise; GH secretory pulses were shorter in duration but greater in amplitude. The authors concluded that exercise is able to overcome GH auto-negative feedback. Meeusen et al (213) found the same exaggerated GH response after the second bout of incremental exercise test till exhaustion after a 4-h rest period in trained athletes. However, Stokes et al (308) reported that the GH response was attenuated after a repeated sprint (30s cycle ergometer sprint repeated after 60 minutes) and Brenner et al (30) reported unchanged GH responses after two 30-min bouts of exercise at 50% peak VO_2 max. Their results indicate that not every exercise bout is able to overcome negative feedback and that a certain level of activity and/or duration is necessary to overcome the auto-negative feedback threshold. A GH pulse prior to exercise might have induced autonegative-feedback in individuals in some studies, which might

explain the great deal of inter-individual variability in the GH responses to exercise of low intensity or short duration. In the study of Pritzlaff et al (256) no differences were reported among conditions for baseline GH during the 2-hour period before exercise started.

In addition, Galassetti et al (104) describe a gender difference in response to multiple bouts of exercise (90 min 50% cycle ergometer test, separated by a 3-h interval) on the same day. Comparison of these results with the work of others is confounded because Galasetti et al (104) kept euglycaemic levels during the exercise sessions by glucose infusions in their athletes. However, they concluded that women are less sensitive to the GH auto-negative feedback than men which corresponds to the gender difference in functioning of the GH-IGF-I axis reported by others (336, 341) after a single bout of exercise or at rest. The general pattern of exercise-induced GH release is similar in men and women. Veldhuis et al (341) reported the same absolute peak concentration (although men started from a lower baseline value) and the same GH AUC after exercise for both genders, where women attained peak GH concentrations more rapidly after exercise compared to men (341). So far, the exact mechanism behind the sexual dimorphism in the GH-IGF-I axis is not yet understood.

Recovery phase and response of GH axis

In men, several studies describe the effect of acute exercise on 24-hour GH release as a change in pulsatility patterns during the recovery with no change in the total amount of GH released.

A lower GH release was determined during the first half of sleep and a greater GH release during the second half of sleep compared to control conditions after a bout of heavy resistance exercise as well as after 120-150 km biking in the late afternoon in men (176, 230). The total amount of GH secreted was the same for both conditions.

One study has been performed in horses in order to determine the effect of exercise on 32 h GH release. The authors did find a significant increase of GH 4 hours after exercise. In this study, the irregular sampling intervals hinder determination of small nuances in GH pulsatility (115)

Further research is needed to see if the pulsatility changes during recovery after intensive exercise occur in horses as well.

In summary, to induce a significant plasma GH concentration elevation post-exercise, exercise of at least 10 min with intensity above lactate threshold (50-70% VO_2 max) should be performed to overcome autonegative-feedback, in both horses and humans. Repeated bouts of exercise exaggerate this response. Gender differences play an important role in interpreting GH pulsatility. Further research is needed into the role of

class-1 and class-2 isoforms of IGF-I and binding proteins during exercise and their responses to training.

Chronic exercise and response of GH axis

Data in the literature concerning the effect of training on GH levels in humans are contradictory. Weltman et al (356) did not find an effect on resting GH pulse amplitude in young women when exercise training for 12 months was performed below the lactate threshold. When training occurred above the lactate threshold pulsatile release of GH over 24 hours changed by augmenting GH pulse amplitude. A comparable study with training above lactate threshold was done in elderly people (both sexes) and no significant changes in 24-h integrated GH concentrations were found (361). However, the GH-IGF-I axis functions less in older people and might respond slower/differently than for young women. Eliakim et al (86) found no measurable change in mean GH or GH pulsatility during the night after 5 weeks of endurance training in adolescent males. They reported significant reductions in GHBP, IGF-I and IGFBP3, a significant increase in IGFBP-2 and no change in IGF-II during rest. The authors speculated that there are at least two phases in the GH-IGF-I response to a training programme: the first is an acute catabolic-type response initiated by lower GHBP. During this initial phase, local muscle IGF-I might actually increase as circulating IGF-I falls, reflecting an important mechanistic role for autocrine/paracrine effects of IGF-I in the response to training. At some later point (presumably after 5 weeks) a chronic anabolic adjustment of the GH-IGF-I axis occurs (86).

In addition, an increase of the nocturnal pulsatile secretion after regular endurance exercise has been described for the hamster as well (174).

Studies that have examined training and GH responses in humans suggested that training blunts (308), increases (36, 195) or does not affect (369) the GH response to acute exercise. Several studies have shown that endurance training reduces the exercise-induced GH release in response to acute constant load exercise when the absolute workload was unchanged (112, 215, 357, 361). However, in these studies, the exercise test was set at the same absolute power output before and after training. Therefore, the relative intensity would have been lower after training and this may contribute to explain the blunted response of GH. Four months of progressive endurance training in middle-aged and young cyclists did not alter the GH response or serum IGF-I concentrations in response to maximal aerobic exercise on a cycle ergometer (369).

As far as we know, there is no information available regarding the effects of training on GH levels in the equine athlete.

Thus, no obvious training adaptation of the GH-IGF-I axis in the response to acute exercise is found so far. Studies tend toward suggesting that endurance training results in an increased resting GH and a blunted exercise-induced GH response. More studies are needed to determine the effect of long-term training on 24 hour GH pulsatility.

Neuroendocrine aspects of overtraining

A hypothalamic-pituitary dysfunction is suggested as the underlying cause for OTS (10, 16, 185). Several studies provide evidence that overtraining is associated with a hypothalamic dysfunction. However, a lot of studies fail to show any dysfunction. The evidence for hypothalamic dysfunction comes from referred cases suffering for longer periods (months/years) from OTS, while cases that were deliberately overtrained for research goals fail to show a dysfunction. The use of short term intensified training (e.g. 4-6 wks, which is often used for research, because there are ethical limitations associated with intentionally overtraining athletes) may produce different responses/symptoms than those produced after the long periods athletes typically suffer from OTS (e.g. months to years) (143).

Barron et al (16) examined the hypothalamic pituitary function in 4 overtrained athletes (3 runners, 1 competitive walker; 1 developed symptoms during 4-month study and 3 were referrals) and 5 control marathon runners by IV administration of insulin after an overnight fast. Insulin induced hypoglycaemia acts by altering the secretion of hypothalamic factors which stimulate the release of ACTH, GH and PRL from the anterior pituitary. The 4 overtrained athletes had significantly decreased GH, ACTH, cortisol and prolactine responses compared to the control group. Basal cortisol levels were significantly elevated in the overtrained athletes. Four weeks of rest normalized the response to insulin induced hypoglycaemia in the overtrained athletes. The pituitary gland was also stimulated with TSH and LHRH, which evoked normal responses in the overtrained group. Therefore, the dysfunction was hypothesized to be hypothalamic with perhaps a mild degree of pituitary insensitivity in the overtrained athletes (16).

Urhausen et al (321) induced a short-term overtraining state in endurance athletes and investigated the hormonal response at rest and during exercise consisting of a short-term exhaustive endurance test on a cycle ergometer. They found an impaired response of GH and ACTH to exhaustive exercise in the overtrained athletes compared to a control group. At rest, the concentrations of the pituitary hormones were comparable with those during normal training states. Beyer et al (23) reported a low level of GH at rest in track and field athletes when they were close to the state of overtraining.

OTS may be present in different ways, possibly reflecting various stages of overtraining and individual variation in expression of overtraining. This might explain the

different results found during the short-term overtraining. The early detection of signs or markers is important in the prevention of OT/OR and, eventually, OTS. Therefore, we need to look for subtle changes in the overreached athlete. A multiple-exercise test whereby the athletes have to respond to repeated stress situations might be able to detect the subtle changes in an earlier phase than a single-exercise stress test. In addition, a multiple exercise trial gives the opportunity to measure the recovery capacity of the athlete as well. Several studies described the GH and ACTH/cortisol response to multiple exercise bouts in healthy athletes (169, 172, 274) as an increasing response.

Meeusen et al (213) described the hormonal response after multiple exercise bouts in healthy, overreached and one overtrained athlete. In this study, cyclists were tested before and after a 10 day training camp and compared to a motocross athlete who was diagnosed to be overtrained and referred for testing. The training camp was supposed to induce overreaching in the athletes as was proven by a 6% decrease in performance at the end of the 10 days. The athletes underwent two consecutive maximal exercise tests separated by 4 hours. The trained group showed an exaggerated response after the second test for GH, ACTH and cortisol. The overreached group showed an equal response to the second bout of exercise for ACTH and cortisol, with cortisol slightly decreased after both bouts compared to resting values, combined with a significantly blunted response for GH to the second bout of exercise. The overtrained athlete showed a blunted GH increase after both trials compared to the trained and overreached group, a much higher increase of ACTH after the first exercise bout compared to both groups and a slight decrease after the second bout of exercise, with cortisol slightly increased after both exercise bouts. Baseline values for ACTH and GH were much higher in the overtrained athlete compared to the other groups. Cortisol values were the same for the three groups. Although, the values obtained for the overtrained person are based on one individual, the study shows the difficulty of diagnosing overreaching in the individual. During the first exercise test the trained and overreached group had similar results and only during the second exercise bout differences between both groups occurred, which emphasizes the usefulness of repeated bouts of exercise in diagnosing early overtraining. The overtrained person shows striking differences in basal hormonal levels as well as after the first exercise bout. The most interesting finding is the exaggerated response of the ACTH during the first bout of exercise and a complete suppression in the second one. The authors suggest a hypersensitivity of the pituitary followed by exhaustion afterwards.

The cortisol/cortisone (C/Cn) ratio seems to change in overtrained athletes (11, 121). One swimmer showed a gradual increase in the C/Cn ratio from the intensified training period to the recovery period (0.43 and 0.74 respectively) in contrast with the significant decreases reported in other trained swimmers (1.1± 0.7 and 0.57±0.2, respectively; baseline value 0.65±0.3)) (11). Two triathletes developed an overtraining

syndrome and presented an increased urinary C/Cn ratio (>1) due to lower cortisol inactivation compared with the triathlete group (121).

Thus, during OR and OTS an impaired hormonal response to exercise occurs. To detect this subtle hormonal changes in the OR athlete an exercise test with repeated bouts of exercise seems helpful.

Conclusions and Clinical relevance

This review provides a summary of previous literature examining the response of the HPA axis and the GH-IGF-I axis to exercise and training in both humans and horses. Hormonal responses are influenced by variability in age, gender, exercise intensity, body composition, etc, which hampers the interpretation of data of different studies. Setting reference values for diagnosing OR/OTS is difficult; since absolute values differ between individuals. However, most studies show similar hormonal trends in athletes. Therefore repeated bouts of exercise could be (more) helpful in diagnosing OR/OTS.

The first bout shows the hormonal response for an individual to exercise, the second bout gives information about the trend which seems to be rather different in trained, OR and OT individuals. However, more research is needed to confirm these trends and to specify the exercise intensity of the bouts and the time of rest between the exercise bouts to optimize the hormonal responses. The determination of hormonal responses to acute multiple exercise bouts should be combined with measurements of basal concentrations of for instance ACTH (and cortisol), a CRH stimulation test, and a pulsatile GH analysis. The latter is more practical for researchers than for practitioners due to the multiple sampling that has to be done. Nevertheless, detailed information about the changes in GH pulsatility could provide more understanding in normal adaptation of the axis to training and pathologic adaptations in OTS. Pulse analysis (e.g. deconvolution analysis) could be of help to determine the differences in basal GH secretion and changes in pulse parameters. However, as mentioned above, frequent sampling as well as an assay with a very low detection limit is needed for pulse analysis. The horse could serve as a good model for humans in these cases. As shown in the review, most hormonal responses to exercise and training are the same for both species. Due to the unique anatomy of the horse, it is possible to measure pituitary venous samples in a less invasive manner than in humans (148). In pituitary venous samples hormone concentrations are much higher; therefore the detection limit of the assay needed could be higher, which makes application of commercial assays feasible. Another advantage of the horse as a model is the bigger amount of blood that can be sampled, necessary for studying GH pulsatility.

Understanding normal hormonal responses to exercise and training gives also the possibility to enhance welfare as well as detecting abuse of hormonal supplements in competition horses and athletes. Since the introduction of reGH, it is used as a potential performance improver in competition horses (66). To detect abuse, reference ranges for GH values after exercise or at rest in the equine athlete are necessary.

Chapter 4

Assessment of endogenous GH pulsatility in the horse using deconvolution analysis

Ellen de Graaf-Roelfsema[1]
Paula P. Veldhuis[2]
Klien G. van Dam[1]
Paul P.C.A. Menheere[3]
Hans A. Keizer[4]
Michael L. Johnson[2]
Eric van Breda[5]
Inge D. Wijnberg[1]
Johannes H. van der Kolk[1]

[1]Department of Equine Sciences, Medicine Section, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands. [2]University of Virginia Health Sciences Center, Department of Pharmacology, Charlottesville, Virginia (USA). [3]University Hospital Maastricht, Department of Clinical Chemistry, Maastricht, The Netherlands [4]Department of Human Physiology and Sportsmedicine, Free University of Brussels, Brussel, Belgium. [5]Department of Movement Sciences, Faculty of Health Sciences, University of Maastricht, The Netherlands

Abstract

Objective: To quantify GH secretion in Standardbreds.
Animals: Twelve yearlings and two mature Standardbreds
Procedures: Endogenous equine (e)GH half-life was determined after administration of Growth Hormone Releasing Hormone. Exogenous eGH half-life was determined after administration of recombinant eGH with and without suppression of endogenous GH secretion by somatostatin infusion. Pulse detection algorithm (Cluster) as well as deconvolution analysis was used to quantify GH secretory dynamics based on GH concentration-time series sampled every 5 minutes from 2200 till 0600 hours. In addition, reproducibility, impact of sampling frequency and influence of altering initial GH half-life on parameter estimates were studied.
Results: A mean endogenous eGH half-life of 17.7 ± 4.4 min and mean exogenous half-life of 26.0 ± 2.9 min were found. Parameters of GH secretion as well as elimination as analyzed by deconvolution analysis are described. In summary, the mean number of secretion peaks in 8 hours was 12, 99.1 % of the total amount of GH secreted occurred in pulses, basal secretion was very low (0.012 µg/L/min) and half-life was 8.9 min.
Conclusions and Clinical Relevance: The deconvolution model used was of Veldhuis et al (335) and is applicable to equine GH time series. From this study, it can be concluded that as in man, the equine pituitary gland secretes GH in volleys consisting of multiple secretory bursts, without measurable intervening tonic secretion. Defining normal GH secretory dynamics in the horse will make it possible to detect alterations in the GH axis due to (patho)physiologic mechanisms as well as abuse of reGH in the future.

Introduction

GH is secreted by the pituitary gland in a pulsatile fashion and is regulated mainly by hypothalamic Growth Hormone Releasing Hormone (GHRH) and somatostatin (SS). The episodic release of GH is apparently random in nature and is influenced by many metabolic and environmental cues, like age, gender, sleep and exercise, which makes evaluation of episodic GH release difficult. Nonetheless, the development of computerized algorithms to quantify episodic GH release has offered new insights into the pathophysiological regulation of GH secretion in humans (216, 335). Pulse detection algorithms and deconvolution analysis were developed to aid in the unraveling of GH release dynamics. A pulse detection program such as Pulsar (216) is only designed to describe concentration peaks in a data series. It does not provide information about the two events that together determine the concentration peak, namely hormonal secretion and clearance, which may be obtained from deconvolution analysis. Deconvolution analysis will render a quantitative description of GH secretory and clearance dynamics for the number, amplitude, duration, and temporal locations of all significant underlying secretory bursts and the half-life of endogenous GH disappearance. In this respect, it is therefore a more favourable approach for analyzing hormone data series than other pulse detection programmes (161).

In order to use these methods with accuracy, it is necessary to fulfill the following criteria in order to obtain a usable GH concentration curve: a) a sensitive GH assay with a known precision of low, middle and high hormone concentrations, b) a long enough sampling period in which to detect pulses and, c) optimally, the sample frequency should be approximately 0.25 times the hormone half-life. In order to fulfill the last criteria, the disappearance half-life of the hormone should be known.

The half-life of endogenous as well as exogenous GH is described for both humans and rats, but not for horses (46, 90, 106, 140, 142, 225). Disappearance half-life times were determined after administration of Growth Hormone Releasing Hormone (endogenous half-life) or recombinant GH (exogenous half-life), sometimes combined with somatostatin infusion or injections for the suppression of endogenous GH secretion (46, 140, 225).

Several studies investigated GH pulsatility in horses (49, 62, 71, 304, 307, 313, 314) using a pulse detection programme. As stated previously, unlike deconvolution analysis, these programs are unable to provide direct information about secretory event characteristics and its half-life. To our knowledge, deconvolution analysis has not been utilized to study GH pulsatility in horses yet. This novel approach might enhance understanding of GH dynamics in the horse in health and disease and is therefore worthwhile to investigate.

The work reported here aimed to quantify GH secretion in the horse by deconvolution analysis. In order to obtain usable GH time series data, a validated homologous equine GH assay was used, a precision profile determined and the half-life of exogenous as well as endogenous equine GH estimated. In addition, reproducibility of deconvolved parameter estimates, the impact of sampling frequency and influence of altering initial GH half-life of parameter estimates were evaluated.

Material and Methods

Animals
Twelve healthy young Standardbred geldings (aged 14-16 months) (Group A) and two healthy mature Standardbred horses (one mare and one gelding, aged 3 and 4 years, resp.) (Group B) were used for the different experiments.

Approval
The experiments were approved by the Committee for Animal Welfare at the Faculty of Veterinary Medicine, Utrecht University.

GH assay
GH concentrations were assayed by an automated enzyme-linked-immuno-sorbent assay (ELISA) designed for measurement of equine GH (eGH) (Diagnostic Systems Laboratories, Inc., Webster, TX, USA). The assay was validated with respect to specificity, parallelism, recovery, and linearity. The sensitivity of the assay was 0,11 ng/ml, and the mean intra- and interassay coefficients of variation were 4,5 and 6,5%, respectively. In addition, uncertainties (SD) of each GH concentration were estimated empirically considering variances were associated with assay response and standard curve evaluations. Subsequently, confidence limits for the standard curve parameters were calculated. Briefly, ten standard solutions (range 1,5-50 ng/ml) were obtained from the manufacturer. These control solutions were assayed ten times. From the resulting absorbance values, a precision profile (CV against concentration) over the dynamic range of the assay was calculated according to Ekins (85) and Hunter (146). With the resulting mathematical function, CV's for all of the measured GH data points were calculated.

Determination of endogenous GH half-life
Eight Standardbred horses, aged 14-16 months (part of group A), were used to study endogenous GH half-life after GHRH administration. Somatoreline, a synthetic GHRH (Ferring B.V., Hoofddorp, The Netherlands) (1.0 µg/kg BW) was administered for this

study. The lyophilized GHRH powder was dissolved in the accompanying solvent solution and injected as a rapid bolus via a catheter in the left jugular vein. Blood samples were collected at 15 and 0 minutes before injection and at 15, 30 and 45 minutes after injection via the same catheter, after careful flushing with 20 ml 0.9% NaCl. Horses that showed a spontaneous endogenous GH peak coincided with the administration of GHRH were excluded from the study. Detection of an endogenous peak was based on increasing GH concentrations between 15 and 0 minutes before administration of GHRH and/or GH concentrations above 2 µg/L.

The fractional turnover rate (GH disappearance rate) κ was calculated over the interval from 30 to 45 minutes after GHRH administration by use of the following formula (44): κ (%/min) = $(\ln[GH]_1 - \ln[GH]_2) / \text{interval}_{min} \times 100$, where $\ln[GH]_1$ represents the natural logarithm ($\log^e$) of the GH concentration at the beginning of the interval and $\ln[GH]_2$ represents the natural log of the concentration at the end. Plasma half-life of eGH was determined for this interval by use of the following formula: Half-life (min) = $\ln 2/\kappa \times 100$.

Determination of exogenous GH half-life

Two healthy Standardbred horses (Group B), weighing 442 and 446 kg, respectively, were used to determine the exogenous GH half-life. The horses were kept in stables all day except for a one-hour walk in the walking machine. The study was performed as described by Hindmarsh et al (140). Following an overnight fast, a catheter was inserted into each jugular vein; one of these catheters was subsequently used for the infusion of somatostatin (S1763-1MG, Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany) and for the administration of the bolus injection of exogenous GH (Equigen^{TM}, Bresagen, Thebarton, South Australia) while the other was used for recovering blood samples with which to measure plasma GH concentrations. An infusion of somatostatin (50 µg/m^2/h) was begun at 0900 h and samples for the assessment of serum GH concentration were drawn at 10-min intervals for the succeeding hour. At 1000 h an intravenous bolus of methionyl equine somatotropin was administered in a dose of 20 µg/kg BW. Blood was then drawn at 2-min intervals for 30 min, at 5-min intervals for the following 30 min, and at 10-min intervals for a final 30 min. On another occasion each (with at least 13 days between sample days), the study was repeated with an infusion of saline instead of somatostatin. Horses were allotted randomly to the initial infusion.

The program MLJ_LS (UVA Pulse Analysis Software, Charlottesville, VA, USA) was used to calculate the half-life. MLJ_LS performs global nonlinear least-squares parameter estimations to a series of generic fitting functions. A Monte-Carlo method was used to determine the standard deviation of the determined parameter t1/2.

Determination of GH time series
A pilot study was performed with two healthy mature Standardbred horses (Group B) in order to obtain a time frame during the night in which GH secretion peaks could be detected. Blood was collected with an interval of 10 minutes for 12 hours from 2000 till 0800 h.

To study GH pulsatility, twelve healthy young Standardbred geldings (group A) were used. Blood was drawn via an indwelling catheter in the jugular vein at 5-min intervals for 8 hours from 2200 till 0600. On the experimental day, the horses performed an exercise test in the morning for other purposes. These results will be described elsewhere. The horses were then allowed to relax for the rest of the day in their stables. The last feeding of grass silage occurred at 19.30 h. During the experiment the horses were kept in their own stables to allow them to perform their normal nightly activities. Blood samples for determination of GH were drawn into lithium heparin tubes (Venoject, Terumo, Leuven, Belgium) on ice, and centrifuged (Hettich Zentrifugen, Tuttlingen, Germany) at 4°C for 10 minutes at 4000g. The resulting plasma was stored at -20^{o}C until assayed.

Quantification of GH release by pulse detection algorithm
Cluster 8, a pulse detection algorithm (UVA Pulse Analysis Software, Charlottesville, VA, USA), was utilized to provide descriptive information regarding observed GH concentrations as a function of time, such as the number of peaks in 12h, the mean interval between peaks, the mean peak width (i.e. duration), -height (i.e., amplitude), and -area (332).

The Cluster program was configured in a 3x3 fashion, for the 5-min interval GH curves, defining significant peaks with three samples and signifcant nadirs with three samples and a t-statistic of 3.0 for the upstroke and downstroke. For the 10-min interval GH curves, the Cluster program was configured in a 2x2 fashion and a t-statistic of 2.0 as advised by Johnson (164). The outlier T-score was set at 4.0, minimum peak size was set at 0.0 and half-life was set at 20 mins.

Quantification of GH release by deconvolution analysis
To analyze spontaneous GH secretion, Autodecon, a multi-parameter deconvolution analysis (UVA Pulse Analysis Software, Charlottesville, VA, USA) was applied to the GH concentration time series (162, 333). The software was programmed to fit to basal secretion of zero, a secretion s.d. of 2.5 min and a half-life of 12 minutes. The outcome variables estimated by deconvolution analysis included the following burst parameters: the number of secretory bursts per 8 h, the mean interval between bursts, a mean burst mass, and a mean burst amplitude (i.e., maximal secretory rate). Additional calculations included the basal GH secretion rate, 8-h basal GH secretion (basal secretion rate x

480 min), half-life, 8-h pulsatile GH secretion (mean burst mass x number of secretory bursts), 8-h total GH secretion (i.e., the sum of basal and pulsatile secretion), and the ratio of pulsatile to total GH secretion. The deconvolution analysis program uses iterative nonlinear least squares parameter estimation with 95% statistical confidence intervals to quantify all aforementioned parameters of secretion.

The stability of the GH pulse parameter estimates were evaluated by testing the reproducibility of the parameters and the influence of decreasing the sampling frequency and of altering the initial half-life on the parameter estimates were studied.

The reproducibility of estimates of GH secretory and clearance parameters was evaluated by comparing the two constituent 10-min GH concentration-time series by applying the deconvolution analysis to the even-numbered samples (i.e., 0-, 10-, and 20- min samples) or the odd-numbered samples (i.e., 5-, 15- and 25-min samples).

The sampling frequency was decreased from a 5-min serie to a 10-, 15-, 20- and 30-min serie by deleting appropriate values in the original serie of one horse.

The deconvolution program (Autodecon) needs an estimate of four parameters (basal secretion, initial concentration at time 0, secrection SD, and hormonal half-life) before it starts the actual fitting. In order to study the influence of the estimated initial half-life on the pulsatility parameters, the initial GH half-life was changed stepwise (from 2.5, 5.0, 7.5, till 40 minutes) before applying deconvolution analysis to the same data serie.

Results

Precision Profile GH assay

The precision profile is shown in Figure 1. The covariance could be calculated following the formula: $CV = a + bx + cx^2 + dx^3 + ex^4 + fx^5 + gx^6$,
whereby x represent the logarithmic (log^{10}) of the GH concentration, a = 24.85064745, b = -54.36000976, c = 83.01580674, d = -84.61192516, e = 50.37733207, f = -15.70949226, and g = 2.124299021. Correlation coefficient of the fitted curve is 0.999980.

Determination of endogenous GH half-life

Characteristics of the GH peak after administration of GHRH of the eight individual horses are shown in Table 1. GHRH injection induced an increase in plasma GH concentrations in all horses. Peak GH concentrations were achieved 15 or 30 minutes post-injection. One horse (horse 8) was excluded from the study, because it appeared to be in the middle of an endogenous GH peak at the time of GHRH administration. The mean ± s.d. half-life of endogenous GH was 17.2 ± 4.4 for the 7 horses left. Horses with

the highest basal GH concentrations (horse 6 and 8) showed the lowest relative GH increase after stimulation.

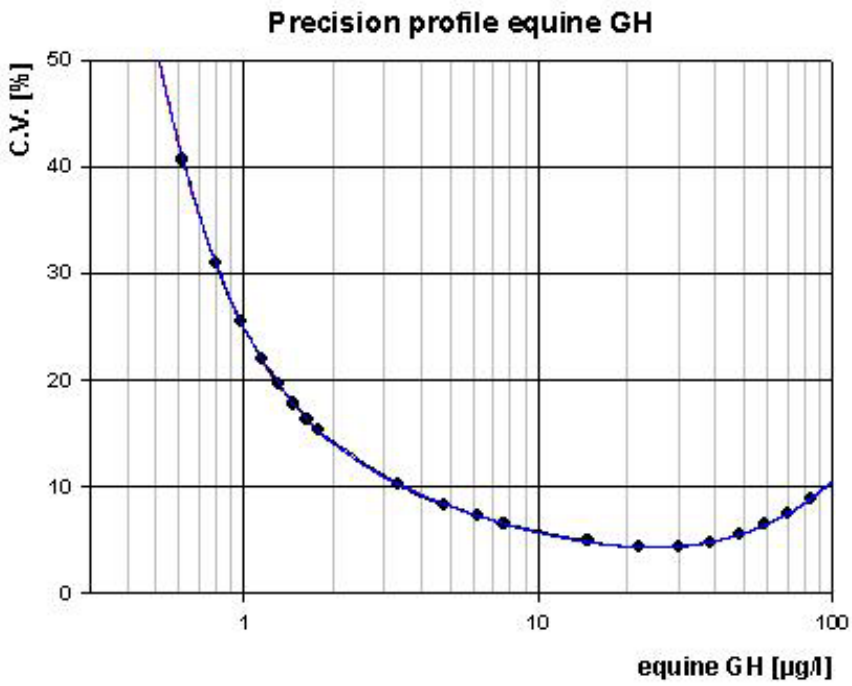

Figure 1. Precision profile equine GH

Determination of endogenous GH half-life

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Table 1. Characteristics of the pulsatile release of endogenous GH in response to 1 µg/kg BW of GHRH given IV at time 0 in eight Standardbred horses.

Horse	*Basal concentration (µg/l)*	*Peak amplitude (µg/l)*	*Time of peak (min)*	*Increase (10^2%)*	*Half-life GH (min)*
1	0.24	3.66	15	15.2	10.2
2	0.08	25.56	30	319.5	19.4
3	0.04	11.29	30	282.2	15.1
4	0.08	7.48	15	93.5	16.3
5	0.99	11.4	15	11.5	21.7
6	1.3	9.9	15	7.6	15.0
7	0.16	1.53	30	9.6	23.0
8 *	6.5	25.5	15	3.9	12.3
Total**	0.41 ± 0.51	10.1 ± 7.8	21 ± 8	105.6 ± 137.2	17.2 ± 4.4

* horse was excluded from the study due to the high baseline values at time 0.
**mean ± sd of horses 1-7

Determination of half-life of exogenous eGH

The characteristics of the GH peak after administration of exogenous GH (peak GH concentration, half-life and half-duration (e.g. the duration of the peak at half height)) are presented in Table 2. Mean half-life was 26.0 ± 2.9 (mean ± SD) minutes. Mean half-life determined during somatostatin infusion (25.8 ± 4.9 min) did not differ from mean half-life determined during NaCl infusion (26.2 ± 0.49 min). As is shown in Table 2, higher GH peak values are associated with longer half-life times. Figure 2 shows the two decay curves of horse A with and without somatostatin. The curves are almost similar. Figure 3 shows the curve of the pooled data (mean ± s.d.) of the two horses on a semi-logarithmic scale. The GH decay curves were lineair on a semi-logarithmic scale. This was true for both the curves of the animals individually and for the pooled results of the two animals.

Sensitivity study

The reproducibility of the estimates of GH secretory and clearance parameters was evaluated. There were no differences in any of the secretion or clearance parameters between these two series (data not shown).

Table 2. Half-life of exogenous GH with or without suppression of endogenous GH release by continuous somatostatin infusion in two Standardbreds.

	Half-life (min)	*Somatostatin*	*Peak GH (µg/L)*	*Half-duration (min)*
Mare	22.3 ± 0.33	Yes	280	0.79
	25.9 ± 0.38	No	320	0.90
Gelding	29.3 ± 0.26	Yes	450	0.62
	26.6 ± 0.22	No	375	0.52

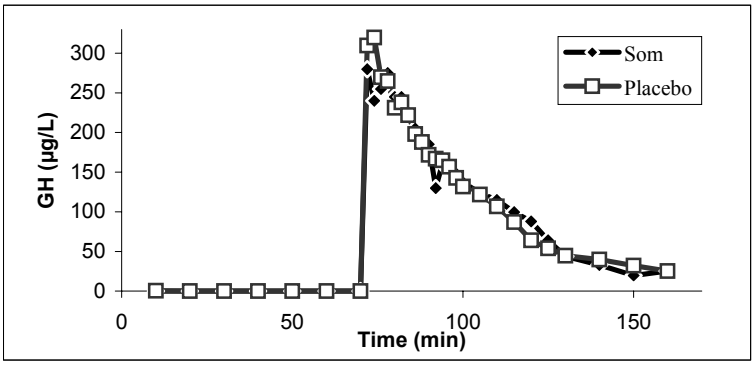


Figure 2. GH concentration curve of a mare after administration of reGH with suppression of endogenous GH secretion by somatostatin or placebo (NaCl 0.9%).

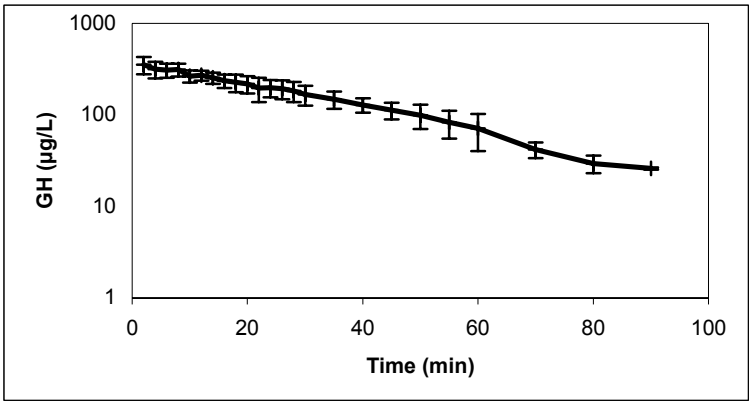


Figure 3. GH decay curve plotted on a semi-logarithmic scale for the pooled data (mean ± s.d.) of two Standardbreds studied for GH half-life times after administration of reGH with suppression of endogenous GH secretion by somatostatin or placebo (NaCl 0.9%).

Table 3. Changes in deconvolution parameters as fitted half-life was changed.

Estimated Half-life (min)	*Basal secretion (µg/l.min)*	*Half-duration (min)*	*Half-life (min)*	*Burst freq*	*Interpulse interval (min)*	*Mass (µg/l)*	*Amplitude (µg/l.min)*
2.5	1.3 E-02	3.4	6.7	15	32.9	3.1	0.87
5.0	1.3 E-02	3.3	6.8	16	30.7	3.1	0.88
7.5	1.4 E-02	2.8	6.9	15	32.9	3.2	1.10
10.0	1.1 E-02	2.9	9.3	12	41.3	3.0	0.96
12.5	1.3 E-02	3.2	10.6	10	50.5	3.1	0.91
15.0	7.3 E-03	2.9	16.7	7	74.2	2.9	0.95
17.5	7.0 E-03	3.0	17.1	6	85.9	3.3	1.00
20.0	5.1 E-03	2.9	21.7	4	135.3	4.1	1.30
22.5	5.0 E-03	2.8	21.7	4	135.3	4.1	1.40
25.0	5.1 E-03	2.8	21.6	4	135.3	4.1	1.40
27.5	5.1 E-03	3.0	21.7	4	135.3	4.1	1.30
30.0	5.1 E-03	3.1	21.6	4	135.3	4.1	1.30
32.5	5.1 E-03	2.6	21.7	4	135.2	4.1	1.50
35.0	5.0 E-03	3.0	21.7	4	135.3	4.1	1.30
37.5	5.1 E-03	2.7	21.4	4	135.3	4.1	1.40
40.0	5.1 E-03	2.8	21.7	4	135.3	4.1	1.40

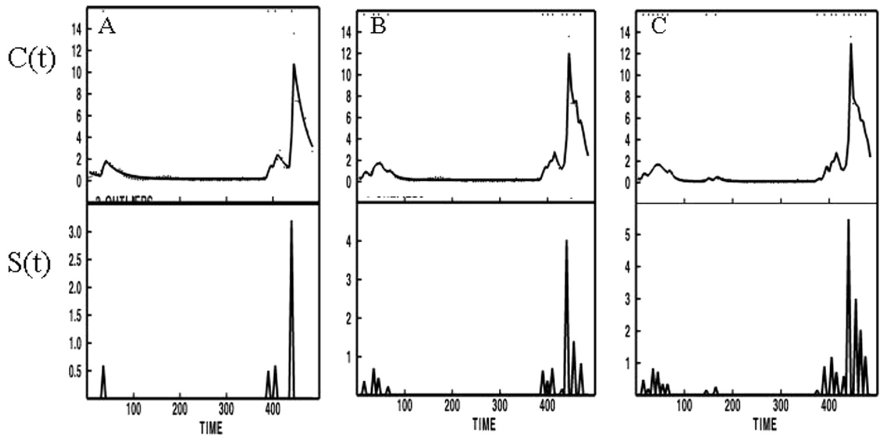


Figure 4. Upper level graph shows the GH concentration curve measured at 5 min intervals over 8 h [C(t)] with units in µg/l. Lower graph GH secretion rate plotted versus time [S(t)] with units in (µg/lv.min). Graphic A, initial estimated half-life was 2.5 min; Graphic B initial estimated half-life was 12.5 min; Graphic C initial half-life was 40 min.. Denote the difference in Y-axis of the S(t) graphs.

Results of the impact of sampling frequency on the analysis of endogenous GH secretion are shown in Table 4. Fewer GH secretory bursts per 24 h were identified with coarser sampling. Compared with a 5-min sampling frequency, 20- and 30-min sampling underestimated the number of secretory events by 45 and 100%, respectively.

Table 4. Influence of sample frequency on GH secretion and elimination parameters determined by deconvolution analysis

Sample frequency / ***Parameter***	***5 min***	***10 min***	***15 min***	***20 min***	***30 min***
Basal secretion (µg/L/min)	3.0E-05	8.0E-05	2.0E-04	1.0E-04	2.5E-01
Half duration (min)	6.5	23.8	17.6	20.6	122.4
Half-life (min)	14.6	12.3	15.5	11.7	3.5
Burst frequency	9	5	5	5	0
Interpulse interval (min)	57.2	111.5	109.3	111.1	0
Mass burst (µg/L)	4.9	10.3	9.2	10.6	0
Amplitude burst (µg/L/min)	0.71	0.41	0.49	0.48	0
% pulsatile secretion	100.00	99.99	99.98	99.99	86.50
mean GH concentration (µg/L)	1.7	1.57	1.82	1.63	1.74
AUC (µg/L)	810.5	767.3	901.2	817.9	891.3

The results of the impact of starting deconvolution analysis with different half-life are shown in Table 3 and Figure 4. Starting the analysis by fitting to a very short half-life (compared to the known half-life) increases the burst frequency, the bursts become smaller as shown by less mass and lower amplitude. A longer half-life decreases the number of secretion bursts and the bursts become larger. The number of GH secretion bursts detected was maximal with the shortest half-life and decreases rapidly with changing the initial half-life estimate. Visual inspection of the curve fitting showed that the most accurate fitting occurred with the shortest half-life (Figure 4).

Quantification of GH release by deconvolution analysis

The parameters (mean ± sd) of the pulse detection analysis are presented in Table 5. The parameters (mean ± sd) of the deconvolution analysis are presented in Table 6. Secretion peaks (12.0 ± 3.2) occur more frequently than GH concentrations peaks (2.2 ± 0.8). Basal secretion is low, most GH is secreted in pulses (99.1%).

Table 5. Mean pulse parameters of 8-h plasma GH concentration profiles (Group A) and 12-h GH concentration profiles (Group B) as determined by Cluster analysis.

Parameter	*Group A*	*Group B*
Mean GH (µg/L)	1.5 ± 0.8	0.4 ± 0.3
Total GH AUC (µg/L)	741 ± 409	318 ± 206
Number of GH concentration peaks	2.2 ± 0.8	4.5 ± 2.1
Mean interval between peaks (min)	195 ± 99	114 ± 0.7
Mean duration of peaks (min)	81 ± 26	98 ± 49
Mean amplitude of peaks (µg/L)	6.0 ± 5.9	1.5 ± 0.2
Mean AUC of peaks (µg/L)	180± 196	45 ± 10
Mean nadir GH concentration (µg/L)	0.13 ± 0.17	0.02 ± 0.01

Data are expressed as mean ± SD. Venous blood samples were withdrawn at 5-min intervals in 12 young geldings (Group A) and at 10-min intervals in two older Standardbreds. (Group B, one mare and one gelding). Cluster analysis was used with a cluster test configuration of 3x3, and a *t*-statistics of 3.0 for Group A. For Group B a cluster test configuration of 2x2, and a *t*-statistics of 2.0 were used.

Table 6. Mean pulse parameters of 8-h plasma GH concentration profiles (Group A) and 12-h GH concentration profiles (Group B) as determined by deconvolution analysis.

Parameter	*Group A*	*Group B*
Basal secretion (µg/L/min)	0.012 ± 0.014	0.004 ± 0.003
Half duration (min)	6.3 ± 4.1	5.5 ± 0.1
Half-life (min)	8.9 ± 2.6	13.9 ± 5.2
Burst frequency	12.0 ± 3.2	5.0 ± 0.0
Interpulse interval (min)	38.0 ± 11.7	86.0 ± 27.1
Pulse Mass (µg/L)	5.0 ± 3.3	2.1 ± 0.7
Pulse Amplitude (µg/L/min)	0.9 ± 0.5	0.4 ± 0.1
mean GH concentration (µg/L)	1.5 ± 0.8	0.5 ± 0.3
AUC (µg/L)	741 ± 409	318 ± 206
% pulsatile secretion	99.1 ± 0.9	99.3 ± 0.1

Data are expressed as mean ± SD. Venous blood samples were withdrawn at 5-min intervals in 12 young geldings (Group A) and at 10-min intervals in two older Standardbreds. (Group B, one mare and one gelding).

Discussion

The aim of the current study was to begin quantifying GH release in the horse by using a pulse detection programme and by deconvolution analysis. In order to use these algorithms, the half-life of GH should be known. Exogenous as well as endogenous GH half-life was estimated. The mean estimated endogenous GH half-life was 17.2 ± 4.4 minutes, which wasl comparable to a mono-exponential half-life of 18.9± 0.8 min (90) reported for men. Due to the long sampling interval in the current study, it was not possible to distinguish between mono-exponential or multi-exponential decay. In humans, mono-exponential as well as bi-exponential half-life(s) for hGH is reported (90, 142). In rats, only a bi-exponential GH half-life is reported (46).

The mean estimated exogenous GH half-life was 26.0 ± 2.9 min, which is much longer than the half-life of 8.9 ± 1.5 min (140), 9.8±0.8 (294) and 13.6 min (range 11.9-19.4) (225) described for men. At present we have no explanation for these differences, but it might be related to longer GH exposion in the circulation. In a later study, Hindmarsh et al (1990) showed that the duration of exposure to GH in the circulation is an important factor in determining GH half-life. In the current study, administration of reGH resulted in supra-physiologic plasma GH concentrations. Peak GH values of 280 – 450 ng/ml were reached. At the end of the experiment baseline values (<0.5 ng/ml) were not achieved. Mean GH concentration 90 minutes after administration of reGH

was 26.0 ± 0.7 ng/ml. The longer exposure to GH in the circulation might explain the longer half-life. Our observations correspond well with the results of Shah et al (294) who demonstrated a GH half-life of 23 ± 2.3 min in men after cessation of constant infusions and then calculated the half-life from decay after equilibrium. The reGH dosage used in the current study was based on the recommended dosage of 20 µg/kg BW once a day by the manufacturer, which is much higher than the dosages used in human studies (0.5 - 4 µg/kg). For further research, a lower dosage is advisable for determination of exogenous GH half-life.

The GH decay curves were linear on a semi-logarithmic scale, indicating that the decay was mono-exponential. Suppression of spontaneous GH release by somatostatin infusion did not alter the GH half-life significantly in horses as was also described for humans (90, 140).

Up to fourfold variability has been recognized in the t1/2 of exogenously infused GH (range 15-51 min.) (90), and more recently of endogenously secreted GH in healthy individuals (20-28 min) (90, 338). This is caused by the amount of free and bound GH in the circulation. In response to a single pulse of GH, the plasma concentration of free GH increases rapidly, and decays rapidly initially because of removal by irreversible metabolic clearance as well as by association with binding protein (BP) in plasma. Disappearance rates of 2-12 minutes have been described for free GH (338).

Table 7. Summary of GH pulse parameters analysed by pulse detection algorithms in equine literature

	Thompson '92	Thompson '94			Davicco '93		DePew '94		Stewart '93		Sticker '95	Cahill '89
Sample freq (min)	10	15	15		20	20	15	15	15	15		20
Sample duration (h)	6	4	4	4	24	24	12	8	8	14	24	24
# horses	12	10	10	10	5 foals	5 lact	16	6 foals	6	4	4 lact	5 lact
Sexe	F	F	M	G	3 F, 2 M	F	8F, 8M	3F, 3M	3F, 3M	F	F	F
# peaks	2.4 ± 0.3	0.6	1.4	1.6	10.0 ± 0.7	5 ± 2	2.9	2.3	M: 4.3 F: 1.3	2.0	10.0 ± 0.7	5 ± 2
Peak amplitude (µg/l)	19.9 ± 6.3	1.7	28.6	28.2	16.1 ± 1.2	7.9 ± 0.4				3.51		7.9 ± 0.4
Mean Peak duration	60 min											
Mean GH conc (µg/l)		2.4	8.6	8.5	7.4 ± 0.4	3.4 ± 0.3				1.15		3.4 ± 0.3
Baseline GH conc (µg/l)		1.8	3.0	2.4				8-10	3-5	1.00		
Remarks							No sex difference	No sex difference	Sex difference	Control group		

F=female, M=male, G=gelding, Lact=Lactating mare

The concentration of bound GH increases almost as rapidly, but decays considerably more slowly because molecules of GH dissociate from the BP at a finite rate while some additional molecules of GH continue to associate with the BP. This prolongs the half-life considerably until around 18 minutes (338). In disease states disappearance rates of 21-50 min have even been described. In conclusion, the GH half-life can differ under different circumstances within individuals.

With the knowledge of both half-lives, the sampling interval for the collection of GH time series could be chosen. In general, the optimal sampling frequency ranges from three to five measurements per hormone half-life (18, 19). Based on the estimates of the endogenous half-life of 17.2 min and the exogenous half-life of 26.0 min, a sampling interval for the collection of GH time series was chosen to be 5 minutes (25% of 20 min). Sampling was performed at night based upon the fact that in humans the majority of GH pulses are formed during the onset of slow wave sleep. Several equine studies described a greater incidence of GH pulses occurring with the onset of, or during the dark period of the day, which might indicate that the majority of GH pulses are formed at night in horses as well (40, 49). The pilot study with two older Standardbreds confirmed that detectable pulses could be found at night. A duration of 8 hours appeared adequate for the detection of GH pulses for analysis in the horse.

A peak detection algorithm was used in the current study to make comparisons possible to the available literature on equines as summarized in Table 7. A mean GH concentration is lower in the current study compared to the other studies. This is probably caused by differences in the assays utilized. The current study used a homologous ELISA for the detection of eGH, while the other studies used heterologous RIA's validated for horses for the detection of eGH (49, 62, 71, 304, 307, 313, 314). The number of concentration peaks was comparable to the findings of Stewart et al (304) who found 2.3 peaks during 8 hours in foals and 4.3 peaks in adult stallions during 8 hours. The programme Cluster only uses complete peaks for its calculations. In the present study, nine out of twelve horses (75%) were in the middle of a peak at the beginning or at the end of the sampling period. Cluster underestimates the number of peaks in these cases. The mean peak duration was comparable to Thompson et al (314). The peak amplitude, peak AUC and basal GH concentration are difficult to compare due to the divergence of assays used. Differences between group A (12 Standardbred geldings of 1.5 years old) and B (one mare and one gelding, 3.5 years old) were only indicative due to the difference in sampling frequency and sampling duration combined with the difference in age and number of horses. Nevertheless, it seems that the amount of GH secreted was higher for group A than for group B, which appeared to have a higher mean GH concentration, a larger area under the GH curve, higher peak number, amplitude and mass. However, the number of peaks was lower for group A, which means that the amount of GH secreted per peak was much higher for group A. An interesting finding was the change that might be caused by the difference in age. A gradual decrease during aging is described for the mean pulse amplitude and

duration and the fraction of GH secreted in man; the pulse frequency however remains the same during aging in man, which is comparable to the findings in our horses (58). During mid-s to late puberty, the increase in GH production is due to both enhanced pulse amplitude and increased mass of GH per secretory burst rather than to a change in pulse frequency (200).

Deconvolution analysis was used to obtain information about the secretion as well as the elimination characteristics of endogenous equine GH for young Standardbreds. The main parameter for the elimination characteristics of GH is the half-life of GH. The estimated half-life of 20 min was changed to a half-life of 12 min for the initial guess for the deconvolution analysis, because the concentration time series appeared to have a lower GH half-life as was calculated by Pulse 2 and the fitting became better using the lower half-life as initial guess for the deconvolution algorithm.The final GH half-life as calculated by deconvolution analysis was 8.9 minutes for the young Standardbreds, which is lower than the GH half-life of 17.0, 17.7 and 17.8 min described for man after deconvolution analysis of endogenous GH time series (130, 271, 316). However, the half-life is in good agreement with the values found after stimulation with GHRH in humans as shown above (140, 225, 294).

The conventional approach of GH administration or stimulation with GHRH would be expected to overestimate endogenous half-life, since the descending limbs of concentration peaks are augmented to various degrees by underlying secretory bursts (335). However, in this case the overestimation was very large and probably due to the longer exposure to GH caused by the high administration dosage of eGH as discussed above.

Deconvolution revealed the existence of approximately 80% more pulses than detected in the plasma profiles by pulse detection in the young Standardbreds. This is partly caused by the fact that Cluster8 (pulse detection algorithm) only uses complete peaks for its calculations. The other reason is that large peaks of plasma GH concentrations often reflect the occurrence of a succession of secretory pulses. Hartman et al (130) found that only 4% of GH secretion occurred as isolated single bursts, and these secretory episodes had very low amplitudes.

As is in humans, there seems to be no tonic secretion in equines too as shown by the amount of GH secretion as pulsatile, almost 100%.

Sensitivity studies

As in humans, in horses GH is released within major secretory episodes, which are themselves interspersed among periods of secretory quiescence (89). Intensive sampling demonstrates that these major secretory episodes are formed by high frequency GH pulsations, which is consistent with the concept that major GH secretory events comprise multiple bursts of GH secretory activity. Inappropriately long sampling

intervals lead to an underestimation of the true number of peaks of GH secreted over the desired period of time. Short sampling intervals are expensive and require significant amount of blood from the subject studied (140). For the study of GH pulsatility in the horse a sampling interval of 5 minutes seems to be appropriate.

Conclusion

The deconvolution model of Veldhuis et al (335) is applicable to equine GH time series. We found that a sampling interval of 5 minutes appears to be adequate to quantify the parameters of secretion and elimination of GH pulsatility. From this study, it can be concluded that as in man, the equine pituitary gland secretes GH in volleys consisting of multiple secretory bursts, without measurable intervening tonic secretion.

Chapter 5

The influence of intensified exercise on nocturnal Growth Hormone secretory dynamics as assessed by deconvolution analysis and ApEn in Standardbreds

Ellen de Graaf-Roelfsema[1]
Paula P. Veldhuis[2]
Hans A. Keizer[3]
Mireille E. van Ginneken[1]
Klien G. van Dam[1]
Michael L. Johnson[2]
Ab Barneveld[1]
Paul P.C.A. Menheere[4]
Eric van Breda[5]
Inge D. Wijnberg[1]
Johannes H. van der Kolk[1]

[1]Department of Equine Sciences, Medicine Section, Faculty of Veterinary Medicine, Utrecht University, The Netherlands. [2]University of Virginia Health Sciences Center, Department of Pharmacology, Charlottesville, Virginia, USA. [3]Department of Human Physiology and Sportsmedicine, Free University of Brussels, Brussel, Belgium. [4]University Hospital Maastricht, Department of Clinical Chemistry, Maastricht, The Netherlands. [5]Department of Movement Sciences, Faculty of Health Sciences, University of Maastricht, The Netherlands.

Abstract

The objective of this study was to determine the influence of intensified training and detraining on nocturnal GH secretion and elimination dynamics in young Standardbred geldings. A longitudinal training study with ten 1.5 year old horses was performed on a treadmill for a total of 32 weeks. Training was divided into four periods: (1) acclimatisation, (2) training, (3) intensified training, and (4) detraining. In period 3, the horses were randomly divided into 2 groups: control (C) and intensified training group (IT). At the end of period 2, 3 and 4, blood was sampled every 5 min for 8 h (2200 to 0600) for assessment of GH secretory dynamics using pulse detection (Cluster8) as well as deconvolution analysis. To test for regularity (orderliness), a scale-and model-independent statistic, Approximate Entropy (ApEn) was used. The main results show a significant increase in the number of concentration peaks (3.6 vs. 2.0, resp.), a smaller peak secretion pattern with a prolonged half-life (15.2 vs. 7.3 min, respectively) and an increased ApEn (0.89 vs. 0.49, respectively) during period 3 for the intensified trained group compared to the control group. Four weeks of detraining did not lead to the full recovery of the intensified trained group. Performance was still decreased in some horses and some parameters of the GH pulsatility were not yet comparable to the control group. It is hypothesized that the increase in GH frequency might be beneficial for the intensified trained horses to restore homeostatic balance.

Introduction

In humans, growth hormone (GH) is secreted in a pulsatile fashion with the majority of pulses secreted at night during slow wave sleep (331). In contrast, GH levels during the day are, in general, very low and sometimes undetectable (130). It has been shown that pulsatile delivery of GH is more effective than continuous administration in inducing certain specific tissue responses to GH in muscle, bone and liver (150, 154). While exercise, stress, and some neurogenic factors can stimulate nocturnal GH secretion, and consequently linear growth and protein metabolism ((25, 113, 120, 300, 366), emotional deprivation and too much stress can inhibit its release (32, 182), which is closely related to retarded linear growth and protein metabolism (183, 144, 238).

Albeit there are many reports on the effect of acute exercise on GH secretion in humans, very few report the impact of exercise or training on nocturnal pulsatile GH secretion, which might be an important clue for adaptation and recovery (86, 172, 176, 230, 231, 316, 356). Therefore, analysis of pulsatile GH secretion during sleep can provide more information on the impact of a stressor such as exercise than perhaps the direct effects of acute exercise.

It has been suggested that the influences of exercise on nocturnal hormonal secretion are strongly dependent on the intensity of the exercise (176, 356). Training at higher intensities of exercise shows a greater increase in the pulsatile release of GH than those who have trained less intensively (272). However, training with increased intensities can lead to the state of overreaching and overtraining, the latter of which might be associated with a decrease in GH pulsatility, as have been suggested from the works of Kern and colleagues (176) and Barron and colleagues (16) who both reported decreased nocturnal GH secretion.

The relationship between sleep and GH secretion has not yet been investigated in horses, however, some studies found the majority of GH pulses during the dark period at night (40, 49).

Two methods have been used to objectively analyze hormone concentration time series, e.g. pulse detection algorithms (216, 332) and deconvolution analysis (333). The first provides information about GH peak values and amplitudes; the latter is able to provide information about the secretion as well as the elimination kinetics of each pulse. Changes in GH secretory events cannot be directly inferred from GH time series because of the continuing influence of ongoing metabolic clearance. Changes induced by intensified training in circulating GH concentrations may be attributable to either a change in GH secretion or a change in the metabolic clearance of GH or both (131). Deconvolution analysis is therefore a more favorable approach for the analysis of GH concentration-time series.

Pulsatility is modulated by feedforward and feedback signals within the neuroendocrine axis, thereby conferring physiological regulation that sometimes results in strikingly irregular hormone concentrations over time (334). Clear pulse identification as well as interpretation of the consequences of altered pulsatile hormone secretion can be extremely difficult in irregular GH time series. Approximate entropy (ApEn) can assist in those situations, since it quantifies the regularity or orderliness of time series without the need to identify discrete pulses (244, 339). ApEn was developed from a different perspective on hormone secretion as the pulsatility algorithms and is a valuable secondary tool to understand GH secretion dynamics. While the pulsatility algorithms analyze individual pulses, ApEn analyzes patterns in the complete data series representing more complex and integrated feedforward and feedback signals. Therefore, ApEn will detect changes in underlying episodic behaviour not visible and/or reflected in the mean or variance of hormone concentrations or in peak occurrences or amplitudes.

In conclusion, scant information is available about nocturnal GH secretion in man and horses particularly the influence of intensified training on nocturnal pulsatile GH secretions. Unlike rodents, the equine athlete is very suitable for standardized exercise according to the study of Bruin et al (34), who hypothesized it to be a good model for studying the effects of training and overtraining. Therefore the purpose of the current investigation was to characterize the effect of an increased training load and detraining on nocturnal GH secretion patterns in Standardbreds. In the present study, we utilized multiparameter deconvolution analysis to detect changes in GH secretion and elimination parameters. Additionally, we utilized ApEn to calibrate possible changes in the orderliness of GH secretion as a function of intensified training.

Material and Methods

Animals

Twelve healthy Standardbred geldings were included in the study after a two-month quarantine period at the research centre. During this time they walked daily for 45 mins on the walking machine. Two horses were prevented from completing the study due to injuries. The data from the remaining ten horses will be presented. At the start of the experiment, horses were aged 20 ± 2 (mean ± SD) months and had a body weight of 368 ± 45 kg (mean ± SD). The horses had no known history of health and exercise problems and had not previously been involved in any kind of organized exercise or training regimen. The horses were individually housed in boxes, and their diet consisted of grass silage supplemented with concentrated feed according to the daily estimated

energy requirements of 58 MJ NE (range 54-66). Salt blocks and water were available ad libitum.

For practical purposes, the study was performed in two different groups consisting of six (first year) and four (second year) horses in two successive years. At the beginning of each year, the horses were divided into three pairs based on age. Of every pair, one horse was randomly selected for the intensified training program in period three.

The experiments were approved by the Committee for Animal Welfare at the Faculty of Veterinary Medicine, Utrecht University.

Experimental set up

Prior to the start of the experiment, the horses were acquainted with the Equine Exercise Laboratory and were acclimatized to running on the high-speed treadmill (Mustang 2000, Kagra, Graber AG, Fahrwangen, Switserland). The training period consisted of a total of 32 weeks divided into four periods. A schematic representation of these periods is provided in Figure 1. At the end of period 1, 2, 3 and 4, the horses performed a standardized exercise test in the morning between 0730 and 1230 h in order to monitor performance improvement. GH profiles were collected on the same day from 2200-0600 h at the end of periods 2, 3 and 4.

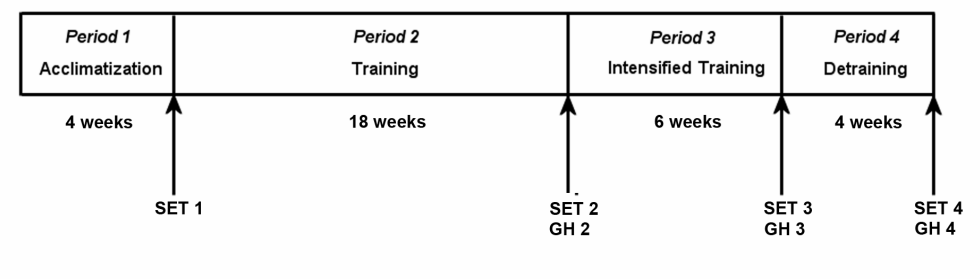


Figure 1. Schematic view of the training schedule and performed tests. (SET = standardized exercise test; GH = Growth hormone profile sampling)

Monitoring training

In order to standardize training to the individual exercise capacity of the horses, training and exercise intensity were adapted to the maximal individual heart frequency. For this

purpose, an incremental exercise test was performed at the end of period 1. After thirty minutes of walking in a horse-walker, horses performed an incremental exercise test on a high-speed treadmill. The incremental exercise test began with a warm-up after which the horses trotted for 2 mins of 5 m/s, followed by 2-mins at 6 m/s. Intensity was increased by 1 m/s every 2 minutes until the horses reached fatigue, which was defined as the speed where the horse could not keep up with the treadmill despite humane encouragement. Heart frequency was monitored with an on-line ECG recording (Cardio Perfect Stress 4.0, Cardio Perfect Inc, Atlanta, GA, USA) and an external heart rate meter (Polar S610, Polar Electro Oy, Kempele, Finland). The incremental exercise test was difficult to perform for the young, relatively untrained horses because of coordination problems on high speeds. In hopes of minimizing the risk of injuries during the incremental exercise test, the test was not performed in the second year group. In the first year, all six horses performed this test at both the end of period one and period two, and reached a mean maximal heart frequency of 221±17 beats per minute (bpm). A plateau in heart frequency at maximal exercise intensity was, however, not observed in all animals. Therefore the measured heart frequency can only be considered as the average peak heart frequency, and not as the 'maximal' heart frequency. Based on the maximal heart frequency of horses that did reach a plateau in maximal heart frequency during the current study, and based on earlier work of Bruin and colleagues (34) with 2-year old Standarbred stallions an estimated maximal heart frequency ($HF_{est\text{-}max}$) of 240 bpm was predicted. The $HF_{est\text{-}max}$ was used to guide training intensity (speed and inclination) on the treadmill, and was adjusted on a weekly basis to the measured peak heart frequencies during training.

Training protocol

After an acclimatization period of two months, basic endurance training was initiated to further introduce the horses to the high-speed treadmill for four weeks (period 1). Directly thereafter, the horses received an 18-week training program of mixed endurance training (ET) and high intensity training (HIT) (period 2). Days of ET were alternated with HIT. Each training session was preceded by 30 mins warm-up at the walking machine followed by 8 mins warm-up (4 mins at 1.6 m/s and 4 mins at 4.5 m/s) at the treadmill. The endurance running included 20-24 min of continuous level running at 60% $HF_{est\text{-}max}$ or 16-18 min at 75% $HF_{est\text{-}max}$. The interval training consisted of three 3-min or four 2-min bouts at 80-85%HF_{max}, interspersed with 3-min or 2-min periods at 60% $HF_{est\text{-}max}$. Each training session ended with a cooling down which consisted of a 5 min walk at the treadmill followed by a 30 mins walk on the walking machine. The horses exercised 4 days/wk throughout period 2.

In period 3, the horses were randomly divided into a control group (C) and an intensified trained group (IT). The control group continued training at the volume and

intensity they received in the second period for six weeks. For the IT, the intensified training regimen consisted of alternating days of HIT and ET for 6 days/week during the first 3 weeks followed by 7 days/week for the last 3 weeks. Each training session was preceded by a 30 mins warm-up on the walking machine followed by an 8 mins warm-up (4 mins at 1.6 m/s and 4 mins at 4.5 m/s) at the treadmill. Exercise intensity and volume during the ET was gradually increased to 24-35 mins 60-75% $HF_{est\text{-}max}$. High intensity exercise gradually increased to five 3-min or six 2-min bouts at 80-85% $HF_{est\text{-}max}$ interspersed with 2-min periods 1-min or 2-min periods at 60% $HF_{est\text{-}max}$.

In period 4, the horses received a 4-week training program of light endurance exercise for detraining. The horses performed endurance training for 20 min at 60% $HF_{est\text{-}max}$ for 3 days and 70% $HF_{est\text{-}max}$ for 1 day a week.

On the resting days the horses walked for 60 minutes at the walking machine throughout all periods.

Standard Exercise Test (SET)

Before the SET started, horses walked for 30 minutes in the horse walker followed by a warming up on the treadmill that consisted of 4 mins walking (1.6 m/s) and four minutes of slow trotting (4.5 m/s) and one minute walking (1.6 m/s). This was immediately followed by the time trial procedure where the horses trotted for 20 minutes at a speed and inclination that elicited a heart frequency of approximately 80% of the HF_{estmax}. The cooling down consisted of walking on the treadmill for 5 minutes (1.6 m/s) followed by 30 minutes of walking in the horse walker. Heart frequency was monitored constantly with a Polar S610 and on-line ECG measurement. Speed and inclination of the treadmill could be adjusted to achieve the desired heart frequency. In period 3 and 4, the speed and inclination were not further increased. This made a comparison between the tests possible. Venous blood was drawn from the jugular vein before the test (t=0 mins), after the warming up (t=9 mins), every 5 minutes during the SET (t=14, 19, 24, 29 mins), and after the cooling down (t=34 mins). Samples were kept on ice until heparinized whole blood lactate had been analyzed (ABL-605 Radiometer Copenhagen, Westlake, Ohio, USA).

GH-profiles

To study pulsatile GH secretion, blood was drawn via an indwelling catheter in the jugular vein at 5-min intervals for 8 hours from 2200 till 0600 h at the end of period 2, 3, and 4. During the experiment the horses were kept in their own stables allowing them to follow their normal nightly activities such as sleeping. The last feeding of concentrates was at 1800 h and of grass silage at 1930 h. Blood samples for GH were drawn into lithium heparin tubes on ice, and centrifuged (Rotina 48R, Hettich zentrifugen,

Tuttlingen, Germany) at 4°C for 10 minutes at 4000g. The resulting plasma was stored at -20°C until assayed for hormone concentrations.

IGF-1
Serum was sampled for the determination of IGF-1 concentrations at the end of each period on the same day as the GH profiles were collected. Serum was stored at -20 °C until assayed for hormone concentration.

Assay
- GH assay
GH concentrations were quantitated by an automated enzyme-linked-immuno-sorbent assay (ELISA) designed for measurement of equine GH (eGH) (Diagnostic Systems Laboratories, Inc., Webster, TX, USA). The assay was validated with respect to specificity, parallelism, recovery, and linearity. Standard curves were evaluated by linear regression. The sensitivity of the assay was 0.11 µg/L, and the mean intra- and interassay coefficients of variation were 4.5 and 6.5%, respectively. In addition, uncertainties (SD) of each GH concentration were estimated empirically considering that variances were associated with assay response and standard curve evaluations. Subsequently, confidence limits for the standard curve parameters were calculated. Briefly, ten standard solutions (range 1,5-50 µg/L) were obtained from the manufacturer. These control solutions were assayed ten times. From the resultant absorbance values, a precision profile (CV against concentration) over the dynamic range of the assay was calculated according to Ekins (85) and Hunter (146). With the resulting mathematical function, CV's for all measured GH data points were calculated.

- IGF-1 assay
Serum IGF-1 concentrations were measured using a RIA kit, following extraction with acid ethanol. The efficiency of extraction was 85-90%. Curves obtained with serial dilutions of equine plasma spiked with IGF-1 were parallel to the standard curve, and the intra-assay and inter-assay coefficients of variation were 3.2% and 15.6%, respectively (64, 229).

Pulse detection algorithm
The Cluster analysis program (332) was used to analyze the overall mean GH concentration (µg/L), 8-h integrated AUC, and characteristics of GH concentration peaks, such as the number of concentration peaks in 12h, the mean interval between peaks, the mean peak width (i.e. duration), the mean peak height (i.e., amplitude), and the mean area under the peaks. For the 5-min interval GH curves the Cluster program was configured in a 3x3 fashion, defining significant peaks with three samples and

significant nadirs with three samples and a t-statistic of 3.0 for the upstroke and downstroke as advised by Johnson (164).

Deconvolution analysis

The multi-parameter deconvolution analysis (Autodecon, UVA Pulse Analysis Software, Charlottesville, Virginia) was used to analyze secretion as well as elimination characteristics of the GH concentration time series (333). The parameters estimated by deconvolution analysis in the present study included the number of secretory bursts per 12 h, half-duration of burst (duration at half-maximal amplitude), mean interval between secretory bursts, mean secretory burst mass, mean secretory burst amplitude (i.e., maximal secretory rate), basal GH secretion rate and an elimination half-life. Additional calculations included the 8-h basal GH secretion (basal secretion rate x 480 min), 8-h pulsatile GH secretion (mean burst mass x number of secretory bursts), 8-h total GH secretion (i.e., the sum of basal and pulsatile secretion), and the ratio of pulsatile to total GH secretion. Initially the PULSE2 program was used to estimate the number and position of secretory bursts that might comprise the final GH concentration profile. The file generated by PULSE2 was then utilized in the deconvolution analysis program, where iterative nonlinear least squares parameter estimations at 95% statistical confidence intervals were utilized to quantify all aforementioned parameters of secretion and elimination. Values below the minimal detectable concentration of the assay were reported by the laboratory and used for the analysis with an uncertainty based on the precision profile as advised by Johnson (163).

Approximate Entropy (ApEn)

The ApEn statistic was used as an estimate of the regularity of the GH release process in each condition. ApEn is a single value calculated for a hormone time-series where a higher ApEn denotes greater process irregularity or greater disorderliness of hormone release (242, 243, 244). The ApEn analysis was applied with parameters of m=1 (i.e., run length), r=0.20 (i.e., tolerance window), and 1000 Monte Carlo simulations per series.

Statistics

All data are expressed as mean ± SD. Statistical analysis was performed using a linear mixed-effects model with a one-step autoregressive process (SPSS version 14.0 for Windows, SPSS Inc, Chicago, Ill). Fixed factors used were horse, couple, group and period. Interactions for group and period were calculated. P value < 0.05 was considered significant.

Results

The SETs were performed throughout the experiment to monitor performance. During SET 1 horses trotted on a speed of 6.5-7.0 m/s with no incline, during SET 2, 3 and 4 horses trotted on a speed of 7.5-8.5 m/s with a treadmill inclination of 1-4%. Set 2, 3 and 4 were performed on the same incline and speed to make valid comparisons possible.

In the intensified trained group, the mean duration of SET 3 was decreased compared to SET 2 with 12% as shown in Table 1B and therefore lactate concentrations were not present for all horses after 20 minutes. Therefore only data of the first 15 minutes of the SETs are compared and presented. At period 3, all five horses of the IT group showed changes in behavior during training and/or the SET. Three horses (A, B, and C) of the IT group were unable to maintain trot at high speeds during training or the SET and started galloping. One horse (D) of the IT group was not able to complete SET 3 and stopped after 10 minutes. One horse (E) of the IT group only wanted to trot at the end of treadmill. We were unable to keep him in front of the treadmill during the training at the end of phase 3 and SET 3. At period 4, the behavioural changes were only observed during SET 4 and not during the training sessions. Horse D was still unable to complete the SET and ceased after 13 minutes of trotting. Horse B and C started galloping again during the SET. For the control horses, no differences in behaviour were observed during the different SETs.

A right shift of the lactate curve during SET 3 compared to SET 2 was observed for the IT group (see Figure 2) with a significant difference in blood lactate concentration at t=14 min ($P = 0.034$), t = 19 min ($P = 0.043$) and t = 24 min ($P = 0.011$) (see Table 1A). There were no significant changes found between period 3 and period 4, however the difference in blood lactate concentration at t = 14 min almost reached significance ($P = 0.052$). The maximal lactate values reached at the end of the time trial are shown in Table 1B. A significant decrease is found for the maximum blood lactate concentration for the intensified trained horses between period 2 and 3. During period 4 the maximum lactate level is significantly lower for the IT group compared to the control group.

The parameters describing the concentration peaks as calculated by the pulse detection program (Cluster8) are presented in Table 2. The secretion and elimination parameters of the GH data series as calculated by the deconvolution program together with the results of the ApEn and the mean IGF-1 concentrations are shown in Table 3 and Figure 4.

Table 1A. Plasma lactate concentrations (mmol/l) before (t=0), after warming up (t=9) and during the first 10 minutes (t=14, t=19 and t=24) of the SETs for the control (C) and intensified trained (IT) group.
Table 1B. Maximal lactate concentrations (mmol/l) and duration (min) of the SET.

A.

	SET 2		*SET 3*		*SET 4*	
	C	*IT*	*C*	*IT*	*C*	*IT*
T=0	0,6 ± 0,1	0,6 ± 0,1	0,6 ± 0,3	0,8 ± 0,3	0,8 ± 0,3	0,7 ± 0,2
T=9	0,5 ± 0,1	0,4 ± 0,1	0,4 ± 0,1	0,4 ± 0,1	0,5 ± 0,2	0,4 ± 0,1
T=14	3,1 ± 1,0	3,5 ± 1,1[a]	2,4 ± 1,0	1,9 ± 1,3[b]	3,6 ± 1,3	2,4 ± 1,1
T=19	4,6 ± 3,2	4,8 ± 1,5[a]	3,1 ± 1,3	3,0 ± 1,4[b]	4,4 ± 1,9	3,5 ± 1,3
T=24	7,0 ± 4,1	6,2 ± 1,1[a]	4,9 ± 2,2	3,2 ± 1,6[b]	5,9 ± 2,5	4,2 ± 2,0

B.

	C	*IT*	*C*	*IT*	*C*	*IT*
Lactate max	7.7 ± 3.2	8.3 ± 1.5[a]	6.6 ± 3.1	4.3 ± 2.2[b]	9.1 ± 4.8[c]	5.1± 2.1[ab]
Duration SET	17.8 ± 3.0	18.0 ± 2.7	19.6± 0.90	17.4 ± 4.3	19.6± 0.90	17.6 ± 2.9

[a,b]Within a row, values with different superscript letters differ significantly ($P < 0.05$).

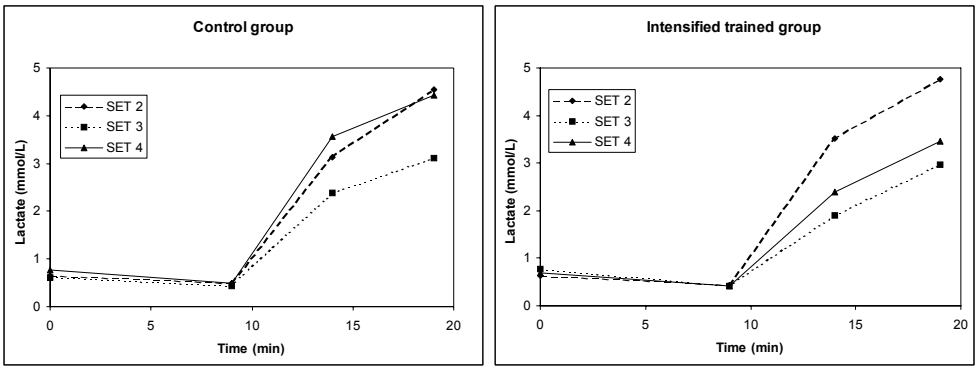


Figure 2. Lactate curves of standardized exercise test 2, 3 and 4 presented as mean concentrations at the first 10 minutes of trot (t = 14, 19 and 24 mins) in the control and intensified trained group.

Table 2. Results of peak detection analysis (Cluster) of nocturnal GH time series for control (C) and intensified trained (IT) Standardbreds after a period of training, intensified training and detraining.

	Training		**Intensified training**		**Detraining**	
Parameter	*C*	*O*	*C*	*O*	*C*	*O*
8-h mean GH (µg/L)	0.91 ± 0.47	0.96 ± 0.34	0.95 ± 0.71	1.36 ± 0.91	0.97 ± 0.67	1.16 ± 1.05
Total GH AUC (µg/L)	441 ± 229	464 ± 165	463 ± 346	659 ± 439	472 ± 325	561 ± 510
# GH concentration peaks/ 8 h	2.6 ± 0.6	2.4 ± 1.1[a]	2.0 ± 0.0[a]	3.6 ± 1.1[b]	2.0 ± 0.7	1.8 ± 0.4[a]
Mean interval between peaks (min)	126 ± 83	186 ± 116	206 ± 152	124 ± 73[a]	189 ± 78	236 ± 73[b]
Mean duration of peaks (min)	66 ± 29	85 ± 26	58 ± 23	72 ± 20[a]	80 ± 38	109 ± 35[b]
Mean amplitude of peaks (µg/L)	4.3 ± 4.3	5.2 ± 3.6	5.2 ± 4.7	4.6 ± 2.4	5.2 ± 2.3	8.2 ± 7.1
Mean AUC of peaks (µg/L)	81.9 ± 92.8	168.8 ± 119.2	137.0 ± 125.3	100.1 ± 57.0	147.1 ± 119.5	236.6 ± 232.5
Mean nadir GH concentration (µg/L)	0.26 ± 0.35	0.25 ± 0.31	0.09 ± 0.11	0.74 ± 0.92	0.36 ± 0.34	0.04 ± 0.00

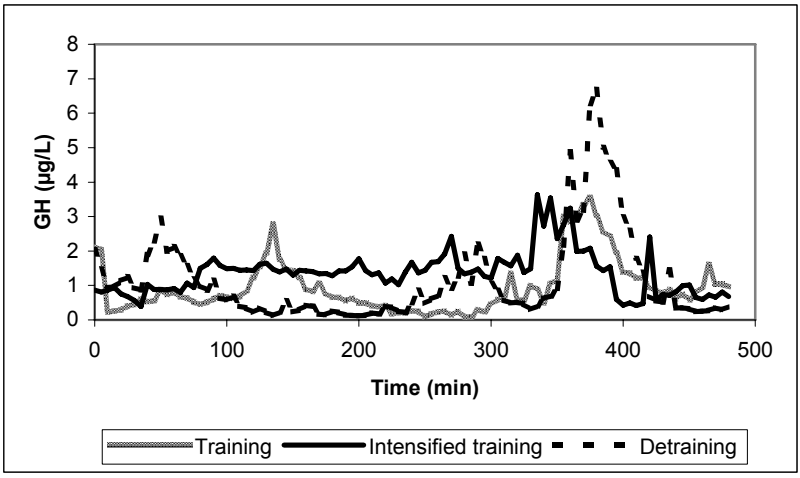
[a,b]Within a row, values with different superscript letters differ significantly ($P < 0.05$).

Table 3. Results of nocturnal GH secretory and elimination parameters analyzed by deconvolution analysis (Autodecon) for control (C) and intensified trained (IT) Standardbreds after a period of training, intensified training and detraining.

	Training		**Intensified training**		**Detraining**	
Parameter	*C*	*OT*	*C*	*OT*	*C*	*OT*
Basal secretion (µg/L/min)	0.014 ± 0.017	0.014 ± 0.025	0.008 ± 0.005	0.010 ± 0.008	0.008 ± 0.006	0.009 ± 0.009
Half-duration (min)	3.1 ± 0.6[b]	2.8 ± 0.4	5.7 ± 3.9	4.6 ± 4.9	7.9 ± 4.4[a]	3.3 ± 1.6[b]
Half-life (min)	9.6 ± 4.2	14.3 ± 2.3[c]	7.3 ± 4.4[ac]	15.2 ± 1.2[bc]	10.8 ± 6.2	9.1 ± 3.7[a]
Number of bursts per 8-h	8.6 ± 2.9	10.6 ± 0.9	9.4 ± 2.7[a]	10.0 ± 5.4	6.2 ± 1.8[b]	9.2 ± 4.1
Mean amplitude burst (µg/L/min)	0.86 ± 0.35	0.63 ± 0.22	0.96 ± 0.30	0.86 ± 0.72	0.72 ± 0.67	1.12 ± 0.43
Mean mass burst (µg/L)	2.8 ± 1.1	1.9 ± 0.8	6.3 ± 5.8	2.5 ± 1.2	4.5 ± 2.8	4.3 ± 3.5
Mean Interval between bursts (min)	49.4 ± 15.1	39.1 ± 7.6	51.6 ± 15.1	49.1 ± 25.2	70.4 ± 28.9[a]	38.9 ± 19.9[b]
Mean GH concentration (µg/L)	0.90 ± 0.46	0.95 ± 0.34	0.97 ± 0.73	1.37 ± 0.92	0.96 ± 0.66	1.15 ± 1.04
8-h total secretion (µg/L)	441 ± 229	464 ± 165	462 ± 346	659 ± 439	472 ± 325	561 ± 510
8-h basal secretion (µg/L)	6.5 ± 7.9	6.9 ± 11.8	3.6 ± 2.4	4.6 ± 3.9	3.7 ± 2.8	4.2 ± 4.2
8-h pulsatile secretion (µg/L)	434.7 ± 228.2	456.7 ± 154.0	458.8 ± 346.6	654.4 ± 439.9	467.9 ± 326.4	557.0 ± 506.0
Ratio pulsatile/total secretion (%)	98.4 ± 1.7	98.9 ± 1.6	98.7 ± 1.2	99.1 ± 0.8	98.8 ± 0.9	99.3 ± 0.2
Approximate Entropy	0,63 ± 0,26	0,56 ± 0,24	0,49 ± 0,1[a]	0,82 ± 0,29[b]	0,6 ± 0,22	0,54 ± 0,42[a]
IGF-1 (ng/ml)	164 ± 69	157 ± 42	189 ± 74	162 ± 17	145 ± 57	179 ± 33

[a,b,c]Within a row, values with different superscript letters differ significantly ($P < 0.05$).

A.



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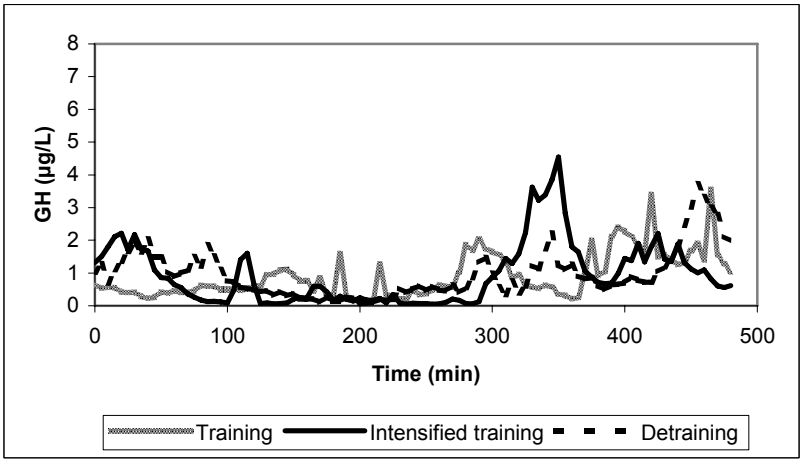

Figure 3. Mean nocturnal GH (µg/L) data series after period of training (2), intensified training (3) and detraining (4) for the intensified trained group (A) and the control group (B). Control group performed the same training during period 2 and 3.

Figure 3 depicts the GH data series of the different periods for both groups based on the mean plasma GH concentration at each time point. An increase in basal levels of GH is seen for the IT group during period 3.

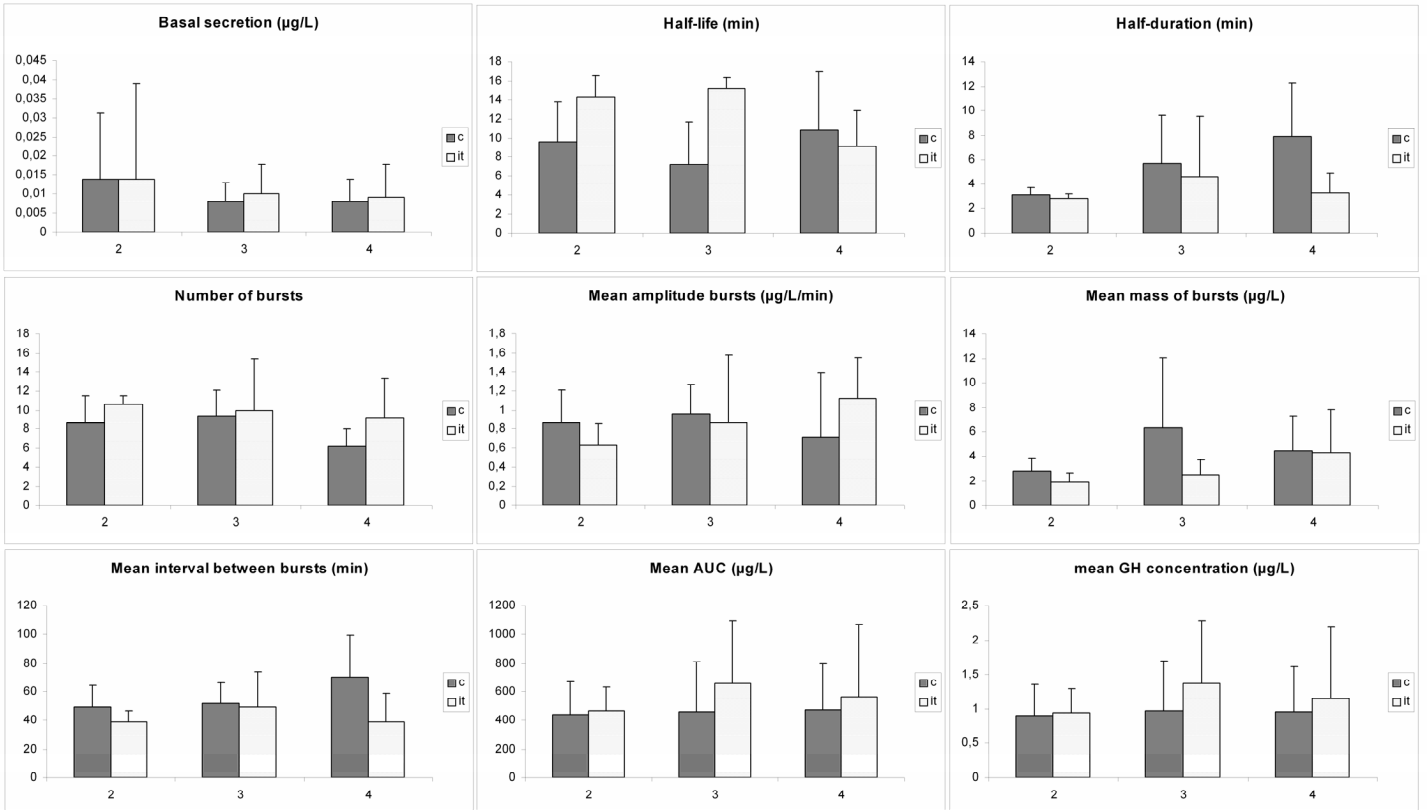

Figure 4. Results of nocturnal GH secretory parameters analyzed by deconvolution analysis for control (C) and intensified trained (IT) Standardbreds after a period of training (2), intensified training (3) and detraining (4).

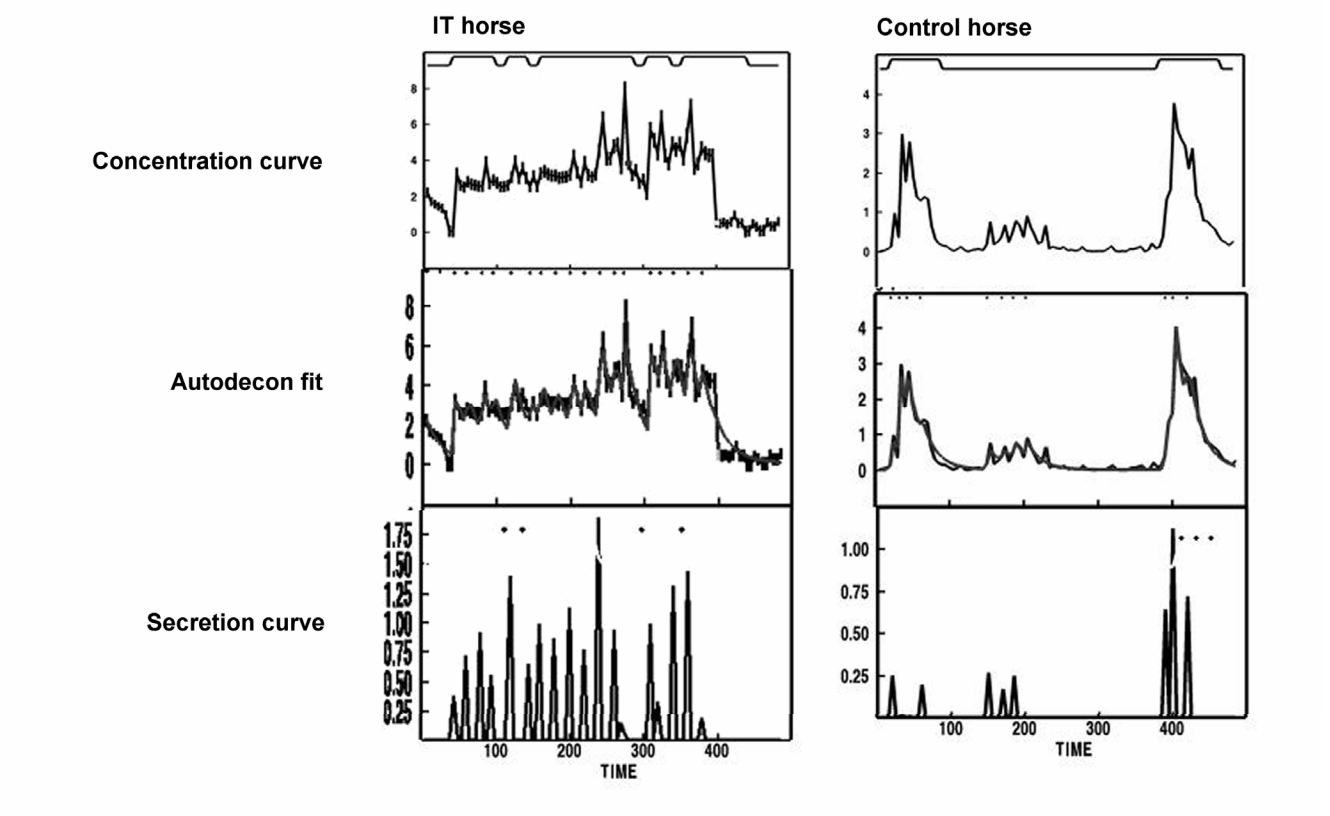

Figure 5. GH concentration curve and secretion curve as assessed by Cluster and Autodecon of a control horse and an intensified trained horse during intensified training period.

Visual inspection of the deconvolution analysis parameters (Figure 4) shows a trend towards an increase in the amount of GH secreted for the IT group during period 3 (8-h mean GH concentration, and total AUC GH show an increase), however statistically this was not significant. Figure 5 shows the GH concentration curve and the calculated GH secretion curve for an intensified trained horse and control horse during period 3.

No significant differences between the two groups during period 2 were found. During period 3 the number of concentration peaks, half-life and ApEn value were increased in the IT group compared with the C group (P = 0.004, 0.011 and 0.039, respectively). During period 4 significantly lower levels were found for the IT group compared to the control group for half-duration (P = 0.031) and the interval between secretion bursts (P = 0.020). For the intensified trained group a significant increase was found for the number of peaks (P = 0.042) between period 2 and 3. Between period 3 and 4 the ApEn values (P = 0.023) and the number of concentration peaks (P = 0.000) increased. The half-life (P = 0.045) significantly decreased, while interval between the concentration peaks (P = 0.036), half-life) and the duration of the concentration peaks (P = 0.042) significantly increased. Between period 2 and 4 the half-life (P = 0.040) was significantly decreased.

What was found for the control group was a significant decrease between period 3 and 4 for the number of secretion burst (P = 0.043) and a significant increase between period 2 and 4 for the half duration of the secretion peaks (P = 0.026).

Some parameters nearly attained significance like the mass of secretion peaks (P = 0.064) and the nadir GH concentration (P = 0.093) during period 3 between both groups, the ApEn (P=0.064) between period 2 and 3 for the IT group, and the AUC of the concentration peaks between period 3 and 4 for the IT group (P = 0.065).

Discussion

In this prospective study, we were able to quantitate the influences of prolonged exercise-stress on nocturnal GH pulsatility modalities and regularity. We showed, for the first time, an increase in approximate entropy (ApEn) combined with an increase in GH pulse frequency and half-life in intensified trained horses indicating that the GH-IGF-1 axis shows pathophysiological adaptation to increased exercise stress.

An important finding was the increase in ApEn after the intensified training period for the IT group. ApEn quantities the orderliness of the subordinate (= non-pulsatile) secretory GH patterns. A larger GH ApEn value denotes greater irregularity and has been reported during puberty and in patients with acromegaly and Cushing’s disease

(243, 324). Decreased regularity could reflect impaired coordinate controle of GH secretion by GHRH, somatostatin, GH, and/or IGF-1.

In the present study, the main difference found with the peak analysis program (Cluster8) is an increase in GH peak frequency for the IT group at the end of the intensified training period. This increase is accompanied by a decrease in mean peak duration and interval as well as an almost significant decrease in the mean AUC of the concentration peaks as shown by the differences occurring between the intensified training and the detraining period. Thus, the GH pulsatility pattern changed to more frequent, smaller concentration peaks for the intensified trained horses.

The results from the present study differ from literature in that there were no significant differences in the total amount of GH secreted and the amplitude of the concentration peaks observed. Studies have reported increases in both parameters as a result of training or acute metabolic stress (25, 231, 356). However, in the current study comparisons were made between moderately trained and highly intensified trained subjects, while Weltman et al (356) compared nocturnal GH secretion between untrained individuals before and after one year of (moderately intensive) endurance training above lactate threshold. They found an increase in GH peak amplitude, nadir GH concentration, GH peak area and 24-h GH concentration due to this training. When comparisons between the group trained at lactate threshold and the group trained under lactate threshold were made, only significant differences in the nadir GH concentration and the integrated GH concentration were found, which is in agreement with our findings, where nadir GH concentration showed an increasing trend during the intensified training as compared to the control group during the same period. These data might suggest that an increase in amplitude and GH peak area are early adaptations of the GH-axis obtained by a moderate training. Further adaptations of the GH-axis to increased training load, leading to too much stress, might result in an increase in number of concentration peaks. This hypothesis is also suggested in fasting studies where pure fasting increases GH pulse frequency (45, 131) and partial fasting resulted in increases in amplitude (231).

The plasma GH concentration peak can be divided into relevant contributions by GH secretory burst amplitude and duration, burst frequency, basal secretion and half-life (334). In the current study, deconvolution analysis revealed an increased half-life of GH after intensified training, suggestive of a decrease in metabolic clearance rate. An increase in GH half-life is not often observed. This phenomena is described in patients with chronic liver disease due to decreased production of IGF-1 by the liver and hence an impaired negative feedback (221) and in renal disease due to impaired clearance of GH by the kidney (315). Another explanation is an increased concentration of binding proteins, and consequently an increase in the amount of bound GH (338). However, an

underestimation of secretory pulses will also lead to an increased GH half-life as shown below.

The increased frequency in concentration peaks after intensified training was not accompanied by an increase in secretion bursts. This is in contrast with our expectations, because large peaks of plasma GH concentrations often reflect the occurrence of a succession of secretory pulses. Hartman et al (130) found that only 4% of GH secretion occurred as isolated single bursts, and that these single secretory episodes had very low amplitudes. This is also the case in our horses, because the deconvolution analysis revealed the existence of approximately 80% more pulses than detected in the plasma profiles by pulse detection with the Cluster program in both groups. An explanation for the discrepancy found during the intensified training period for the IT group might be the fact that the deconvolution program had more difficulty in fitting the curves of the IT group due to the increase in disorderliness as shown by the increase in ApEn. This could have led to an underestimation of the GH pulse frequency. In addition, Figure 3 shows that the interpeak (basal) serum GH concentrations rose simultaneously with increasing GH secretory pulse frequency, probably because the decay of the serum GH concentration from previous peaks was not complete before the next high-amplitude pulse occurred. This has already been described for exceptionally irregular GH patterns associated with acromegaly (337). When identification of the individual secretory pulses fails, secretory pulses are combined which leads to longer half-lifes.

Four weeks of detraining were integrated into the current experiment to evaluate the duration of the effects induced by the intensified training. For the intensified trained group, the number of concentration peaks decreased combined with increased intervals between the concentration peaks as well as the duration of the peaks, half-life became shorter and ApEn normalized after detraining as compared to the intensified training period. Parameters were not different from the control group during detraining. Two parameters showed an opposite response at the end of the detraining for the control and the IT group. Half-duration of the secretion peaks as well as the interval between secretion peaks are significantly longer for the control group compared to the IT group at detraining. This is in agreement with the significant decrease in the number of secretion peaks found for the control group between detraining and intensified training. In addition, visual inspection of the integrated GH data series showed that for the control group the curves of training and detraining were almost identical. The curves of the IT group, however, were still different. In conclusion, the nocturnal GH profile of the IT group was changing in the same direction as the control group during detraining, however secretion pulses were still smaller and more frequent.

The combination of changes in GH secretion dynamics drove us hypothesize about which pathophysiologic mechanism could cause these alterations. Based on the model

that GHRH predominantly increases GH pulse amplitude whereas somatostatin primarily controls GH pulse frequency (131), the current results suggest that intensified training in young Standardbred horses primarily increases somatostatin withdrawal intervals so that GH burst frequency is increased, while less demanding training predominantly influences GHRH by increasing GH pulse amplitude. This corresponds readily to the decrease in serial regularity of GH release shown by the higher approximate entropy values in the intensified trained horses, suggesting a relative loss of coordinated somatostatin and GHRH release, which normally would give rise to organized pulses of GH release (340).

An increase in frequency might be of more benefit for the response of target tissues in the intensified trained horses because GH pulses tend to promote optimal linear and muscular growth, while continuous GH stimulation modulates carbohydrate and lipid metabolism via induction of hepatic IGF-1 synthesis and GH receptor as well as low-density lipoprotein (LDL)-receptor expression (334). The increased training load for the IT group during the intensified training period did not allow them to recover between exercise sessions as was shown by the decrease in performance at the end of this period and might have even induced alterations in GH pulsatility to optimize recovery between training sessions. In addition, endogenous opiates as well as catecholamines stimulate GH secretion by inhibiting somatostatin release in hamsters (25). It is hypothesized that the intensified training might have induced increased levels of stress hormones like catecholamines with a result being decreased levels of somatostatin allowing high frequency GH pulsatility. As visualized in Figure 3, the IT group has longer intervals of increased GH levels resembling a more continuous GH stimulation that might promote restoring homeostatic balance.

Interestingly, Tuckow et al (316) found similar changes in GH secretory dynamics after acute intensive resistance exercise at the end of the day in man: an increase in secretory burst frequency combined with a decrease in mean interval between bursts, mean burst mass and mean burst amplitude as well as an increase in ApEn. Total secreted GH and half-life remained the same. Several studies describe the influence of acute exercise on nocturnal GH secretory dynamics in man (172, 176, 230). In general, it appears that exercise of sufficient duration and intensity at the end of the day can influence the temporal release of overnight GH release. The total amount of GH secreted is not changed, but there is a shift of GH secretion towards the second half of the night possibly caused by an increase in the stress hormone cortisol during early sleep induced by intensive exercise at the end of the day. This impairs GH secretion during early hours of sleep and diminishes the anabolic response (176, 230). Based on the similar changes found in the current study, it seems that acute exercise stress and chronic exercise stress elicit the same endocrinological response except that chronic stress increases the half-life and possibly increases the amounts of GH secreted.

These differences may indicate that the normal endocrinological response to stress fails to restore homeostatic balance during overloading and further adaptations need to be made. Additional research is needed to verify this hypothesis.

An expected but nonetheless important finding was that no significant change in any parameter determined between both groups was found at the end of the training period. During this training period, the horses performed exercise based on a general training scheme adapted to their individual capacities with the goal to make up an equally and comparable trained group. During the intensified training period the control group (C) performed the same training as in period 2. For the intensified trained group (IT) the training load was considerably increased and the number of resting days successively decreased to zero. The increased training load combined with insufficient rest increased the training stress for the horses in the IT group as shown in the changes in behavior and decreases in performance of the training and the SET. During detraining, the training load was decreased for both groups and the resting days were increased to 3 days, which allowed the horses to recover from the preceding training period. The detraining period enabled us to objectively measure whether changes during the intensified training period normalize after a period of relative rest.
The SET's were performed throughout the experiment to monitor performance. The performance of the horses increased during the training period, as observed from the higher speeds and inclination at the end of training period (SET 2) compared to acclimatization period (SET 1) needed to reach 80% HF_{estmax}. In the intensified trained group, the mean lactate concentration of SET 3 was decreased compared to SET 2 during the first 15 minutes of trot, which indicates a significant right shift of the lactate curve during SET 3 compared to SET 2. In addition, there was a significant decrease in the maximum lactate levels at the end of SET 3 compared to SET 2. Maximum lactate levels were still decreased compared to the control group after detraining. The right shift of the lactate curve did not change after detraining, however it almost reached a significant difference after 5 minutes of trot, which implies that the horses were not fully "recovered" yet or were able to keep their performance with minimal training for longer than four weeks as is shown by the control group. The lactate curves of the control group stayed the same during the three periods. Notwithstanding the fact that the duration of the time trial was not significantly decreased between the training and the intensified training period, the intensified trained horses showed a loss of performance while the training load was increased. One horse stopped before the end of the SET and three horses were not able to trot and had to change their gait to fulfill the required SET.

A right shift of the blood lactate curve is often associated with an improvement in performance, but both optimal training and overtraining can induce a right shift of the lactate curve (26, 156). A decrease in submaximal blood lactate can result from a

decreased production, or an increased utilization by the muscle and other organs (26). Endurance training improves lactate utilization (26, 75), while overtraining seems to decrease muscle lactate production capacity (26, 156). Several mechanisms have been proposed to explain this decrease. It is suggested that not the amount of substrate in the muscle, but the capacity to mobilize the substrate available is the critical factor. Mobilization of substrate is influenced by several hormones among them the stress hormones adrenalin/noradrenalin, corticosteroids and GH (2, 16, 26).

Chronic training stress (= chronic overload) results in a delayed adaptation process of the body and a loss of performance (179). Prolonged overloading results in overreaching or eventually leads to the overtraining syndrome (OTS). Recovery from overreaching will occur after 2-3 weeks of rest. Recovery, if any, of the OTS will take months of rest (179). The signs and symptoms occurring at the end of the intensified training period (namely, the diminished coordinated GH secretion, the alterations in GH secretory dynamics, loss of performance in spite of increased training, behavioural changes, decrease in maximal lactate concentrations, the right shift of the lactate curve at the end of the intensified training period) and the persistance of some of the signs and symptoms during the detraining might indicate that the IT group was not fully recovered at the end of detraining. Thus, overreaching or perhaps an early stage of overtraining was induced during the intensified training period for the IT group.

Conclusion

In summary, to the best of our knowledge we have demonstrated for the first time, that intensified training for 6 weeks alters nocturnal GH pulsatility in young Standardbred horses compared to a control group via a more frequent, smaller peak secretion pattern with a prolonged half-life possessing an increased ApEn. The prolonged half-life is likely caused by the increase in disorderliness of the GH data series and the difficulty associated with fitting irregular curves by the deconvolution program instead of an actual change in GH kinetics. After four weeks of detraining certain parameters remained different from the control group, which might lead to the conclusion that an early stage of overtraining was induced during this experiment. It is hypothesized that the change in nocturnal GH pulsatility pattern is of benefit for the intensified trained horse by promoting a reestablishment of homeostatic balance, which improves recovery of and adaptation to the increased training load/stress. Longer periods of somatostatin withdrawal are expected to be the underlying mechanism for the observed changes in GH pulsatility pattern.

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Chapter 6

The effects of short-term recombinant equine Growth Hormone (eGH) and hydrocortisone and long-term recombinant eGH administration on tissue sensitivity to insulin in horses.

Ellen de Graaf-Roelfsema[1]
Theerawat Tharasanit[1]
Klien G. van Dam[1]
Hans A. Keizer[2]
Eric van Breda[2]
Inge D. Wijnberg[1]
Tom A.E. Stout[1]
Johannes H. van der Kolk[1]

[1]Department of Equine Sciences, Faculty of Veterinary Medicine, Utrecht University, Utrecht; The Netherlands. [2]Department of Movement Sciences, Faculty of Health Sciences, University of Maastricht, The Netherlands.

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Abstract

Objective: To determine the effects of short-term IV administration of hydrocortisone or equine growth hormone (eGH) or long-term IM administration of eGH to horses on tissue sensitivity to exogenous insulin.
Animals: 5 Standardbred and 4 Dutch Warmblood horses.
Procedure: The euglycemic-hyperinsulinemic clamp technique was used to examine the sensitivity of peripheral tissues to exogenous insulin 24 hours after administration of a single-dose of hydrocortisone (0.06 mg/kg), eGH (20 µg/kg), or saline (0.9% NaCl) solution and after long-term administration (11 to 15 days) of eGH to horses. The amounts of metabolized glucose (M) and plasma insulin (I) were determined.
Results: Values for M and the M-to-I ratio were significantly higher 24 hours after administration of a single-dose of hydrocortisone than after single-dose of eGH or saline solution. After long-term administration of eGH, basal I concentration was increased and the mean M-to-I ratio was 22% lower, compared with values for horses treated with saline solution.
Conclusions and clinical relevance: Increases in M and the M-to-I ratio after a single dose of hydrocortisone imply that short-term hydrocortisone treatment increases glucose use by, and insulin sensitivity of, peripheral tissues. Assuming a single dose of hydrocortisone improves sensitivity of peripheral tissues to insulin, it may be an interesting candidate for use in reducing insulin resistance in peripheral tissues of horses with several disease states. In contrast, long-term administration of eGH decreased tissue sensitivity to exogenous insulin associated with hyperinsulinemia. Therefore, increased concentrations of growth hormone may contribute to insulin resistance in horses with various disease states.

Introduction

Insulin resistance can be defined as the metabolic state in which physiologic concentrations of insulin stimulate a lower-than-anticipated biological response (cellular glucose uptake) (170). As such, insulin resistance can be quantified by examining glucose tolerance, a parameter that can be measured most accurately by use of 2 clamp techniques (ie, the hyperglycemic clamp and the euglycemic-hyperinsulinemic clamp technique) (7), both of which have been validated for use in horses (87, 268). The hyperglycemic clamp technique is primarily a method for quantifying the sensitivity of the pancreas to exogenous glucose, whereas the euglycemic-hyperinsulinemic clamp technique is a method for quantifying tissue sensitivity to insulin.

Insulin resistance in horses is of interest because it has been associated with several disease states, including obesity, hyperadrenocorticism, equine metabolic syndrome or peripheral hyperadrenocorticism, laminitis, hyperlipemia, and excessive amounts of growth hormone (GH) (97, 105, 155, 158, 236, 262). Although it has been suggested that both cortisol and growth hormone are responsible for increased insulin resistance in horses with some of these conditions, the exact cause of insulin resistance has not been documented for any of them, primarily because insulin resistance in most studies (48, 105, 261, 262) was only estimated by use of indirect measurements, such as a single plasma glucose or insulin concentration, oral or IV glucose tolerance tests, or an insulin tolerance test. A more precise investigation of the possible cause or causes of increased insulin resistance can be conducted by measuring the effect of proposed causal agents on tissue sensitivity to exogenous insulin by use of the euglycemic-hyperinsulinemic clamp technique. During the steady-state condition of the euglycemic-hyperinsulinemic clamp technique, endogenous glucose production by the liver and endogenous insulin production by the pancreas are suppressed, and it is therefore possible to directly quantify the metabolism of glucose by peripheral tissues (69).

The objective of the study reported here was to use the euglycemic-hyperinsulinemic clamp technique to assess the effects of short-term administration of hydrocortisone or equine GH (eGH; experiment 1) or long-term administration of eGH (experiment 2) to horses on the sensitivity of peripheral tissues to exogenous insulin. The testable hypotheses were that eGH and hydrocortisone would be completely inert and have exactly the same effect as physiologic saline (0.9% NaCl) solution on insulin resistance of peripheral tissues. To our knowledge, the relationships between eGH and insulin sensitivity of peripheral tissues as well as between hydrocortisone and insulin sensitivity of peripheral tissues have not been examined in horses by use of the euglycemic-hyperinsulinemic clamp technique.

Materials and Methods

Animals

Nine horses were used in the 2 experiments of the study reported here. Five healthy Standardbred horses (4 mares and 1 gelding) were used in experiment 1. Horses ranged from 3 to 13 years of age (mean ± SD, 5.6 ± 4.2 years) and weighed between 411 and 457 kg (mean ± SD, 435 ± 14 kg). The horses were housed in stables all day except for 1 hour of exercise (ie, walking controlled by use of a walking machine). For experiment 2, we used 2 of Standardbred horses (mares) from the first experiment and 4 Dutch Warmblood mares. Horses ranged from 4 to 13 years of age (mean ± SD, 8.3 ± 3.7 years) and weighed between 477 and 736 kg (mean ± SD, 557 ± 94 kg). Body condition score of the horses ranged from 4 to 5 (mean ± SD, 4.5 ± 0.55). The horses were maintained on pasture all day and used for light riding.

The medical history for each horse was collected. A clinical examination and hematological evaluation were performed, with emphasis on body condition score and indications of insulin resistance (basal plasma glucose and insulin concentrations). Horses were excluded from the study on the basis of a body condition score < 4 or > 5 (136), hyperglycemia, hyperinsulinemia, or clinical evidence of systemic disease. Horses were also excluded when they had received medications within the month preceding onset of the study.

The experiments were approved by the Committee for Animal Welfare at the Faculty of Veterinary Medicine, Utrecht University.

Experiment 1

The effects of a single-dose of eGH, hydrocortisone, or saline (control) solution on the sensitivity of peripheral tissues to exogenous insulin were examined by use of a euglycemic-hyperinsulinemic clamp technique in 5 horses allotted randomly to treatment order for a 3-way crossover design. The 3 treatments were hydrocortisone (Solu-Cortef Act-O-Vial (100 mg/2 ml), Pharmacia & Upjohn Co, Woerden, the Netherlands; 0.06 mg/kg), recombinant eGH (EquiGen (10 mg eST), BresaGen, Adelaide, Austalia; 20µg/kg), and control solution (5 mL of saline solution). All treatments were administered IV 24 hours before measurement of variables by use of the euglycemic- hyperinsulinemic clamp technique. Successive treatments in each horse were separated by an interval of at least 7 days. In each case, food was withheld from the horses beginning 12 hours before and until completion of the euglycemic-hyperinsulinemic clamp technique.

Experiment 2

Six mares were used in an experiment in accordance with a 2´2 Latin-square design to investigate the effects of long-term administration of eGH on sensitivity of peripheral tissues to exogenous insulin, as measured by use of the euglycemic-hyperinsulinemic clamp technique. The 2 treatments were eGH and control solution, with horses allotted randomly to the initial treatment. As recommended in the data sheets of the manufacturer for prolonged treatment with eGH, each horse was treated with 5 mg of eGH dissolved in 2 mL of saline solution for the first 2 days and thereafter with 10 mg of eGH dissolve in 4 mL of saline solution. Correspondingly, the control treatment consisted of 2 mL of saline solution for the first 2 days and 4 mL of saline solution thereafter. All treatments were administered IM once daily for a minimum of 11 days (range, 11 to 15 days; mean ± SD, 13 ± 1.7 days). Variation in duration of treatment resulted from attempts to control for effects of reproductive hormones because insulin resistance has been associated with disturbances in the duration of the estrous cycle (53, 293). Therefore the administration of eGH or saline solution was started on the day of ovulation in each mare and continued until the dominant follicle of the subsequent estrus reached 35 mm in diameter, which caused variation in duration of eGH or control treatment. The subsequent estrus was induced by IM administering a prostaglandin F2a analogue (0.375 µg cloprostenol sodium, Estrumate, Schering-Plough Animal Health, Brussels, Belgium) on day 6 after ovulation that coincided with the onset of treatment. The IM injections were rotated among 8 injection sites in each horse (ie, neck, pectoral, gluteal and semimembranosus-semitendinosus muscles on the left and right side).

The euglycemic-hyperinsulinemic clamp technique was performed 24- to 72 hours after the last dose of eGH or saline solution. The variation in time between end of treatment and onset of testing by use of the clamp technique was primarily caused by the fact that treatment was completed concurrently in some horses and we were unable to perform measurements of all horses 24 hours after completion of treatment. To verify that eGH was still effective in horses which measurements were performed > 24 hours after completion of treatment, basal serum concentrations of insulin-like growth factor (IGF)-1 and basal plasma concentrations of insulin were measured before testing by use of the clamp technique.

Horses were allowed a recovery period of at least 1 estrus cycle between the 2 courses of treatment to avoid possible carryover effects of eGH treatment. Basal plasma insulin concentration (I) and basal serum IGF-1 concentration measured before testing by use of the clamp technique in each horse were used to verify that there were no carryover effects.

Euglycemic-hyperinsulinemic clamp technique

In both experiments, in vivo insulin sensitivity was examined by use of the euglycemic-hyperinsulinemic clamp technique described elsewhere (268). To perform the clamp, a catheter was inserted into each jugular vein. One of these catheters was subsequently used for infusion of glucose (50 %-solution) and insulin (Actrapid recombinant hyman insulin (100 U/mL), Novo Nordisk A/S, Bagsvaerd, Denmark), whereas the other catheter was used for collection of blood samples to monitor circulating glucose and insulin concentrations. The first blood sample (time 0) was used to establish the basal glucose concentration. Within 10 minutes after the collection of the first blood sample, a priming dose of insulin (45mU/kg dissolved in 50 mL of saline solution) was administered IV as a bolus injection. This bolus injection of insulin was followed immediately by constant rate infusions of insulin (6 mU/[kg X min]) and glucose (mean infusion rate, 8.6 µmol/[kg X min]) (268). Constant rate infusions were accomplished by use of a pump (Volumetric pump, model Argus 414, Argus Medical AG, Heimberg, Switzerland). Throughout the period of insulin and glucose infusion, blood samples were collected at 10-minute intervals into heparinized syringes. Within 2 minutes after collection, glucose concentration in each sample was measured by use of an automated analyzer (ABL-605, Radiometer Copenhagen, Westlake, Ohio). Glucose was maintained at the basal concentration measured in each horse (range 3.9 to 5.6 mmol/L). When the blood glucose concentration differed from the basal concentration, the glucose infusion rate was adjusted to compensate. A steady-state condition was presumed to exist when the plasma glucose concentration and the glucose infusion rate were concurrently held constant for at least 30 minutes and the plasma glucose concentration was within the range for euglycemic values.

Values for I were determined before the clamp technique was started (time 0) and in 3 samples collected at 10-minute intervals during the euglycemic-hyperinsulinemic steady-state condition into lithium heparinised tubes. These blood samples were centrifuged (Rotina 48R, Hettich zentrifugen, Tuttlingen, Germany) for 10 minutes at 6,000 X g; the resulting plasma was harvested and stored at -20oC until insulin concentrations were analyzed by use of a radioimmunoassay (Coat-A-Count TKIN2 836, Diagnostic Products Corp, Los Angeles, California) validated for use in samples obtained from horses (328).

Calculations

For plasma glucose concentrations to exist in a steady-state condition, the amount of glucose infused must equal the amount of glucose being removed from the glucose space (i.e. amount of metabolized glucose [M]), provided that endogenous glucose production is suppressed completely. However, in reality, the plasma glucose concentration is never absolutely constant (69), and a space correction factor must be

included to account for glucose that is added or removed from the glucose space by means other than metabolism. Thus, M during steady-state conditions during use of the clamp technique can be expressed by the following equation (69):

$$M = INF – SC,$$

where INF is the amount of glucose infused, and SC is the space correction factor. The space correction was calculated by use of the following equation (69):

$$SC = ([G2-G1] \bullet GS) / ([t2-t1] \bullet BW),$$

where G1 and G2 are the glucose concentrations at time points t1 and t2, respectively; GS is the glucose space, which is calculated as 0.19 L/kg • BW of the horse in kg; and BW is the body weight of the horse (in kg). In this study, values of M were calculated for 10-minute intervals.

Values for I were calculated for the 3 samples recovered during the steady state condition. Those concentrations were used to calculate the mean M-to-I ratio, which reflects the quantity of glucose metabolized per unit of plasma insulin and is a good index of tissue sensitivity to exogenous insulin (69).

In addition, for results of 3 clamp-technique measurements of relatively long duration performed in 3 horses, 2 different steady-state conditions were identified. The M and I values for each steady state were measured as described previously.

Analysis of blood samples

For experiment 1, additional blood samples were collected into coagulant-free (ie, serum) tubes (Vacuette, Z Serum Sep. Clot Activator, Greiner Bio-One GmbH, Kremsmünster, Austria), before the experiment began and at the beginning of measurements by use of the clamp technique. These samples were used to measure IGF-1 concentrations. In addition, blood samples were collected into lithium-heparinized tubes (Vacuette, LH Lithium Heparin, Greiner Bio-One GmbH, Kremsmünster, Austria), at the beginning of measurements obtained by use of the clamp technique and at the end of the steady-state; these samples were used for analysis of nonesterified fatty acid (NEFA) and cortisol concentration.

For experiment 2, blood samples were collected at the beginning of measurements obtained by use of the clamp technique (for use in measuring IGF-1, NEFA and ß-hydroxybutyrate concentrations) and at the end of the steady state (for use in measuring NEFA and ß-hydroxybutyrate concentrations).

Assays

Plasma cortisol concentrations were measured by use of a radioimmunoassay (Coat-A-Count, Diagnostic Products Corp, Los Angeles, California) validated for use in samples

obtained from horses (328). Serial dilutions of equine plasma with a high cortisol concentration yielded results that were parallel to the standard curve. The addition of measured amounts of cortisol to samples of plasma resulted in a mean ± SD recovery of 101 ± 7%. The detection limit of the assay was 1.1 nmol/L, and the intra-assay and interassay coefficient of variation (CV) was 6% and 8%, respectively (328). Cross-reactivity for the immunoassay (calculated at 50% of the initial binding) included cortisol (100%), 21-deoxy-cortisol (62%), corticosterone (11%), prednisolone (3%), cortisone (2%), 11-deoxy-cortisol (1.3%), dexamethasone (0.3%), deoxycorticosterone (1,3%), 17-a-OH-progesterone (0.1%) (328).

Plasma insulin concentration was measured by use of a commercial radioimmunoassay kit (Coat-A-Count TKIN2 836, Diagnostic Products Corp, LA, California) that had been validated for use in samples obtained from horses. Curves obtained with serial dilutions were parallel to the standard curve. The detection limit of the assay was 8.6 pmol/L. The intra-assay and interassay CV were 5% and 7%, respectively. The insulin antiserum cross-reacted with proinsulin (approx 40%) and had a particularly low cross-reactivity to compounds other than insulin and proinsulin (327, 328).

Serum IGF-1 concentrations were measured in samples extrated by use of acid ethanol; extraction efficiency was 85% to 90%. Concentrations were measured by use of a radioimmunoassay kit that had been validated for use in samples obtained from horses (229, 296). Curves obtained with serial dilutions were parallel to the standard curve. The intra-assay and interassay CV were 3.2% and 15.6%, respectively. Plasma NEFA concentrations were measured by use of a commercial kit (Randox kit NEFA, FA 115, Randox Laboratories Ltd, Antrim, UK) that had been validated for use in samples obtained from horses. The intra-assay and interassay CV were 7.3% and 16.0%, respectively. Curves obtained with serial dilutions were parallel to the standard curve.

Serum ß-hydroxybutyrate concentrations were measured by use of a commercial kit (Randox kit Ranbut, RB1007, Randox Laboratories Ltd, Antrim, UK) that had been validated for use in samples obtained from horses. The intra-assay and interassay CV was 7.8% and 10.9%, respectively. Curves obtained with serial dilutions were parallel to the standard curve.

Plasma glucose concentrations were analyzed by use of an automated analyzer (ABL-605, Radiometer Copenhagen, Westlake, Ohio).

Statistical analysis

All values were recorded as mean ± SD and analyzed by use of 2-way, repeated measures ANOVAs (SPSS, version 10.0 for Windows, SPSS Inc, Chicago, Ill) in which horses, treatment order, and treatment were the variables. Post hoc analysis was

performed by use of the Bonferroni test. Paired-sample t tests were used to compare values for steady-state conditions reached after short versus longer duration of the clamp technique. In all cases, differences were considered significant at values of $P<0.05$.

Results

Experiment 1

Mean ± SD values for M, I, basal I, the M-to-I ratio, cortisol concentration, and IGF-1 concentrations were determined (Table 1). The first hour after onset of the clamp technique is considered an equilibration period; therefore, only steady states achieved at least 60 minutes after starting the insulin infusion were considered useful for comparison. In experiment 1, all clamp tests fulfilled this criterion, with the steady-state condition reached after a mean of 125 ± 55 minutes. No significant between-treatment variations in duration of the clamp technique were found.

Table 1. Mean ± SD values for insulin concentrations and glucose metabolism for 5 Standardbred horses after IV injection of a single dose of saline (0.9% NaCl) solution, hydrocortisone, or equine growth hormone (eGH) administered 24 hours before measurements were obtained by use of a euglycemic-hyperinsulinemic clamp technique.

Variables	***Saline solution***	***Hydrocortisone***	***eGH***
M (mmol/[kg X min])	0.010 ± 0.0026[a]	0.015 ± 0.0047[b]	0.011 ± 0.0026
Isteady state (pmol/L)	4,079 ± 1,010	4,925 ± 462	3,928 ± 710
M-to-I ratio (X 10–6)	2.5 ± 0.8[c]	3.2 ± 0.9[d]	2.6 ± 1.1
Ibasal (pmol/L)	37.4 ± 8.9	37.8 ± 19.2	48.4 ± 32.4
$Cortisol_{basal}$ (nmol/L)	171.3 ± 50.9	183.6 ± 17.1	200.0 ± 56.0
$Cortisol_{steady\ state}$ (nmol/L)	202.0 ± 34.1	213.7 ± 38.7	206.0 ± 54.0
$IGF\text{-}1_{basal}$ (ng/mL)	222 ± 45	214 ± 47	220 ± 60

M = Amount of metabolized glucose. Isteady state = Plasma insulin concentration (I) at steady state during testing by use of the euglycemic-hyperinsulinemic clamp technique. I_{basal} = I before onset of testing by use of the euglycemic-hyperinsulinemic clamp technique. $Cortisol_{basal}$ = Plasma cortisol concentration before onset of testing by use of the euglycemic-hyperinsulinemic clamp technique. $Cortisol_{steady\ state}$ = Plasma cortisol concentration at steady state during testing by use of the euglycemic-hyperinsulinemic clamp technique. $IGF\text{-}1_{basal}$ = Serum concentration of insulin-like growth factor-1 before onset of testing by use of the euglycemic-hyperinsulinemic clamp technique.

[a-d]Within a row, values with different superscript letters differ significantly ([a,b]$P < 0.001$ and [c,d]$P = 0.009$).

Hydrocortisone treatment resulted in mean values for M and M-to-I ratio that were significantly higher than for the control or eGH treatments. However, there were no significant differences found before onset of testing and at steady state during testing by use of the clamp technique in plasma hydrocortisone concentrations or serum IGF-1 concentrations. Furthermore, treatment order did not significantly affect M or the M-to-I ratio. Mean ± SD value for space correction factor for the 3 clamp applications was 0.0 ± 0.04 L (mean CV, 1.82 ± 0.55 %).

Mean plasma NEFA concentrations at the beginning of measurement by use of the clamp technique did not differ significantly among treatments (hydrocortisone, 0.38 ± 0.13 mmol/L; eGH, 0.46 ± 0.08 mmol/L; and saline solution, 0.57 ± 0.17 mmol/L). However, in all groups, mean plasma NEFA concentration during steady state was significantly lower (hydrocortisone, 0.08 ± 0.01 mmol/L; eGH, 0.08 ± 0.01 mmol/L; saline, 0.08 ± 0.02 mmol/L) compared with the values before use of the clamp technique.

Experiment 2

Mean ± SD values for M, I, basal I, M-to-I ratio, NEFA concentration, ß-hydroxybutyrate concentration, and IGF-1 concentration for mares treated for >11 days with eGH or saline solution were calculated (Table 2). Similar to experiment 1, the first hour after onset of the clamp technique was considered an equilibration period; therefore, only steady states achieved at least 60 minutes after starting the insulin infusion were considered useful for comparison. For experiment 2, all clamp tests fulfilled this criterion, with the steady state condition reached after a mean of 149 ± 48 minutes. There were no significant between-treatment variations in duration of the clamp technique.

Mean ± SD value for the space correction factor for the 2 clamp applications was 0.0 ± 0.1 L (mean CV, 1.96 ± 0.95 %). Long-term administration of eGH led to significant increases in the basal I and basal IGF-1 concentration and to a significant decrease in the mean M-to-I ratio. The horses were randomly allotted to the treatments; however, comparison of the data for horses treated with saline solution first and eGH second with that for horses treated with eGH first and saline solution second revealed a significant ($P<0.001$) decrease in the M-to-I ratio when saline solution was used as the second treatment. No significant changes were found for M. The NEFA concentrations decreased significantly during testing by use of the clamp technique but did not differ significant between treatments. A similar pattern was observed for ß-hydroxybutyrate concentrations, although the decrease in concentrations from values before onset of the clamp technique to values at the steady-state condition was only significantly different for the control treatment.

Table 2. Mean ± SD values for metabolic variables for 6 horses after an IM injection of eGH or saline solution administered once daily for 11 to 15 days and tested by use of a euglycemic-hyperinsulinemic clamp technique 24 to 72 hours after the final daily injection.

Variables	*Saline solution*	*EGH*	*Reference range*
M (mmol/[kg X min])	0.019 ± 0.0067	0.016 ± 0.0042	NA
$I_{steady\ state}$ (pmol/L)	2,720 ± 581	2,933 ± 294	NA
M-to-I ratio (X 10–6)	7.3 ± 2.9[a]	5.7 ± 1.6[b]	NA
I_{basal} (pmol/L)	62.0 ± 52.7[a]	163.0 ± 77.7[b]	< 409.0
$NEFA_{basal}$ (mmol/L)	0.78 ± 0.21[A]	0.65 ± 0.11[C]	< 0.66
$NEFA_{steady\ state}$ (mmol/L)	0.11 ± 0.05[B]	0.11 ± 0.05[D]	
IGF-1basal (ng/mL)	200 ± 50[a]	420 ± 125[b]	
ß-$Hydroxybutyrate_{basal}$ (mmol/L)	0.21 ± 0.02[E]	0.16 ± 0.06	< 0.39
ß-$Hydroxybutyrate_{steady\ state}$ (mmol/L)	0.09 ± 0.05[F]	0.09 ± 0.03	< 0.39

$NEFA_{basal}$ = Nonesterified fatty acid (NEFA) concentration before onset of testing by use of the euglycemic-hyperinsulinemic clamp technique. NEFAsteady state = The NEFA concentration at steady state during testing by use of the euglycemic-hyperinsulinemic clamp technique. ß-$Hydroxybutyrate_{basal}$ = ß-Hydroxybutyrate concentration before onset of testing by use of the euglycemic-hyperinsulinemic clamp technique. ß-$Hydroxybutyrate_{steady\ state}$ = ß-Hydroxybutyrate concentration at steady state during testing by use of the euglycemic-hyperinsulinemic clamp technique.
[a,b]Within a row, values with different superscript letters differ significantly (P < 0.05). [A,B]Within a column, values with different superscript letters differ significantly (P < 0.05). [C,D]Within a column, values with different superscript letters differ significantly (P < 0.05). [E,F]Within a column, values with different superscript letters differ significantly (P < 0.05).
NA = Not available.
See Table 1 for remainder of key.

Maximum duration of the clamp technique for experiments 1 and 2 was 310 minutes. The maximum duration of the clamp technique that provided values used for the calculations was 280 minutes, which included the steady-state period.

Mean M and the mean M-to-I ratio did not differ significantly between measurements obtained by use of the clamp technique with a short duration (steady-state condition achieved after 80, 80 and 120 minutes, respectively), compared with values for measurements obtained by use of the clamp technique with a relatively long duration (260, 280 and 190 minutes, respectively). Mean CV of the plasma glucose concentration of the measurements obtained by the use of the clamp technique with a short duration was 2.23 ± 0.40%, whereas the corresponding value for the measurements obtained by use of the clamp technique with a relatively long duration was 2.73 ± 0.91%. Mean I for measurements obtained by use of the clamp technique with a short duration was 3,817 ± 1,014 pmol/L, which did not differ significantly from

mean I for measurements obtained by use of the clamp technique with a relatively long duration (4,659 ± 590 pmol/L).

Discussion

Glucocorticoids have many effects on metabolism. Overall, however, their actions on carbohydrate metabolism are considered to be glucose-sparing effects that are typified by increased hepatic glucose production and diminished glucose use by peripheral tissues (222). For this reason, we expected hydrocortisone administration to result in a decrease in M and the M-to-I ratio, indicating increased insulin resistance and diminished glucose use in peripheral tissues. On the contrary, a single-dose of hydrocortisone administered to clinically normal Standardbred horses 24 hours before onset of testing by use of the euglycemic-hyperinsulinemic clamp technique resulted in an increase in glucose metabolism and sensitivity of peripheral tissues to exogenous insulin. This phenomenon of an apparent increase in glucose use as a result of cortisol has been reported in dogs with hyperadrenocorticism and healthy humans (239, 270). However, despite the fact that absolute glucose use was increased in both of those reports, it was proposed that there was a relative impairment of glucose clearance because the increase was less than would have been expected on the basis of the size of the concurrent increase in circulating glucose concentrations. In short, it appeared that cortisol induced a state of hepatic insulin resistance that led to increased endogenous hepatic glucose production, with the subsequent hyperglycemia triggering hyperinsulinemia (73). The combination of hyperglycemia and hyperinsulinemia could mask inhibitory effects of cortisol on glucose use by peripheral tissues because hyperglycemia stimulates increased glucose use by tissues and hyperinsulinemia helps overcome insulin resistance in peripheral tissues. In fact, the glucocorticoid-induced increase in insulin secretion in human patients compensates for the anti-insulin effects of the glucocorticoids and thereby helps reestablish euglycemic circulating glucose concentrations and homeostasis. In the study reported here, hyperglycemia or compensatory increases in basal I were not evident after administration of a single-dose of hydrocortisone.

It must be kept in mind that insulin concentrations are supraphysiologic during testing by use of the euglycemic-hyperinsulinemic clamp technique. Therefore, it is assumed that endogenous glucose production by the liver and endogenous insulin production by the pancreas are suppressed during the steady state (22, 69). This hyperinsulinemia may have overcome any insulin resistance induced by the liver and therefore may also have suppressed any increase in endogenous glucose production by the liver. Of course, the infused insulin could also have overcome some insulin

resistance in the peripheral tissues, but this could not explain the increases in glucose metabolism, compared with the results for the other treatments, because the same hyperinsulinemia was induced after each of the other treatments. Thus, it is suggested that increases in glucose metabolism in the study reported here were purely a reflection of an increase in hydrocortisone-induced glucose use in peripheral tissues.
In GH-deficient humans, treatment with GH is associated with insulin resistance, and GH-treated patients have an increase in the incidence of type 2 diabetes mellitus (59, 292). In horses, treatment with GH also is associated with hyperglycemia, hyperinsulinemia, and insulin-resistance (297). For this reason, it was expected that eGH would induce insulin resistance in peripheral tissues of horses in both experiments. In the study reported here, a single dose of eGH did not induce insulin resistance because values for M and the M-to-I ratio did not differ from those for horses when administered the control treatment. In addition, serum IGF-1 and I measured before use of the clamp technique did not differ significantly from corresponding values when the horses received the control treatment. However, after long-term eGH administration, basal I and basal serum IGF-1 concentrations were significantly increased, which indicated that long-term eGH administration had a measurable biological effect. In addition, the M-to-I ratio decreased significantly, which indicated a decrease in sensitivity of peripheral tissues to exogenous insulin. However, the absolute M was not influenced significantly by long-term eGH administration, possibly because the induced hyperinsulinemia during testing by use of the euglycemic-hyperinsulinemic clamp was sufficient to compensate for insulin insensitivity in the peripheral tissues. This hypothesis could be examined further by use of the hyperglycemic clamp technique (ie, hyperglycemia is induced by infusion of glucose solution) to allow measurement of pancreatic sensitivity to exogenous glucose and determine the rate of glucose metabolism when there is a lack of extreme hyperinsulinemia.

In experiment 2, there was a significant decrease in the M-to-I ratio when eGH was administered as the first treatment and saline solution was administered as the second treatment. Consequently, the effect of the long-term eGH treatment obviously persisted throughout 1 estrous cycle in association with I and IGF-1 concentrations within the reference range, which illustrated the sensitivity of the euglycemic-hyperinsulinemic clamp technique. As a result, it can be suggested that the effect on tissue sensitivity may persist at the receptor or postreceptor level for at least 28 days. Additional studies will be needed to test this hypothesis.

Hyperinsulinemia that lasts more than 5 to 7 hours may enhance insulin action itself which could be expressed by an increase in the rate of glucose infusion (301). However, no significant differences were found when mean values of M and the M-to-I ratio were compared during 2 steady-state conditions during the same test in 3 horses.

This corresponds with the findings of a study in humans in which investigators reported a plateau for the glucose infusion rate at a steady state was reached between 5 and 7 hours after onset of the clamp technique at approximately 30% above the glucose infusion rate reported during the same test at only 2 hours after starting the infusions (299). In the study reported here, duration of the longest test by use of the clamp technique was 280 minutes, which included the duration of the steady state.

Uptake and degradation of insulin is a feature of all insulin-sensitive tissues. After the liver and kidneys, muscle plays the major role in insulin removal. Removal of insulin from the circulation does not imply immediate destruction of the hormone. A substantial amount of receptor-bound insulin is released from cells and reenters the circulation. Insulin clearance rates are decreased in patients that are obese or diabetic or have increases in other hormones such as catecholamines and GH (80). The I measured during steady-state conditions revealed an assessment of insulin clearance in horses, which may serve as an interesting basis for future research.

Cortisol and GH both influence lipid metabolism by stimulating lipolysis and both trigger an increase in plasma concentrations of free fatty acids (99, 101). Typically, insulin inhibits this hormone-induced lipolysis by stimulating an increase in activity of lipoprotein lipase and decreasing activity of hormone sensitive lipase; this combination eventually clears the plasma of triglycerides. Withholding of food also enhances the rapid release of free fatty acids from the adipose tissue into the circulation via the activation of hormone-sensitive lipase. Therefore, it was expected that the administration of eGH or hydrocortisone followed by a 12-hour period of food withholding would result in a higher mean plasma NEFA concentration before testing by use of the clamp technique, compared with the concentration recorded when horses received the control treatment. However, in both experiments, mean plasma NEFA concentrations did not differ significantly among hydrocortisone, eGH or saline solution treatments. In experiment 2, this could be explained by inhibition of lipolysis as a result of secondary hyperinsulinemia induced by long-term administration of eGH.

In experiment 1, a single dose of eGH did not induce an increase in NEFA or IGF-1 concentrations or substantially increase hyperinsulinemia, which suggested that a single dose of eGH is not sufficient to induce a measurable biological effect. The failure of administration of a single dose hydrocortisone to induce an increase in NEFA concentrations indicative of lipolysis has been described elsewhere (101).
Finally, the decrease in mean plasma NEFA and mean serum ß-hydroxybuyrate concentrations following the onset of infusions during the clamp technique in both experiments was presumably a result of the antilipolytic action of the infused insulin, as described elsewhere (87).

Conclusion

In the study reported here, we determined that a single dose of hydrocortisone in Standardbreds increased glucose metabolism and increased insulin sensitivity of peripheral tissues to exogenous insulin, whereas a single-dose of eGH did not affect either of those variables. In contrast, long-term eGH administration increased basal I and basal IGF-1 concentrations and decreased tissue sensitivity to exogenous insulin without increasing plasma concentrations of NEFA and ß-hydroxybutyrate. Furthermore, use of the euglycemic-hyperinsulinemic clamp technique appeared to suppress lipolysis and ketogenesis, which was reflected in decreases in plasma concentrations of NEFA and ß-hydroxybutyrate. These findings could help investigators identify underlying mechanisms for disorders associated with insulin resistance and aid in the development of appropriate treatments for animals with these disorders (eg, horses with laminitis).

Chapter 7

The effect of long-term exercise on glucose metabolism and peripheral insulin sensitivity in Standardbred horses.

Ellen de Graaf-Roelfsema[1]
Mireille E. van Ginneken[1]
Eric van Breda[2]
Inge D. Wijnberg[1]
Hans A. Keizer[2]
Johannes H. van der Kolk[1]

[1]Department of Equine Sciences, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands. [2]Department of Movement Sciences, Faculty of Health Sciences, University of Maastricht, The Netherlands.

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Abstract

Reasons for performing the study: To study the possible long-term effect of improved glucose tolerance in horses after long-term training, as the impact of exercise training on glucose metabolism is still unclear in the equine species. It is not known whether there is a direct long-term effect of training or if the measurable effect on glucose metabolism is the residual effect of the last exercise session.
*Objective*s: To determine the chronic effect on glucose metabolism and peripheral insulin sensitivity of long-term training in horses by use of the euglycaemic hyperinsulinaemic clamp technique.
Methods: Eleven Standardbred horses were acclimatised to running on the high-speed treadmill for 4 weeks (phase 1) followed by training for 18 weeks with an alternating endurance (~ 60% HR_{max}) high intensity training programme (~ 80% HR_{max})(phase 2). Training frequency was 4 days/week. At the end of phase 1, a euglycaemic hyperinsulinaemic clamp was performed 72 hours after the last bout of exercise in all horses. At the end of phase 2, the horses were clamped 24 h or 72 h after the last bout of exercise.
Results: Glucose metabolism rate did not change significantly after 18 weeks of training, measured 72 hours after the last exercise bout (0.018 ± 0.009 and 0.022 ± 0.006 mmol/kg bwt/min, respectively). Peripheral insulin sensitivity also did not change significantly following training ($7.6 \pm 5.7 \times 10^{-6}$ and $8.0 \pm 3.1 \times 10^{-6}$, respectively). The same measurements 24 hours after the last bout of exercise showed no significant differences.
Conclusions: Results indicated that long-term training in Standardbreds neither changed glucose metabolism nor insulin sensitivity 72 h after the last bout of exercise.
Potential relevance: The fact that the beneficial effect of increased insulin sensitivity after acute exercise diminishes quickly in horses and no long-term effects on insulin sensitivity after chronic exercise have as yet been found in horses, implies that exercise should be performed on a regular basis in horses to retain the beneficial effect of improved insulin sensitivity.

Introduction

Physical training has been shown to improve glucose tolerance and insulin action in man (17,133, 217). There seems, however, to be inconsistency in literature concerning the persistence of the effect of increased glucose tolerance after cessation of acute exercise and it remains unknown, whether or not regular exercise results in long-term adaptive increases in insulin sensitivity after a period of training (365). It is now generally accepted that an acute bout of exercise leads to an improved insulin sensitivity and/or glucose tolerance. These effects have been recognised after different kinds of exercise (short/long-term, low-to-high intensity exercise, resistance or endurance exercise and even single bouts of exercise) in both man and rats and have been reported to persist for a period of time ranging from 2-48 h post exercise (38, 152, 178, 218, 224).

To overcome the possible residual effects of an acute bout of exercise the long-term effects of regular exercise on the above mentioned parameters require at least a period of rest (preferably > 48 hours) after the last bout of exercise. The long-term effects of exercise have been studied in man and rats and the outcome of the different experiments is diverse. Some studies showed an increase in insulin sensitivity as a long-term effect of training (153, 246). However, most of the studies showed no effect of training 3-6 days after the last bout of exercise on glucose tolerance or insulin sensitivity (196, 284). To our knowledge, no data on the long-term effect of exercise on insulin sensitivity is available for horses.

The aim of the current study was to monitor the persistence of the effect of improved glucose tolerance in Standardbreds after long-term moderate-intensity exercise. Therefore, young, nonobese healthy Standardbreds were tested by use of the euglycaemic hyperinsulinaemic clamp technique 24 or 72 h after the last training session to ascertain if there had been any influence from the last bout of exercise.

Material and methods

Animals

13 untrained Standardbred geldings entered the study, spread over a 2 year period for practical reasons after a 2 month quarantine period at the research centre, during which they walked 45 minutes on the walking machine per day. Eleven horses fully participated in the study (5 horses in 2004 and 6 horses in 2005). The horses served as their own controls during the study. Two horses had to leave the study after the acclimatisation phase because of practical reasons (Phase 1) and stayed at the research centre. Therefore we were able to clamp them again after 18 weeks of only 1

h daily walking and use them as additional controls for the trained horses. All horses were 1.5 years of age and weighed (mean ± s.d.) at the first clamp 384 ± 42 (range; 331-485) kg and 405 ± 37 (range; 360-502) kg at the second clamp. All horses had a body condition score 4 or 5 during the experiments (136). The horses were individually housed, and were fed according to the daily estimated energy requirements of 58 MJ NE (range 54-66) consisted of haylage and concentrate feed. Salt blocks and water were available *ad libitum*. The experiments were approved by the Committee for Animal Welfare at the Faculty of Veterinary Medicine, Utrecht University.

Experimental procedures

Prior to the start of the experiment, the horses were acquainted with the Equine Exercise Laboratory and acclimatised to running on the high-speed treadmill (Mustang 2000, Kagra, Graber AG, Fahrwangen, Switserland) for 4 weeks (Phase 1) followed by the training period, that lasted 18 weeks (Phase 2) (see below). At the end of Phase 1, the horses underwent an exercise test followed by 2 days of relative rest (60 min walking in the walking machine). On day 3, the euglycaemic hyperinsulinaemic clamp was performed in each horse. Horses were than trained for 18 weeks. On the last day of training they underwent a standardized exercise test, followed by a euglycaemic hyperinsulinaemic clamp 24 h (first year) or 72 h (second year) later to assess the long-term adaptive effects of regular exercise. The standardized exercise test was performed for other purposes and therefore not described in this context.

The two additional control horses performed only Phase 1 at the same time as group 2, during Phase 2 these horses walked on the walking machine for 60 min daily for 18 weeks instead of training on the treadmill.

Experimental protocol

The 6 horses of 2004 (group 1) trained longer in Phase 1 which appeared to be too much for the young horses, as they showed fatigue in the beginning of Phase 2, which resulted in a different scheme in 2005 (number in between brackets). The 2 additional control horses followed the scheme of group 2. Each training session was preceded by 30 min warm-up on the walking machine followed by 8 min warm-up on the treadmill, consisting of 4 min at 1.6 m/sec and 4 min at 3.0-4.0 m/sec, no incline. The training programme during Phase 1 consisted of endurance training: week 1, 30%HF_{max} for (20)-30 min 3/week; week 2, 30% HF_{max} for (25)-45 min 4/week; week 3, 40%HF_{max} for (30)-45 min 4/week; and week 4, 50% HF_{max} for (35)-45 min 4/week. Each training session ended with a cooling down consisting of a 5 min walk at the treadmill followed by 30 min walk on the walking machine.

Training period

The training programme (Phase 2) consisted of two types of exercise, endurance and interval running. The days of interval running were alternated with days of endurance running. Each training session was preceded by 30 min warm-up on the walking machine followed by 8 min warm-up on the treadmill, which consisted of 4 min at 1.6 m/s and 4 min at 4.5 m/s, no incline. The exercise intensity was based on fixed percentages of the peak heart rate, as estimated based on the values of the horses of group 1. The endurance running included 20-24 min of continuous level running at 60%HF_{max} or 16-18 min at 75% HF_{max}. The interval training included three 3 min bouts at 80-90%HF_{max} or four 2 min bouts at 80-90% HF_{max} and interspersed with 3 or 2 min periods at 60% HF_{max}. Each training session ended with a cooling down consisted of a 5 min walk on the treadmill followed by 30 min walk on the walking machine. The horses exercised 4 days/wk throughout the entire training period (Phase 2). On the resting days the horses walked for 60 minutes at the walking machine throughout Phases 1 and 2.

Exercise intensity was based on fixed percentages of the peak heart rate. The peak heart rate was the maximal heart rate measured in the 6 individual horses of group 1 under investigation during an incremental exercise test until exhaustion. During the incremental exercise test, after a warm-up, the horses ran at an initial speed of 5 m/s for 2 minutes. Treadmill speed was than increased to 6 m/s for 2 minutes, followed by 1 m/s increases every 2 min until they reached fatigue. Fatigue was defined as the point where the horse could not keep up with the treadmill despite humane encouragement. Heart rate was measured continuously using an ECG (Cardio Perfect Stress 4.0, Cardio Perfect Inc, Atlanta, GA, USA) and an external heart rate meter (Polar S610, Polar Electro Oy, Kempele, Finland).

To enable exercise intensity adjusted to the individual's ability during the whole training period, running speed and accompanying heart rate were measured during each training session. The training intensity was adjusted according to the measurements on a weekly basis. Heart rates (HR) were measured with a Polar Heart Rate monitor during the training sessions. Group 2 horses had an estimated maximal Heart Frequency of 240 beats/min because the incremental exercise test was difficult to perform in the young, relatively untrained horses. They had difficulty with coordination on the treadmill during the high speeds necessary to obtain maximal heart frequencies, which caused injuries in the group of year 1.

Euglycaemic hyperinsulinaemic clamp technique

In vivo insulin sensitivity was examined by use of the euglycaemic-hyperinsulinaemic clamp technique previously described (64, 268) Food was withheld from the horses 12 h prior to the end of the clamp. To perform the clamp technique, a catheter was

inserted into each jugular vein. One catheter was subsequently used for infusion of glucose (50% solution) and insulin (Actrapid recombinant human insulin (100 U/mL), Novo Nordisk A/S, Bagsvaerd, Denmark), the other used for collection of blood samples to monitor circulating glucose and insulin concentrations. The first blood sample (time 0) was used to establish the basal glucose concentration. Within 10 min after collection of the first blood sample, a priming dose of insulin (45 mU/kg bwt dissolved in 50 mL of saline solution) was administered i.v. as a bolus injection. It was followed immediately by constant rate infusions of insulin (6 mu/[kg bwt X min]) and glucose (mean infusion rate, 8.6 □mol/[kg BW X min]) (268). Constant rate infusions were accomplished by use of a volumetric pump (Argus 414, Argus Medical AG, Heimberg, Switzerland).

Throughout the period of insulin and glucose infusion, blood samples were collected at 10 min intervals into heparinized syringes. Within 2 min after collection, glucose concentration in each sample was measured by use of an automated analyzer (ABL-605, Radiometer Copenhagen, Westlake, Ohio). Glucose was maintained at the basal concentration measured in each horse (range, 3.9 to 5.6 mmol/L). When the blood glucose concentration differed from the basal concentration, the glucose infusion rate was adjusted in order to compensate. A steady-state condition was presumed to exist when the plasma glucose concentration and glucose infusion rates were concurrently held constant for at least 30 min and the plasma glucose concentration was within the range for euglycemic values. In addition, the first hour after onset of the clamp technique is considered an equilibration period; therefore, only steady states achieved at least 60 min after starting the insulin infusion were considered useful for comparison.

Insulin concentrations (I) were determined before the clamp technique was started (time 0) and in 3 samples collected at 10 min intervals during the euglycaemic-hyperinsulinaemic steady-state condition into lithium-heparinized tubes. The blood samples were centrifuged (Rotina 48R, Hettich zentrifugen, Tuttlingen, Germany) for 10 min at 6000 *g*; plasma was harvested and stored at –20^{o}C until insulin concentrations were analysed using a radioimmunoassay (Cout-A-Count TKIN2 836, Diagnostic Products Corp, Los Angeles, Calif) validated for horses (328)

Calculations

In order to reach a steady state plasma glucose concentration, the amount of glucose infused must equal the amount of glucose being removed from the glucose space (i.e., amount of metabolized glucose [M]), provided that endogenous glucose production is suppressed completely. However, in reality, the plasma glucose concentration is never absolutely constant, (69) and a space correction factor must be included to account for glucose that is added or removed from the glucose space by means other than

metabolism. Thus, M during steady- state conditions during use of the clamp technique can be expressed by the following equation (69):

$$M = INF – SC,$$

where INF is the amount of glucose infused, and SC is the space correction factor. The space correction was calculated by use of the following equation adopted from man (69):

$$SC = ([G2 – G1] \bullet GS)/([t2 – t1] \bullet bwt),$$

where G1 and G2 are the glucose concentrations at time points t1 and t2, respectively; GS is the glucose space, which is calculated as 0.19 l/kg bwt; In this study, values of M were calculated for 10 min intervals.

Plasma insulin concentrations were determined for the 3 samples recovered during the steady-state condition. Those concentrations were used to calculate the mean M-to-I ratio, which reflects the quantity of glucose metabolized per unit of plasma insulin and is a good index of tissue sensitivity to exogenous insulin (69).

Assays

Plasma insulin concentration was measured using a commercial radioimmunoassay kit validated for horses (328). Plasma glucose concentrations were analyzed by use of an automated analyzer.

Statistical analysis

All values were recorded as mean ± s.d. A multivariate test for repeated measures, the Huynh-Feldt test (SPSS, version 10.0 for Windows, SPSS Inc, Chicago, Ill) was used for analysis of the data between the first and the second group in time as well as differences between both clamps. The 2 additional control horses were not used for statistical calculations due to the low number of horses. Normality of the data was analyzed, using normal P-P Plot by means of Blom method on data transformed into natural logarithms and the Kolomogorov-Smirnov test. P values <0.05 were considered significant.

Results

Mean ± s.d. values for M, I, and M-to- I ratio were calculated for the trained horses of the first and the second group as well as the two additional control horses (Table 1) and shown in Figures 1 and 2. Long-term training did not lead to significant increases in M, I or M to I ratio as shown by the values of the second group in which clamps 1 and 2

were performed 72 h after the last bout of exercise. There was also no significant influence found of acute exercise on peripheral insulin sensitivity when the clamp was performed at 24 h after the last bout of exercise, as shown by the data of the first group. The Huynh-Feldt revealed no significant time-group interaction for both clamps. The two additional control horses performed the same level of exercise during Phase 1 as the second group. However, they performed no treadmill training at Phase 2. No clear difference in glucose metabolism or insulin sensitivity was found between the two clamps.

The first hour after onset of the clamp technique is considered an equilibration period; therefore, only steady states achieved at least 60 minutes after starting the insulin infusion were considered useful for comparison. All clamp tests fulfilled this criterion, with the steady-state condition reached after a mean of 107 ± 39 minutes for clamp 1 and 123 ± 47 for clamp 2. Mean ± s.d value for the space correction factor for both clamps together was -0.02 ± 0.16 l.

Table 1. Mean ± s.d. values for metabolic variables for horses (groups 1 and 2) before and after 18 weeks of intensified training and for 2 additional control horses, which did not perform the intensified training, tested by use of a euglycemic-hyperinsulinemic clamp technique.

Variables	***Group 1***	***Group 2***	***Additional controls***
M (clamp 1)	0.024 ± 0.006	0.018 ± 0.009	0.016 ± 0.003
M (clamp 2)	0.025 ± 0.008	0.022 ± 0.006	0.015 ± 0.005
$I_{steady\ state}$ (clamp 1)	2178 ± 762	2691 ± 588	2905 ± 181
$I_{steady\ state}$ (clamp 2)	3184 ± 697	2857 ± 443	2870 ± 425
M-to-I ratio (clamp1)	13.3 ± 7.1	7.6 ± 5.7	5.6 ± 1.2
M-to-I ratio (clamp 2)	8.6 ± 3.8	8.0 ± 3.1	5.2 ± 1.0

M = Amount of metabolized glucose in (mmol/[kg X min]). $I_{steady\ state}$ = Plasma insulin concentration (I) at steady state during testing by use of the euglycemic-hyperinsulinemic clamp technique in (pmol/L). M-to-I ratio ($x10^{-6}$). The first clamp was performed 72 hours after the last bout of exercise in all horses. The second clamp was performed 24 hours after the last bout of exercise in the first group and 72 hours after the last bout of exercise in the second group and the additional control group.

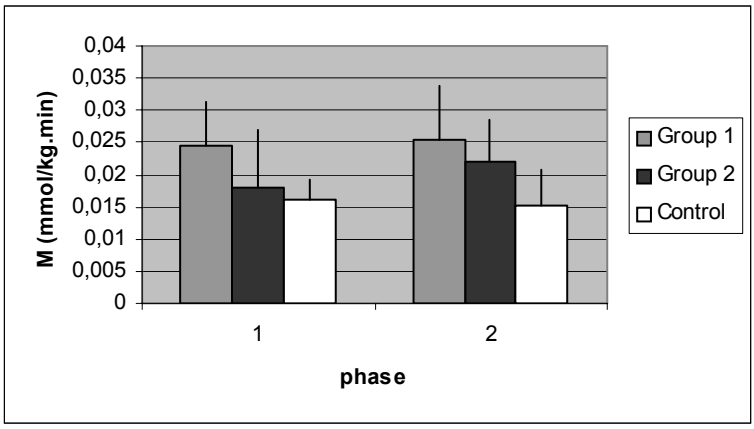

Figure 1. Mean ± s.d. values for the amount of metabolized glucose (M) measured during the steady state of the euglycaemic hyperinsulinaemic clamp before and after 18 weeks of intensified training for groups 1 and 2 and for 2 additional control horses, who did not perform the intensified training. The first clamp was performed 72 hours after the last bout of exercise in all horses. The second clamp was performed 24 hours after the last bout of exercise (Group 1) or 72 hours after the last bout of exercise (Group 2 and the additional control group).

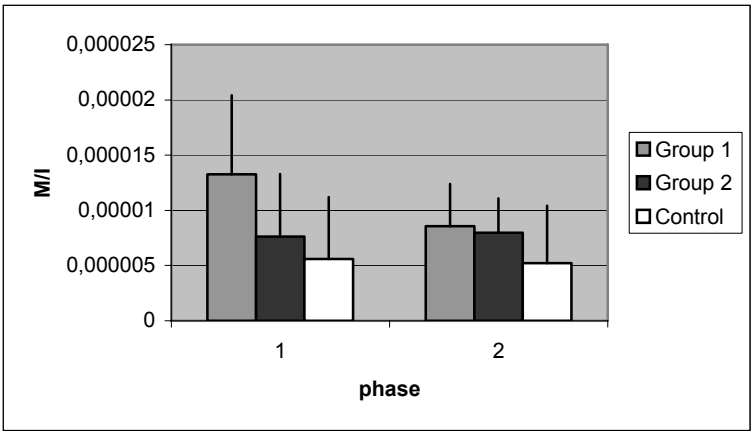

Figure 2. Mean ± s.d. values for the amount of metabolized glucose per insulin unit (M/I) measured during the steady state of the euglycaemic hyperinsulinaemic clamp before and after 18 weeks of intensified training in the first and the second group and for 2 additional control horses, who did not perform the intensified training. The first clamp was performed 72 hours after the last bout of exercise in all horses. The second clamp was performed 24 hours after the last bout of exercise (Group 1) or 72 hours after the last bout of exercise (Group 2 and the additional control group).

Discussion

The main result of the current study is that regular moderate exercise in young, non-obese healthy horses does not affect glucose metabolism or insulin sensitivity measured 72 h after the last bout of exercise. This result might indicate that in horses no direct long-term adaptive increases in insulin sensitivity or glucose metabolism following long-term training exist. The same result was found after short-term light exercise in horses by Powell et al (253). In that study, insulin sensitivity was determined by a euglycaemic hyperinsulinaemic clamp in lean and obese mares 24 h and 9 days after the last training, which consisted of 7 days of light exercise. They reported an increase in insulin sensitivity 24 h post exercise, as reflected by a significant increase in glucose infusion rate (86%) during the clamp, and a decrease in insulin sensitivity 9 days post exercise as shown by a glucose infusion rate which returned to baseline values (253).

In man, the long-term effects of exercise on insulin sensitivity have been previously studied. Although, the outcome of the different experiments is diverse, most studies show no effect of training 3-6 days after the last bout of exercise on glucose metabolism or insulin sensitivity (196, 285). For instance, a euglycaemic hyperinsulinaemic clamp performed 4-5 days after the last exercise section in lean men, obese men and diet-controlled *type 2* diabetic men trained for 12 weeks on a cycle ergometer, showed no significant alterations in insulin sensitivity by training in any group (285).

An explanation given for the diverse results in men is that the long-term impact of exercise training on glucose metabolism is an indirect effect that depends on concomitant reduction in body fat (277). It is shown that diminishing body fat induces a long-term improvement in insulin resistance in man (276). This mechanism is also described in ponies (98). Freestone et al (98) showed a decreased insulin response after an oral glucose tolerance test performed 2 days after the last bout of exercise in obese, insulin resistant ponies after conditioning. The effect remained present for 6 weeks after ending the conditioning period. In the same study, a different obese, insulin resistant group of ponies was trained for 6 weeks combined with conditioning. Exercise combined with conditioning resulted in a significant increase in insulin sensitivity in this experiment only in the first two weeks compared to conditioning only. After two weeks, both groups showed a similar improvement in insulin sensitivity. Therefore it was concluded that the improved insulin sensitivity was mainly due to the weight loss rather than training (98). In our study no weight loss was observed in the horses, which could explain the finding that insulin sensitivity remained the same. In addition, during the study of Powell et al (253) there was no weight loss or body fat reduction in the lean and obese horses.

Another explanation given in literature for the diverse results in man is that the residual effects of the last exercise session might have been influencing the results. The acute effect of exercise is an improvement in insulin sensitivity and an increase in glucose metabolism lasting between several hours and a few days after an exercise session. This acute effect of exercise has been reported to persist for a period ranging from 2-48 h post exercise (152, 178, 218). However, the time-course over which insulin sensitivity decreases after an acute bout of exercise is not fully known in man. It is speculated that this might be longer than 48 hours (246). Poehlman et al (246) reported in nonobese, young women an increase in insulin sensitivity 4 days after the last bout of exercise after 28 weeks of training without any change in total fat. In our study, no significant increase in insulin sensitivity was found in the horses of the second group 24 hours after the last bout of exercise.

In horses, the response with regards to increasing insulin sensitivity and glucose metabolism after acute exercise has been documented as well. This acute effect of exercise in horses is reported to last at least 24 h after the last session (192, 253), which is in contrast with the result of the current study. However, the time-course over which insulin sensitivity decreases after an acute bout of exercise in horses is not known. One study describes a time-course of less than 9 days (253). The current study adds a time-course of less than 24 h assuming that an acute effect of excercise, as defined by an increase in sulin sensitivity, has occurred. However, it is reasonable to expect an increase in insulin sensitivity acutely following exercise after the moderate intensity training sessions in our study, because the same effect has already shown by other authors in man, rat and horses (152, 178, 253), which could lend credibility to our finding.

In the current study, the horses were adapted to the treadmill during Phase 1. The light endurance training performed by the horses during Phase 1 might have lead to changes in insulin sensitivity in itself. A previous clamp study performed in our laboratory in Standardbreds (mean age 5.4 years, range 3-13) housed in stables all day except for 1 h walking on the walking machine indicates that Phase 1 endurance training might have influenced insulin sensitivity (64). In this study a lower M-value as well as M/I-ratio were found (0.010 ± 0.0026 mmol/kg/min and 2.5 ± 0.8 x 10^{-6}) compared to the values found in the current study. However, one has to take into account that these Standardbreds were older which might have influenced the results as well. Interestingly, the two additional control horses showed no clear change in glucose metabolism and insulin sensitivity, although their mean values were lowest compared to the other horses.

Conclusion

In conclusion, the fact that the beneficial effects of an acute bout of exercise on insulin sensitivity diminishes rapidly in horses and that we found no evidence for direct long-term effects on insulin sensitivity of chronic moderate exercise in horses, implies that exercise should be performed on a regular basis. It cannot be excluded that the adaptation to the treadmill itself has led to changes in insulin sensitivity, but no measurements were made accordingly.

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Chapter 8

The effect of intensified training and subsequent detraining on glucose metabolism and peripheral insulin sensitivity in Standardbred horses

Ellen de Graaf-Roelfsema[1]
Hans A. Keizer[2]
Eric van Breda[3]
Inge D. Wijnberg[1]
Johannes H. van der Kolk[1]

[1]Department of Equine Sciences, Medicine Section, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands. [2]Department of Human Physiology and Sports Medicine, Free University of Brussels, Brussel, Belgium. [3]Department of Movement Sciences, Faculty of Health Sciences, University of Maastricht, Maastricht, The Netherlands.

Abstract

The aim of the current study was to assess glucose metabolism and peripheral insulin sensitivity in young Standardbred geldings selected for a longitudinal study of experimentally induced overtraining according to a previously described systematic standardised protocol characterized by insufficient rest days in order to detect potential markers that may indicate overtraining. Overtraining (as reflected by a 19% decrease in performance) in Standardbred horses was associated with the dissociation between the rate of glucose metabolism and the mean glucose metabolism rate-to-plasma insulin concentration ratio during steady-state in the euglycaemic hyperinsulinaemic clamp test. This dissociation persisted after four weeks of detraining. The period of detraining turned out to be essential to reveal the increased rate of glucose metabolism (from 31.1 ± 6.8 to 18.1 ± 3.4 μmol/kg BW/min) as well as the increased mean glucose metabolism rate-to-plasma insulin concentration ratio (from 1.05 ± 0.31 to 0.62 ± 0.17) during steady-state in overtrained Standardbreds at the end of the intensified training period. In conclusion, the used equine model for overtraining in Standardbreds turned out to be valid.

Introduction

In modern sport practice, the principle of overload training is used in order to improve performance level of athletes. When the balance between training stress and recovery is disproportionate, it is thought that overreaching and possibly overtraining may develop. Overreaching occurs as a result of intensified training and is often considered a normal outcome for elite athletes due to the relatively short time needed for recovery (approximately 2 weeks) and the possibility of a supercompensatory effect in contrast with the much longer time needed to recover from the overtraining syndrome (months to years). The symptoms associated with overtraining, such as changes in emotional behaviour, sleep disturbances, and hormonal dysregulations, are indicative of changes in the regulatory and coordinative function of the hypothalamus (125, 174). The available scientific and anecdotal evidence supports the existence of the overtraining syndrome. However, more research is required to state with certainty that the syndrome exists (125).

Since its domestication, the horse plays an important role in human society. One of its specific qualities is endurance capacity, which can be defined as the ability to maintain relatively high speeds for a prolonged time. This ability makes the horse excellently fit for races over shorter and longer distances. Endurance capacity is mainly determined by the maximal amount of oxygen that can be taken up and delivered to the tissues. The cardiorespiratory system of the horse has a high capacity compared with other species including humans. The amount of blood that is pumped through the body determines the amount of oxygen that can be delivered to the tissues. Compared to the resting state the horse is able to enhance cardiac output during maximal exercise eight- to tenfold, and the arteriovenous oxygen difference five-fold (34) and as a consequence being an excellent athletic model.

It has been shown that overtraining can be induced in Standardbreds (33, 114, 115, 127, 206, 317, 318). The earliest sign of overtraining is the inability to complete the intensive training associated with increased irritability and reluctance to exercise (33, 34).

The aim of the current study was to assess glucose metabolism and peripheral insulin sensitivity in young Standardbred geldings selected for a longitudinal study of experimentally induced overtraining according to a previously described systematic standardised protocol characterized by insufficient rest days (33) in order to detect potential markers that may indicate overtraining in horses.

Materials and Methods

Animals

Ten healthy Standardbred geldings (aging 20±2 months (mean ± SD) and weighing 368±45 kg) were included in the study after a two-month quarantine period at the Equine Exercise Laboratory during which they walked daily for 45 minutes in a walking machine. The horses had not been involved in any kind of organized exercise or training regimen previously. They were housed individually in boxes and their diet consisted of grass silage supplemented with concentrate feed and met nutrient requirements for maintenance and performance with estimated daily energy requirements of 58 MJ NE (range 54-66). Salt blocks and water were available *ad libitum*.

For organizational purposes, the study was performed in two different groups of six (first year) and four (second year) horses in two successive years. At the start of each year, the available horses were divided in pairs based on age. Of every pair, one horse was randomly assigned to the intensified training program in period B.

Experimental set up

Prior to the start of the experiment, the horses were acquainted with the Equine Exercise Laboratory and were acclimatized to running on the high-speed treadmill (Mustang 2000, Kagra, Graber HG, Switzerland). The training period consisted of a total of 32 weeks divided into four periods, namely an acclimatization period of 4 weeks (period 1), a training period of 18 weeks with alternating both endurance and high intensity training (period 2), followed by a period of intensified training of six weeks (period 3) and finally detraining period for 4 weeks (period 4). Training of control horses always consisted of exercise 4 days/week, whereas the intensified trained horses were exercised with increasing volume and intensity 6 days/week during the first three weeks of the intensified training period, and 7 days/week during the last three weeks of the same period. At the end of each period the horses performed a standardized exercise test (SET) in the morning between 07.30 and 12.30 h in order to monitor performance improvement.

The experiments were approved by the Committee for Animal Welfare of Utrecht University.

Monitoring training

In order to standardize training to the individual exercise capacity of the horses, training intensity was adapted to the estimated maximal heart frequency. For this purpose, an incremental exercise test was performed at the beginning of period 1 in the first year. After thirty minutes walking in a horse-walker, horses performed an incremental

exercise test on the high-speed treadmill. The incremental exercise test started with a warming-up and thereafter horses trotted for 2 minutes at 5 m/s, followed by 2-mins at 6 m/s. Intensity was increased by 1 m/s every 2 minutes until the horses reached fatigue, which was defined as the speed where the horse could not keep up with the treadmill despite humane encouragement. Heart frequency was monitored with an external heart rate meter (Polar S610, Polar electro Oy, Kempele, Finland). Based on the maximal heart frequency of horses that did reach a plateau in maximal heart frequency during this test and combined with measurements in a previous study with 2-year old Standarbred stallions (34) maximal heart frequency was estimated at 240 bpm. The incremental exercise test was difficult to perform at the end of period 1 because of coordination problems in the young, relatively untrained horses. Therefore, and also to minimize the risk of injuries during the incremental exercise test, the test was not performed in the second year group. The estimated maximal heart frequency was used to guide training intensity (speed and inclination) on the treadmill, and was adjusted on a weekly basis to the measured peak heart frequencies during training.

Training protocol

After an acclimatization period of two months, basic endurance training was started to adapt the horses further to the high-speed treadmill for four weeks (period 1).

Immediately thereafter, the horses underwent an 18-week training program of mixed endurance training (ET) and high intensity training (HIT) (period 2). Days of ET were alternated with HIT. Each training session was preceded by 30 minutes of warm-up at the walking machine followed by 8 minutes of warm-up (4 minutes at 1.6 m/s and 4 minutes at 4.5 m/s) at the high-speed treadmill. The endurance running included 20-24 min of continuous level running at 60% HF_{estmax} or 16-18 min at 75% HF_{estmax}. The interval training included three 3-min bouts at 80-85% HF_{estmax} or four 2-min bouts at 80-85% HF_{estmax} interspersed with 3-min or 2-min periods at 60% HF_{estmax}. Each training session ended with a cooling-down consisting of 5 min walk at the high-speed treadmill followed by 30 minutes walk at the walking machine. The horses exercised 4 days/wk throughout period 2.

In period 3, the horses were divided into a control group (C) and an intensified trained group (IT). The control group continued training at the volume and intensity they received at the end of the first period for another six weeks. For the IT, the training regimen consisted of alternating days of HIT and ET for 6 days/week during the first 3 weeks followed by 7 days/week for the last 3 weeks of period 3. Each training session was preceded by 30 minutes warming-up in the walking machine followed by 8 minutes warming-up (4 minutes at 1.6 m/s and 4 minutes at 4.5 m/s) at the high-speed treadmill. Exercise intensity and volume during the ET was gradually increased to 24-35 minutes at 60-75% HF_{estmax}. High intensity exercise gradually increased to five 3-min bouts at

80-85% HF_{estmax} interspersed with 2-min periods at 60% HF_{estmax} or six 2-min bouts at 80-85% HF_{estmax} interspersed with 1-min or 2-min periods at 60% HF_{estmax}.

In period 4, the horses received a 4-week training program of light endurance exercise for detraining. The horses performed endurance training for 20 minutes at 60% HF_{estmax} for 3 days and 70% HF_{estmax} for one day a week.

On the resting days the horses walked in the walking machine for 60 minutes throughout all periods.

Standardized Exercise Test

Before the SET, horses walked for 30 minutes in the horse walker followed by a warming-up at the high-speed treadmill that consisted of 4 minutes walking (at 1.6 m/s) and four minutes of slow trotting (at 4.5 m/s) followed by one minute walking (at 1.6 m/s). This was immediately followed by the time trial procedure where the horses were aimed to trot for 20 minutes at a speed and inclination that elicited a heart frequency of approximately 80% of the HF_{estmax}. The cooling-down consisted of walking on the high-speed treadmill for 5 minutes (at 1.6 m/s) followed by 30 minutes of walking in the horse walker. Heart frequency was monitored constantly with a Polar S610 and on-line ECG measurement (CardioPerfect, Cardio Perfect Inc, Atlanta, GA, USA). Speed and inclination of the treadmill could be adjusted to achieve the desired heart frequency. In periods 2 and 3, the speed and inclination were not further increased, thereby making comparisons between various tests possible. Venous blood was drawn from the jugular vein before the test (t=0 minutes), after the warming-up (t=9 min), every 5 minutes during the SET (t=14, 19, 24, 29 min), and directly after the cooling-down (t=34 min). Samples were kept on ice until heparinized whole blood lactate had been analyzed (ABL-605 Radiometer Copenhagen, Westlake, Ohio, USA).

Glucose clamp studies

Techniques used in the study reported here were based on the studies of Defronzo et al (69) and Elmahdi (87), although some adjustments were made as reported previously (268). The euglycaemic hyperinsulinaemic clamp test was performed in the morning of the third day after each SET by placing one catheter in each jugular vein after food was withheld for 12 hours. One of the catheters was used for infusion of glucose as a 50% solution and insulin (Actrapid recombinant human insulin (100 U/mL), Novo Nordisk A/S, Bagsvaerd, Denmark), whereas the other catheter was used for obtaining blood samples. A priming dose of 45 mU of insulin/kg BW, dissolved in 50 mL sodium chloride as a 0.9% solution, was given IV within 10 minutes to induce hyperinsulinaemia. Immediately after administration of the priming dose of insulin, insulin infusion was started with a constant infusion rate of 6 mU /kg BW per minute. Glucose infusion was started simultaneously with an infusion rate of 8.6 µmol/kg BW

per minute. During the insulin and glucose infusions, heparinised blood samples were taken every 10 minutes. The blood glucose concentration was assayed within 2 minutes from these 10-minute samples by use of an automated analyzer (ABL-605 radiometer Copenhagen, Westlake, Ohio, USA). Glucose infusion rate was adjusted when the preceding blood glucose value differed from the euglycaemic concentration (range 3.9 to 5.6 mmol/L). The glucose and insulin infusions were stopped after maintaining a *steady-state* of the blood glucose concentration during 30 minutes. Plasma insulin concentration was determined in 3 samples (lithium heparin tube) taken during the *steady-state* of the blood glucose concentration at 10-minute intervals. Plasma was separated and stored at –20 ^{o}C until insulin concentrations were measured by means of a radioimmunoassay kit (Diagnostic Products Corporation, Los Angeles, CA, USA) validated for use in horses (328).

Calculations

During *steady-state* of the blood glucose concentration, the glucose infusion rate must equal the glucose metabolism rate, provided that endogenous glucose production is completely suppressed by hyperinsulinaemia. The glucose metabolism rate was computed as:

$$M = INF – SC,$$

where M is glucose metabolism rate, INF is glucose infusion rate, and SC is the so-called space correction factor, all in mmol/kg per minute (69). During clamp tests, the plasma glucose concentration is not maintained constant perfectly and a correction must be made. The space correction factor adjusts for glucose that has been added or removed from the glucose space i.e. extracellular volume. The space correction is calculated as:

$$SC = ([G2-G1] \bullet GS) / ([t2-t1] \bullet BW),$$

where G1 and G2 are the glucose concentrations at time points t1 and t2, respectively; GS is the glucose space, which is calculated as 0.19 L/kg • BW of the horse in kg. In this study, values of M were calculated for 10-minute intervals.

The plasma insulin concentration (pmol/L) was determined during the *steady-state* of the blood glucose concentration.

Statistical analysis

Linear regression was performed by use of computer software (SPSS version 12.0, Chicago, Il, USA) . Scatter diagrams were plotted and the strength of the linear association was assessed by obtaining the correlation coefficient (*r*), and testing whether it was different from zero by use of the Pearson product moment correlation

test (2-tailed). Differences in M values and M:I ratios were analyzed by means of a linear mixed-effects model with one step autoregressive process. Fixed factors used were horse, couple, group and period. Interaction for group and period was calculated. The significance of differences in trotting time during SET 3 between both groups was assessed by the independent t-test. All data are expressed as mean ± SD. Values of $P < 0.05$ were considered significant.

Results

The SET's were performed throughout the experiment in order to monitor performance. A right shift of the lactate curve during SET 3 compared to SET 2 was observed for IT group with a significant difference in blood lactate concentration at t=14 min (P = 0.034), t = 19 min (P = 0.043) and t = 24 min (P = 0.011) as compared with control horses (data not shown). There were no significant changes found between periods 3 and 4 with reference to heparinized blood lactate concentrations during SET's.

Regarding the IT group, horses maintained trotting at high speeds during SET 3 for 16.1 ± 2.3 minutes only as compared with 19.8 ± 0.4 minutes (P=0.012) in control horses. IT horses started galloping frequently at the beginning of SET 3 despite humane encouragement to trot or stopped. As a consequence, the mean duration of trotting during SET 3 was decreased significantly by 19% in the IT group as compared with control horses.

Overall, the euglycaemic hyperinsulinaemic clamp was stopped after 189 ± 63 minutes associated with a mean space correction of -0.00279 ± 0.01235. The mean (± SD) rate of glucose metabolism (M; µmol/kg BW/min), the mean plasma insulin concentration (pmol/L), the mean glucose metabolism rate-to-plasma insulin concentration ratio (M/I,), and the correlations between the rate of glucose metabolism and the mean glucose metabolism rate-to-plasma insulin concentration ratio during steady-state in various periods is given in Table 1. The overall correlation between M and MI at the end of period 2 (Fig. 1) was 0.964 (P = 0.000). The correlations between the rate of glucose metabolism and the mean glucose metabolism rate-to-plasma insulin concentration ratio during steady-state in various periods for both groups are shown in Fig. 2.

Table 1. The mean (± SD) rate of glucose metabolism (M; µmol/kg BW/min), the mean plasma insulin concentration (pmol/L), the mean glucose metabolism rate-to-plasma insulin concentration ratio (M/I,) the correlations between the rate of glucose metabolism and the mean glucose metabolism rate-to-plasma insulin concentration ratio during steady state in various periods. Different superscripts within the same row indicate significance at $P < 0.05$. Significance of correlations are based on the Pearson product moment correlation test.

	Period 2		Period 3		Period 4	
	C	*IT*	*C*	*IT*	*C*	*IT*
M	24.6 ± 7.5	24.9 ± 5.9	26.8 ± 9.2	31.1 ± 6.8[a]	24.7 ± 6.8	18.1 ± 3.4[b]
Insulin	2971 ± 407	3166 ± 659	3270 ± 259	3083 ± 620	3133 ± 280	3056 ± 757
M/I	0.88 ± 0.37	0.83 ± 0.28	0.84 ± 0.31	1.05 ± 0.31[a]	0.79 ± 0.19	0.62 ± 0.17[b]
Correlation	0.991	0.937	0.987	0.812	0.933	0.701
P correlation	0.009	0.019	0.002	0.095	0.021	0.187

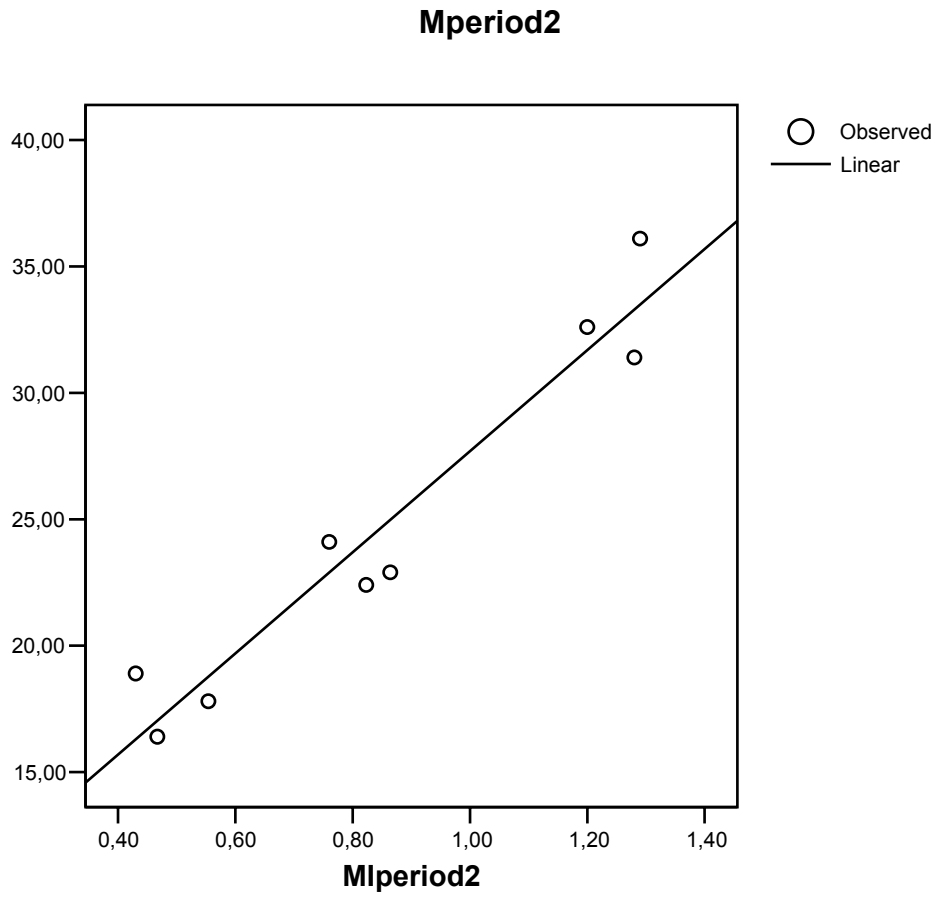

Figure 1. The correlation between rate of glucose metabolism (M; µmol/kg BW/min) and glucose metabolism rate-to-plasma insulin concentration ratio (M/I) after 18 weeks of training in young Standardbreds.

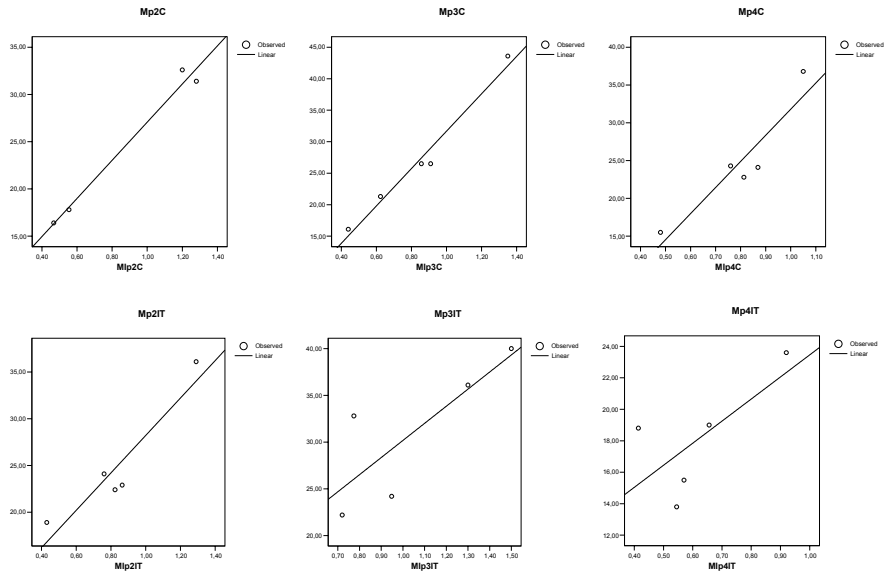

Figure 2. The correlation between rate of glucose metabolism (M; µmol/kg BW/min) and glucose metabolism rate-to-plasma insulin concentration ratio (M/I) in young Standardbreds during steady-state in last three periods (upper panel control group and lower panel intensified trained group).

Discussion

Insulin resistance is defined as a condition in which normal concentrations of insulin produce a subnormal physiologic response (170). The euglycaemic hyperinsulinaemic clamp test is regarded as gold standard with reference to assessment of peripheral insulin resistance. The amount of glucose metabolized (M) divided by the plasma insulin concentration (I) during the steady state of the euglycaemic hyperinsulinaemic clamp technique reflects the quantity of glucose metabolized per unit of insulin in plasma and, as a result, is a reasonable index of the sensitivity of tissues to exogenous insulin (69). In addition, insulin sensitivity measured by the hyperinsulinaemic euglycaemic clamp technique has lower interday variation when compared with the minimal model estimate derived from the frequently sampled intravenous glucose tolerance test in horses (254). Physical training has been shown to improve glucose tolerance and insulin action in man (17, 133, 218). Short-term endurance training for 7 consecutive days in Standardbred horses indeed improved insulin sensitivity and these enhancements in insulin sensitivity were still evident after five days of inactivity (305). However, it has been demonstrated that long-term training for 18 weeks in

Standardbred horses neither changed glucose metabolism nor insulin sensitivity 72 h after the last bout of exercise (63). The latter findings are consistent with the current study also taking into account that the mean space correction calculated here, reflecting the quality of the steady-state, was within a previously published reference range (268).

Overtraining has been defined as an accumulation of training and/or non-training stress resulting in long-term decrement in performance capacity with or without related physiological and psychological signs and symptoms of overtraining in which restoration of performance capacity may take several weeks or months (125).

The intensified trained group in the current study showed a 19% reduction on the average in treadmill trotting-time-to fatigue compared with the control horses. The drop in performance seen in the current study is similar with the reduction on the average in treadmill run-time-to fatigue by 14% as reported by others (115). Extending the trot can have profound energetic requirements that could limit performance as compared with cantering (360). Given the fact that Standardbred horses are bred for trotting and taking into account the lower energetic costs of cantering the intensified group must be regarded as overtrained with reference to the above definition.

During SET 2, there was a significant correlation between the rate of glucose metabolism and the mean glucose metabolism rate-to-plasma insulin concentration ratio during steady-state both overall and within groups. During SET 3 this significant correlation was lost for the overtrained group in contrast with the control group. This finding is in agreement with the fact that when in human subjects mental stress (undergoing five minutes of intense mental arithmetics) is applied while insulin-mediated glucose uptake is already stimulated, sympathetic overactivity (and associated elevated concentrations of both plasma catecholamines) is initially accompanied by increased glucose uptake (220).

Signs of overtraining in Standardbred horses were not related to changes in either maximal aerobic capacity (317), maximal and recovery heart rate (127), differences in skeletal muscle adaptations (318), muscle glycogen utilisation/s (206), plasma gamma-glutamyltransferase or creatine kinase activities, red blood cell concentration, haemoglobin concentration, plasma and red cell volume (127), white blood cell counts, and neutrophil to lymphocyte ratio (127, 319), red cell hypervolaemia (114), basal plasma cortisol (127) as well as adrenocortical function (33, 115) and plasma β-endorphin concentration (115). However, conflicting evidence exists whether overtraining was associated with significant changes in aspartate aminotransferase activity (319) or was not (127) although this activity also increases for several days after regular exercise.

To the author’s knowledge, the dissociation between the rate of glucose metabolism and the mean glucose metabolism rate-to-plasma insulin concentration ratio during steady-state in the euglycaemic hyperinsulinaemic clamp test has not been reported in

overtraining before. Interestingly, this dissociation persisted after four weeks of detraining, which is in agreement with a diagnosis of overtraining rather than overreaching. Furthermore, the period of detraining turned out to be essential to reveal the increased rate of glucose metabolism as well as the increased mean glucose metabolism rate-to-plasma insulin concentration ratio during steady-state in intensified trained horses at the end of the intensified training period (period 3).

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Chapter 9

The influence of training and intensified training on the early plasma glucose and insulin responses during a standardized exercise test in young standardbreds

Marleen M. Jager[1]
Ellen de Graaf-Roelfsema[1]
Eric van Breda[2]
Inge D. Wijnberg[1]
Hans A. Keizer[3]
Johannes H. van der Kolk[1]

[1]Department of Equine Sciences, Medicine Section, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands. [2]Department of Movement Sciences, Faculty of Health Sciences, University of Maastricht, Maastricht, The Netherlands. [3]Department of Human Physiology and Sports Medicine, Free University of Brussels, Brussel, Belgium

Abstract

Reason for performing study: A decline in plasma insulin is important for the rise in glucose production during exercise in a variety of species. However, the early insulin response during exercise has not yet been studied in detail in the equine species.
Objectives: The objective of this study was to monitor the early insulin and associated glucose response in plasma during acute exercise as well as following long-term training, intensified training, and detraining.
Methods: Twelve Standardbred geldings, aged around 1.5 years, were used initially in a longitudinal training study on a treadmill. The training period of 32 weeks in total was divided into four periods: acclimatization, training, intensified training and detraining. In the intensified training period, the horses were randomly divided into 2 groups: control (C) and intensified trained group (IT). At the end of each period, a standardised exercise test (SET) was performed.
Results: A significant ($P < 0.05$) decrease in mean plasma glucose concentration was found five minutes after the start of intense exercise during SET 1 (from 4.6 ± 0.3 to 4.2 ± 0.3 SD mmol/l; n=10) similar to the other SETs. The significant decrease in plasma glucose concentration during SET 2 was associated with a simultaneous decrease in plasma insulin concentration (from 29.8 ± 12.0 to 16.0 ± 5.6 pmol/l; n=6). Insulin concentrations remained low during acute exercise. A post-exercise rebound hyperinsulinaemia was found during all SET's.
Conclusion and clinical relevance: During acute exercise a decline in plasma insulin was associated with an increase in plasma glucose similar to the response in other species. In addition, long-term training significantly increased the glucose response to acute exercise. It is hypothesized that monitoring plasma glucose during a SET might be helpful in determining early overtraining.

Introduction

Besides being the most important energy substrate for the central nervous system, glucose has a very important role in providing energy for muscular contraction as well. Glucose usage and production are, among others, regulated by insulin, which is synthesized and secreted by the beta cells of the islets of Langerhans in the pancreas.

In most species, physical training provokes a large shift in substrate utilisation during exercise. In horses, muscle glycogen, and to a lesser extent plasma glucose, are utilized in substantial amounts during low-, moderate- and high-intensity exercise. Plasma glucose generally increases with all forms of exercise, because of stimulation of hepatic glycogenolysis due to sympathoadrenergic mechanisms operating directly via hepatic sympathetic innervation or indirectly via circulating adrenaline (107, 108). For the rise in glucose during exercise, a decline in plasma insulin is important. This suppression of insulin during exercise is well documented for the horse (97, 107, 108, 210) and appeared to have a threshold of 50% of VO_{2max} (210). To our knowledge, there is no information regarding the early insulin and glucose response following the onset of exercise. The reason might be that most studies describe only few measurements of plasma glucose concentrations during exercise tests and therefore not much quantitative information about these effects is available (109).
For instance, a recent study of Gordon et al (117) showed with only three measurements no differences in plasma glucose levels during a short-term high-intensity graded exercise test and a standing parallel control trial in an experiment involving six untrained Standardbreds.

Carbohydrate availability to skeletal muscle affects maximal exercise performance in humans. This relationship, however, is not very well outlined in horses (165). Horses in training are usually able to consume an appropriate amount of food to replenish depleted energy stores. However, horses that become overtrained might lack sufficient carbohydrates to meet the demands of their excessive training (127). In addition, during overtraining, the hormonal control system acts different, resulting for instance in a blunted response of cortisol to exercise and alterations in catecholamine release (127, 179). Cortisol normally provides a role in the breakdown of protein and fat, when the bodies' stores of carbohydrates are decreased (127) and sympathetic withdrawal (or parasympathetic dominance) is associated with hypoglycaemia in overtrained athletes (179).

The aim of the current study was to determine the early insulin and glucose response during acute exercise and the influences of long-term training, intensified training, and detraining on this response in young Standardbreds.

Materials and methods

Animals
Twelve Standardbred geldings aged about 1.5 years and weighing (mean ± S.D.) 396 ± 47.7 kg were initially trained for 24 weeks and detrained for 4 weeks. The longitudinal study was performed in two successive years with 6 horses per year. Prior to the training period, horses were acclimatized to exercising on the treadmill for four weeks. All training and standardized exercise tests (SET) took place on a high-speed treadmill (Kagra, Graber A, Fahrwangen, Switserland), at a 4% slope at maximum. Following SET 2, one couple was excluded from the experiment because of lameness. The diet consisted of grass hay supplemented with concentrate feed and met nutrient requirements for maintenance and exercise (58 MJ NE (range 54-66)). Grass hay (5-6 kg/horse/day) was fed at 0730 and 1930 h, and a commercially pelleted grain mix (2-3 kg/horse/day) was fed at 0600 and 1800 h. Water was freely available.

Training
Training was divided into four periods: 1) period of acclimatisation to exercise on the treadmill for 4 weeks; 2) training period for 18 weeks with alternating both endurance (~60% HR_{estmax}) and intensity training (~80% HR_{estmax}); 3) intensified training period with increased training volume and intensity for 6 weeks, and 4) detraining period for 4 weeks (~60% HR_{estmax}) according to the protocol by Bruin et al (33). In period 3, the horses were randomly divided into 2 groups: control (C) and intensified training (IT). Training of control horses always consisted of exercise 4 days/week, whereas the intensified trained horses were exercised 6 days/week during the first three weeks of period 3, and 7 days/week during the last three weeks. During period 3, the control group continued training at the volume and intensity they received at the end of period 2. The training for the IT group increased for volume and intensity during period 3. At the end of each period, a standardised exercise test (SET) was performed at ~80% HR_{estmax} for 20 minutes. During SET 1 this matched with a speed of 6.5-7.0 m/s, during SET 2, 3, and 4 this matched with a speed of 7.5-8.5 m/s with a treadmill inclination of 1-4%. HR was monitored using a Polar (S610i, Polar Electro Oy, Kempele, Finland).

Testing protocol
For blood sample collection a 14 gauge catheter, 13cm (Mila, Mila international, Erlanger, KY, USA), was inserted into a jugular vein. A standardised exercise test (SET) was performed on a high-speed treadmill. The SET started with a 9 mins warming up period followed by intense exercise (trotting) of 20 mins and a cool down for 5 mins at 1.5 m/s on the treadmill. Before and directly after the test the horses walked for 30 mins in a horse walker. During the exercise two fans, one in the front and

one on the back of the horse, were used to cool the horses during intense exercise. The study was approved by the Committee of Animal Welfare of the Faculty of Veterinary Medicine, Utrecht University.

Sampling procedures
Blood samples were taken every five minutes from the start of intense exercise at t=0, 5, 10, 15 and 20 minutes and at the beginning of the warming up (t= -9 min). Insulin samples were only collected from the second group of 6 horses, due to generated interest in this parameter.

Assays
Plasma insulin was measured with a commercial radioimmunoassay kit (Coat-A-Count, TKIN2 836, Diagnostic Products Corp, Los Angeles, California, USA), that had been validated for horses. Curves obtained with serial dilutions were parallel to the standard curve. The detection limit of the assay amounted to 1.2 µU/ml. The intra-assay and interassay coefficients of variation were 5 and 7%, respectively. The insulin antiserum showed cross-reactivity with proinsulin of approximately 40% and a particularly low cross-reactivity to compounds other than insulin and proinsulin (328).

Plasma glucose concentrations were measured by an enzymatic spectrophotometer method (ABL-605, Radiometer Copenhagen, Westlake, Ohio, USA).

Statistical analysis
Statistical differences were assessed by the two-way ANOVA with repeated measures (SPSS version 10.0 for Windows, SPSS Inc, Chicago, Ill, USA) for time and exercise vs. control groups, for determination of main effects and interactions. A linear mixed-effects model with a one-step autoregressive process (SPSS version 14.0 for Windows, SPSS Inc, Chicago, Ill, USA) was used to determine the significant differences between periods. P values < 0.05 were considered significant. Results are presented as mean ± SD.

Results

Regarding the IT group, horses maintained trotting at high speeds during SET 3 for 16.1 ± 2.3 minutes only as compared with 19.8 ± 0.4 minutes (P = 0.012) in control horses. IT horses started galloping frequently at the beginning of SET 3 despite humane encouragement to trot or stopped. As a consequence, the mean duration of trotting during SET 3 was decreased significantly by 19% in the IT group as compared

with control horses. Both groups showed an average of 105% increase in body weight as compared with initial weights.

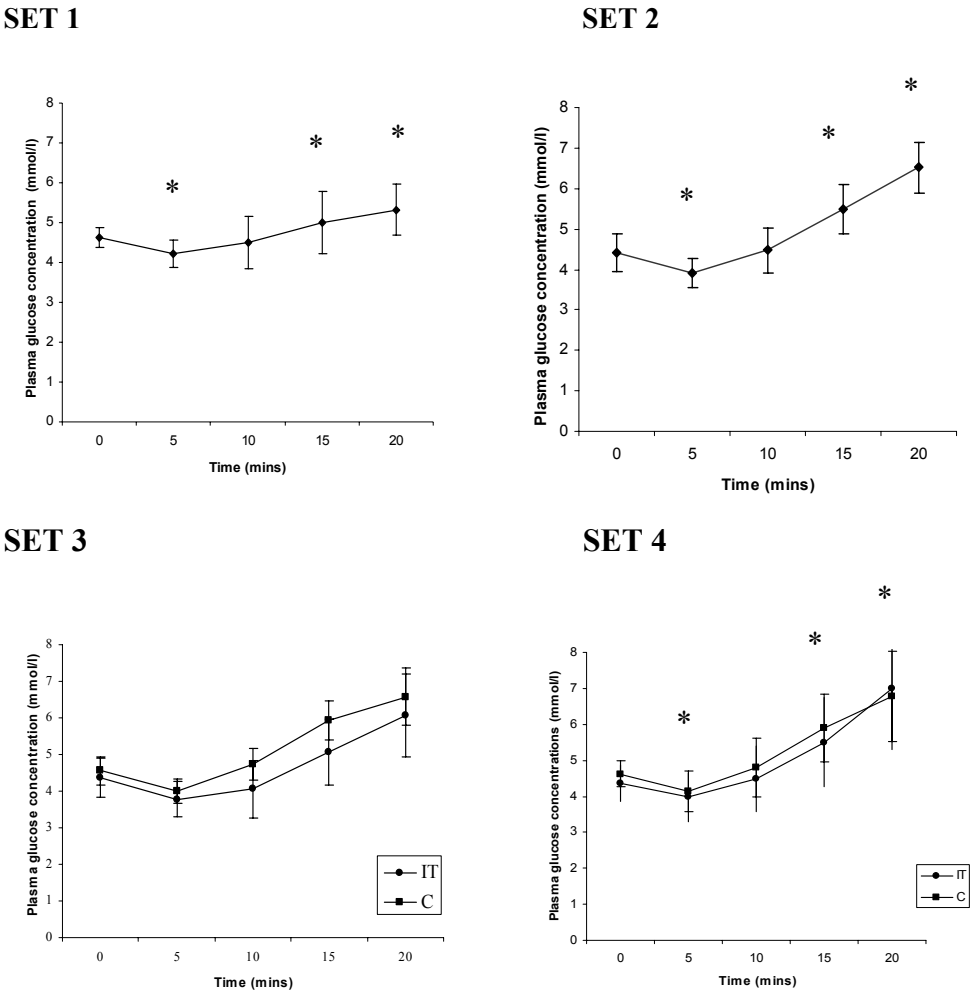

Figure 1. Mean (±SD) plasma glucose concentrations during standardised exercise tests (20 min exercise at ~ 80% HR_{max}; n=10) after acclimatization period (1), training period (2), intensified training period (3) and detraining (4). Horses were randomly divided into control (C) and intensified training group (IT) after training period (2).
* Glucose concentration significant different from preceding glucose concentration ($P<0.05$)

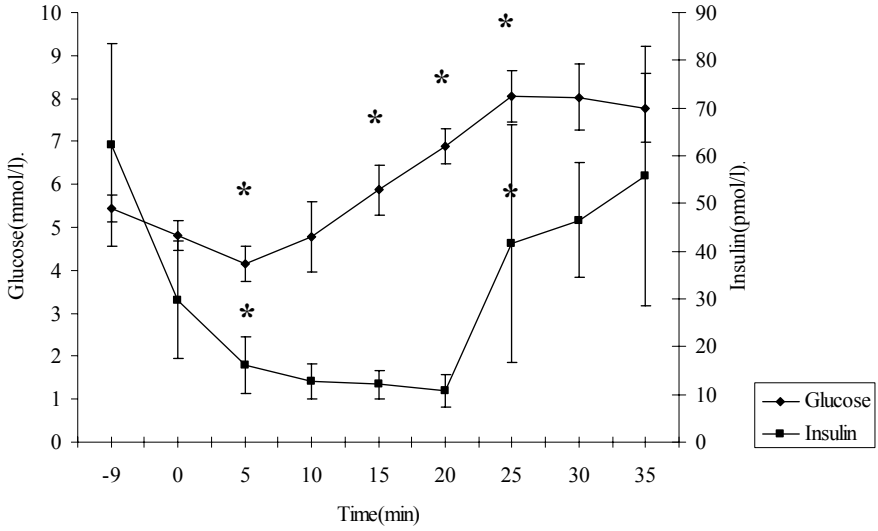

Figure 2. Mean (± SD) plasma glucose and insulin concentrations after 18 weeks of training during SET 2 (20 min exercise at ~ 80% HR_{max}.; n=6) Intense exercise was performed from t=0 until t=20.
* Value significant different from preceding value (P<0.05)

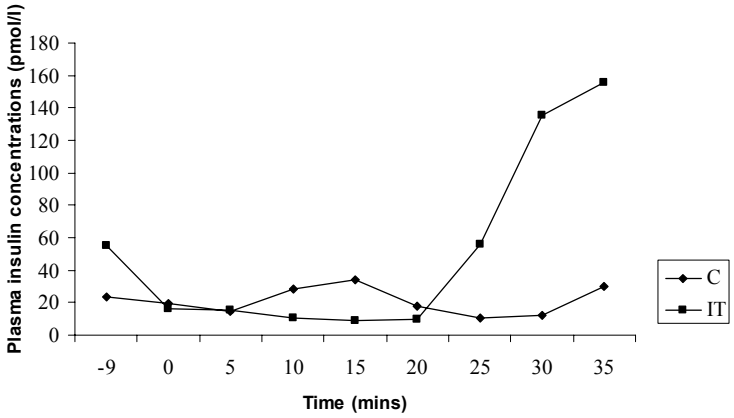

Figure 3. Mean (±SD) plasma insulin concentrations during standardised exercise tests (SET 3) (20 min exercise at ~80% HR_{max}; n=4) after intensified training period for control (C) and intensified trained (IT) group.

Changes in plasma glucose and insulin during acute exercise (SET 1) and after a period of training (SET 2)
Mean plasma glucose decreased significantly at t = 5 (5 minutes after the start of intense exercise) both during SET 1 (from 4.6 ± 0.3 to 4.2 ± 0.3 mmol/l, n=10) as well as during SET 2 (from 4.4 ± 0.5 to 3.9 ± 0.4 mmol/l, n=10) as shown in Table 1 and Figure 1. This significant decrease in plasma glucose concentration after the start of intense exercise at both SET 1 and SET 2 was followed by a significant increase in plasma glucose concentration as exercise continued (Figure 1, n=10).
Glucose concentration reached the highest levels 5 minutes post exercise (t=25) (Figure 2, n=6). This effect occurred in all SET's and in all horses (n=10).

The significant decrease of plasma glucose concentration at the start of intense exercise (t=5) at SET 1 and SET 2 (Figure 2), was associated with a significant decrease of insulin at the start of exercise, in SET 1 (from 91.2 ± 110 to 22.8 ± 12.4 pmol/l; n=6) as well as in SET 2 (from 29.8 ± 12.4 to 16.0 ± 5.9 pmol/l, n=6). A progressively further decrease of insulin concentrations was observed from t=0 until t=15 minutes during intense exercise at SET 2 (Figure 2), associated with an increase of glucose. Significant rises in mean plasma insulin from 23.9 ± 17.3 to 73.9 ± 64.6 pmol/l at SET 1 (not shown) and from 10.8 ± 3.5 to 41.5 ± 24.9 pmol/l at SET 2 (Figure 2), were associated with only small alterations in plasma glucose concentrations.
During SET 1 plasma glucose levels were significantly lower after t=15 (P = 0.039) and t=20 minutes of intensive exercise (P = 0.003) compared to SET 2.

Changes in plasma glucose and insulin after a period of intensified training (SET 3)
Mean (n=5) plasma glucose concentration values of the intensified trained horses showed a non-significant trend in a decreased glucose concentration throughout the whole exercise period compared to the control group (n=5) during SET 3.

Plasma insulin values of SET 3 were obtained from 4 horses (2 IT and 2 C), because one couple of horses could not continue due to lameness. At SET 3, the mean plasma insulin concentrations (n=2) of the IT group showed a non-significant trend in a higher post exercise hyperinsulinaemic response from t=20 (9.32 ± 3.0 pmol/l) to t=25 (55.6 ± 8.6 pmol/l) and t=30 (to 135.6 ± 54.6) in contrast to C group, which showed a slight decrease in insulin concentrations from t=20 on (Figure 2). Plasma glucose levels after 15 minutes of exercise were significantly lower for the intensified trained horses during SET 3 compared to SET 2 (P = 0.06).

Changes in plasma glucose and insulin after a period of detraining (SET4)
During SET 4, mean plasma glucose concentrations in IT group were significantly decreased initially as shown in Table 1 and Figure 1 as compared with C group. During

SET 4, no differences were seen in plasma insulin concentrations between C and IT group.

Table 1. Mean (± SD) plasma glucose concentrations (mmol/L) during 20 minutes of intense exercise in different stages of training and intensified training (C=control group and IT=intensified training; n=10).

Time (min)	*0*	*5*	*10*	*15*	*20*
Glucose (mmol /L)					
SET1	4.6 ± 0.3	4.2 ± 0.3	4.5 ± 0.7	5.0 ± 0.8	5.3 ± 0.6
SET2	4.4 ± 0.5	3.9 ± 0.4	4,5 ± 0.6	5.5 ± 0.6	6.5 ± 0.6
SET3 C	4.6 ± 0.4	4.0 ± 0.3	4.7 ± 0.4	5.9 ± 0.5	6.6 ± 0.8
IT	4.4 ± 0.5	3.8 ± 0.5	4.0 ± 0.8	5.1 ± 0.9	6.1 ± 1,1
SET4 C	4.6 ± 0.4	4.1 ± 0.6	4.8 ± 0.8	5.9 ± 0.9	6.8 ± 1.3
IT	4.4 ± 0.5	4.0 ± 0.7	4.5 ± 0.9	5.5 ± 1.2	7.0 ± 1.7

Discussion

The main result of the present study is the assessment of the early insulin response to acute exercise. The early insulin response to acute exercise in the horse is characterized by a significant decrease in plasma insulin concentration, followed by a significant increase in plasma glucose concentration after 5 minutes of intensive exercise. This results correlate well with the progressive increase in plasma glucose concentration during moderate exercise (55%$V02_{max}$) with the peak at the end of exercise, as described by Geor et al (109). Although other studies have described these effects before, this effect has not been quantified yet. This study shows a clear early insulin and glucose response during acute exercise. It is quantified that in the first ten minutes a balance is found between glucose and insulin, which is important for the exercise performance of a horse. Most studies only describe few measurements of plasma glucose concentrations during exercise tests (100, 117). Freestone et al (100) described a decrease in serum insulin and an increase in glucose concentration during moderate exercise. Glucose was only measured at 2 and 30 minutes after the start of maximal exercise. In addition, serum insulin was decreased 2 min following exercise. The glucoregulatory response in the study of Freestone et al (100) was characterised by a fall in insulin and an increase in adrenaline, noradrenaline and cortisol concentrations. The increase in glucose concentrations during acute exercise, which was also found in this

study during all SET's and in all horses, can be explained by an increase in hepatic glucose output mainly mediated via a decrease in the insulin-glucagon ratio and a decrease in the rate of uptake and utilization in exercising muscle caused by increases in circulating adrenaline (108).

In the current study, the training period seemed to induce higher plasma glucose levels during acute exercise as was shown by the significant increased glucose levels at the end of SET 2 compared to SET 1. In general, endurance training enhances gluconeogenesis by the liver via increased hormonal sensitivity (76, 211). The exact mechanism is not fully known, but for instance the liver can adapt to training stress through modulation of glucagon receptor binding characteristics to enhance the hepatic glucose production responsiveness to glucagon (79, 184). An increased liver sensitivity is also suggested for catecholamines (245). In contrast, training is reported to decrease the secretion of catecholamines as well as glucagon (211, 141). However, this might be a favourable response, because lower circulating hormonal concentrations and increased sensitivity to these hormones might facilitate glycogenolysis as well as lipolysis during exercise.

Plasma glucose concentration in the IT group did not change significantly during SET 3 compared to the C group, although a trend in a decrease of glucose concentrations was observed in the IT group. This was confirmed by a significant lower plasma glucose level 15 minutes after exercise during SET 3 compared to SET 2 for the intensified trained horses. This corresponds well with studies regarding overtraining in human athletes, in which hypoglycemia was observed, in particular during the parasympathetic type of overtraining (102). In contrast, Tyler- McGowan et al (319) found a non significant increase of glucose concentrations in the IT horses compared with C horses in a study seeking for parameters of overtraining. The parasympathetic form of overtraining may be a reflection of an advanced state of overtraining closely associated with exhaustion of the neuroendocrine system, while the sympathetic type may reflect a prolonged stress response preceding exhaustion (102). Following the parasympathetic type of early over-training a decline in plasma glucose concentration as observed in this experiment, can be explained by a decrease of circulating catecholamines and in addition diminished hepatic glucose production.

A postexercise rebound hyperinsulinaemia was seen in all SET's. This effect was also found after 30 minutes of maximal exercise by Freestone et al (100). A rebound hyperinsulinaemia is thought to be caused by either a withdrawal of sympathetic neural inhibition of the pancreatic β-cells or due to elevations in endogenous opioid peptides stimulating insulin release (97). The plasma half-life of catecholamines is short, less than 30 s, and within a few minutes after exercise concentrations have returned to their resting level (299). This withdrawal of sympathetic neural inhibition and a demand of the muscle for glycogen repletion, can be a logical explanation for the increased insulin

levels found in this experiment immediately after exercise. In the present study, we observed a great individual difference in plasma insulin concentrations after exercise. This could be explained by the amount of glycogen repletion in the individuals and the need for glucose uptake. It is described that training appeared to alter the insulin response to exercise, enhancing the ability to synthesize glycogen during recovery (252). In another study, it was shown that insulin sensitivity increased after exercise in humans (173). However, it has been demonstrated that long-term training in Standardbreds neither changed glucose metabolism or insulin sensitivity 72 h after the last bout of exercise (63) in contrast with short-term training in Standardbreds (305).

Conclusion

In conclusion, this experiment showed that long-term training alters the insulin and associated glucose response to acute exercise by increasing plasma glucose levels probably mediated via increased hepatic glucose secretion. Intensified training attenuates this glucose response. It is therefore hypothesized that monitoring plasma glucose during a SET might be useful in determining early overtraining.

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Chapter 10

Intensified training induces ethological effects in Standardbreds

Machteld C. VanDierendonck[1]
Ellen de Graaf-Roelfsema[2]
Eric van Breda[3]
Inge D. Wijnberg[2]
Hans A. Keizer[4]
Johannes H. van der Kolk[2]
Ab Barneveld[2]

[1]Faculty of Veterinary Medicine, Dept. of Animals, Science & Society, Utrecht University, Utrecht, The Netherlands. [2]Faculty of Veterinary Medicine, Dept. of Equine Sciences , Utrecht University, Utrecht, The Netherlands. [3]Faculty of Health Sciences, Department of Movement Sciences, Maastricht University, Maastricht, The Netherlands. [4]Department of Human Physiology and Sportsmedicine, Free University of Brussels, Brussel, Belgium.

Abstract

This is the first systematic study in relation to equine overreaching/training in which the behaviour of the horses was systematically analysed. In human athletes a daily questionnaire (POMS) is the most reliable diagnostic so far, predicting whether an athlete is at risk of overreaching / overtraining. For this study, the human “overtraining” POMS was translated to a series of assessments measuring comparable (and biologically meaningful) aspects during equine training. Two of the three assessments were direct observations. The first assessment was either before, during and after leaving the treadmill (High Intensity Training (HIT)) by means of a standardized protocol. The other assessment was via a remote video camera system, videoing the horses twice a week during six hours (Time Budget). The third assessment was a standardised test, adapted from a Novel Object test. The horses were for 15 minutes in a large arena twice a week. During the middle 5 minutes, they were able to interact with an unknown gelding over a beam (Novel Horse test). The TB and NHT videos were coded later with a predefined ethogram. All three assessments took place during a period of basic training, intensified training (3 weeks), high-intensified training (3 weeks), and detraining (4 weeks). The control horses continued the basic training, except for the detraining period. The horses were their own control.
It was possible to distinguish the control horses from the intensified trained horses, especially during the standardized NHT test. All horses anticipated on the meeting with the novel horse, but the intensified trained horses had shorter interaction with this gelding, explored the arena shorter time before, foraged longer and showed less transitions when the novel horse left. The intensified trained horses also cantered more often during the high-intensified HIT training period. After detraining, most of the parameters were not back to the original levels. So indeed, indications of mental stress or “psychological overload” were found.

Introduction

In order to improve performance in sports, the training load has to be gradually increased over time. This improvement is only achieved if the training load is properly balanced with recovery. However, in both human- and equine elite athletes, the ratio between training load and recovery is often in a precarious equilibrium. When this imbalance occurs over a longer period, it may eventually lead to a severe mal-adaptation to the increased work load defined as overreaching or even, in more severe cases, overtraining (88, 179, 180). In addition, in both horses and humans, several gradual stages of mal adaptation to training can be observed: mechanical overload, metabolic overload (or overreaching) and overtraining (or staleness) (180, 325, 329). Since a full blown overtraining syndrome can lead to a long absence from competition and is a severe welfare issue for the athlete involved, it is important to prevent this.

Most equine studies concentrate on physiological or histochemical indicators associated with overreaching/overtraining (33, 88, 114, 115, 127, 235). However, in humans a full blown overtraining syndrome is often caused by multiple stressors (179, 180, 265) inducing mental as well as physical stress and one of the most consistent symptoms of overtraining is a change in mood state and behaviour. Some horse studies (33, 88) report behavioural changes following intensified training like changes in general behaviour, behaviour while trained, home stable behaviour and reactivity to external stimuli. Albeit, the behavioural parameters were usually not measured as systematically as the physiological counterparts.

Therefore, the current experiment was set up in order to systematically assess the horse's attitude, behaviour and response to intensified training for identifying factors related to early overtraining or overreaching. In human athletes, one of the most effective and sensitive methods of predicting overtraining is the use of a regular questionnaire, assessing the athlete's psychological status (180, 265). Regularly, the Profile of Mood State (POMS) is used: measuring mood changes, emotional instability and motivation (115). The human POMS consists of repeated questions determining mood states like tension - anxiety, depression - dejection, anger - hostility, vigor - activity and fatigue - inertia (265, 359). For use in equines, these mood states need to be translated to behavioural parameters, which are measurable and also biologically relevant.

For a study, undertaken to measure the pathophysiological adaptations to intensified training in Standardbred geldings, a series of behavioural assessments needed to be developed. A combination of the most often reported (behavioural) variables as well as an equine "translation" of the human POMS seemed the most promising to implement in this study. In order to objectively measure the mood state of

the horses, three tests were developed aimed to distinguish between the mood states as described in the POMS.

One test aimed at assessing the horse's motivation (and performance) on the training apparatus itself. This can be measured by means of a standardised pre-defined protocol measuring items related to equine acceptance-resistance, or stress behaviours during preparation before training, during the training itself and post training.

The second test aimed at assessing aspects of "tension", "anger-anxiety", "vigor" and possibly "inertia" represented by several aspects related to basal maintenance like rest and eat patterns, alertness, as well as anticipation towards known positive procedures (food provision), and reactions towards unknown stimuli. These were measured by using remote cameras in the horse's home stable.

The third test aimed to assess the mental attitude ("anger"- "anxiety") towards a presented stimulus. Therefore, a well-known assessment to test anxiety in laboratory animals was adapted: the Novel Object test (326). This test was also used successfully to test reactivity in relation to temperament in young horses (348). It is commonly accepted that a repetition of a Novel Object test can only be performed with at least 4 weeks interval. Since the processes measured here were at a shorter time span it was chosen to replace the Novel Object by a stimulus, which should always be interesting for a horse, namely another unknown (Novel) Horse (NH). Geldings are supposed to be a potentially never-lasting interesting stimulus for another gelding without having reproductive intentions (330).

To make the link between ethological and physiological parameters, heart rate and heart rate variability can be used (191, 349). The heart rate is controlled by the sympathetic and parasympathetic systems. When the animal is not stressed, there is a balance between these two systems represented by a high variation between the beat-to-beat intervals (191, 349). Comparing heart rate variability (HRV) is a suitable method to evaluate the autonomic influence on the system (191). The analysis of various parameters derived from heart rate and variability at different observation intervals or actions gives an indication of the status of autonomic system under these circumstances.

We hypothesized that during intensified training horses will show lower motivation to train and more (biologically relevant for equines) aspects of "tension", "fatigue" and "anger/anxiety" and less "vigor".

Animals, Materials and Methods

Animals

Ten Standardbred geldings aged 1.5 years were trained during a longitudinal study of experimentally induced highly intensified training. For organizational purposes, the study was performed in two different groups of six and four horses in two successive years (2004 and 2005, respectively). The animals were individually housed in standard boxes and were walked in a horse walker daily. They received pellets and silage twice daily. For the basic description of the overall study see De Graaf-Roelfsema et al (63). Animal care and experimentation were performed in accordance with protocols approved by the Science Committee and the local Animal Experimentation Committee (Utrecht University, Utrecht, The Netherlands). Weekly the bodyweight of all horses was determined.

Training set-up

After an acclimatization period of two months, basic endurance training was started to introduce the horses further to the high-speed treadmill for four weeks (period 1).

Directly thereafter, the horses received an 18-week training program of mixed endurance training (ET) and high intensity training (HIT) (period 2). Days of ET were alternated with HIT. Each training session was preceded by 30 mins warm-up at the walking machine followed by 8 mins warm-up (4 mins at 1.6 m/s and 4 mins at 4.5 m/s) at the treadmill. The endurance running included 20-24 min of continuous level running at 60% HF_{estmax} or 16-18 min at 75% HF_{estmax}. The interval training consisted of three 3-min or four 2-min bouts at 80-85% HF_{estmax}, interspersed with 3-min or 2-min periods at 60% HF_{estmax}. Each training session ended with a cooling down consisting of a 5 min walk at the treadmill followed by 30 mins walk at the walking machine. The horses exercised 4 days/wk throughout period 2.

In period 3, the horses were divided into a control group (C) and an intensified trained group (IT). The control group continued training at the volume and intensity they received in the second period for six weeks. For the IT, the intensified training regimen consisted of alternating days of HIT and ET for 6 days/week during the first 3 weeks (period 3a) followed by 7 days/week for the last 3 weeks (period 3b). Each training session was preceded by 30 mins warm-up at the walking machine followed by 8 mins warm-up (4 mins at 1.6 m/s and 4 mins at 4.5 m/s) at the treadmill. Exercise intensity and volume during the ET was gradually increased to 24-35 mins 60-75% HF_{estmax}. High intensity exercise gradually increased to five 3-min or six 2-min bouts at 80-85% HF_{estmax} interspersed 1-min or 2-min periods at 60% HF_{estmax}.

In period 4, the horses received a 4-week training program of light endurance exercise for detraining. The horses performed endurance training for 20 min at 60% HF_{estmax} for 3 days and 70% HF_{estmax} for 1 day a week.

On the resting days the horses walked for 60 minutes at the walking machine throughout all periods. At the end of each phase, a standardised exercise test (SET) was performed at ~ 80% HF_{estmax} (equivalent to 7.5-8.5 m/s and 1-4% incline) for 20 minutes. For logistic reasons one couple of a control and intensified trained horse started in adjacent weeks.

Observations set-up

The observations were divided over three different assessments: the first was a standardized observation protocol before, during and after the training on the treadmill (the “HIT” observations); the second was a specially developed assessment to challenge the horses for a novel situation (Novel Horse Test “NHT”); the third was a remote camera observation within the home stable for 6 continuous hours per observation (Time Budget “TB”).

For all three tests each animal served as its own control. Baseline values were obtained for each individual from multiple recordings during the last three weeks of period 2. During the last three weeks of period 2, 3a, 3b, and 4, the assessments were performed according to the schedule in Table AA. The whole experimental timeline is shown in Figure cc.

Table AA. Test schedule of the three assessments distributed over the different training phases

test	group	Period 2 Basic		Period 3a Intensified		Period 3b Highly intensified		Period 4 Detraining	
	year	2004	2005	2004	2005	2004	2005	2004	2005
HIT	C	6	6	9	9	10	10	7	7
	IT	6	6	6	6	6	6	7	7
NHT	C	2	6	6	6	7	8	6	6
	IT	2	6	6	6	7	8	6	6
TB	C	3	6	6	6	6	6	4	4
	IT	3	6	6	6	6	6	4	4

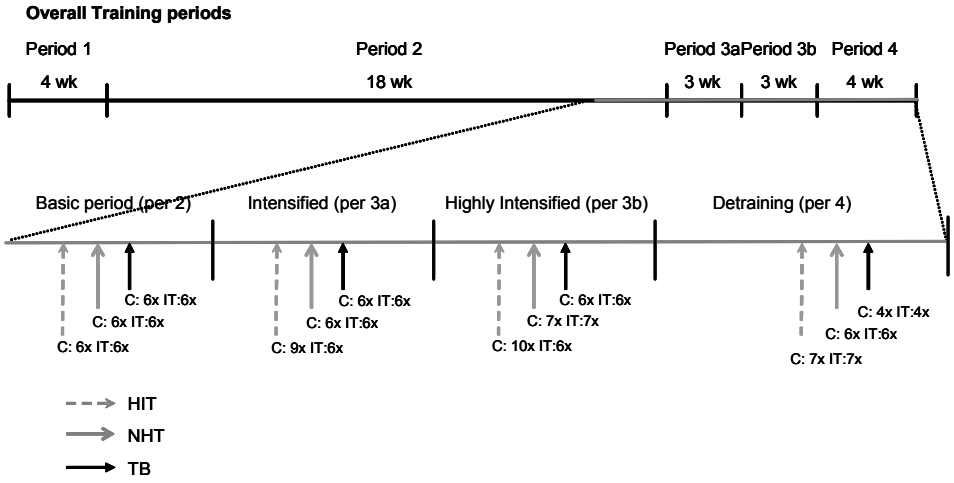

Figure cc. Time schedule of different assessments in different periods. HIT = High Intensity Training; NHT = Novel Horse Test; TB = Time Budget; C = control horses; IT = intensified trained horses.

Assessment of High Intensity Training

During the HIT assessment the horses were observed from the moment they entered the training shed (after a 30 minute warm up at a horse walker). The protocol consisted of items performed during the preparation of the horse for the training (entering the shed; cleaning the horse; application of girth with the remote Heart Rate recording equipment, bridle and safety girth); as well as entering the treadmill. After the training was finished the same type of items were recorded. The items were scored according to a pre-defined 4 point scale standard protocol: 0 meaning “horse performs/undergoes the task without resistant”; 4 meaning “severe stress behaviours and unable to complete/resists tasks”.

The actual training was divided in intervals according to the pre-defined speed changes of that period. During the high intensity training three minutes of a fast speed (~ 80% HR_{max}) were followed by two minutes of a slower speed (~ 60% HR_{max}). During each interval the horse was manually observed according to a standard protocol. The protocol consisted of both frequency items and categorized items (table BB). The categorized items were scored either “never”; “sometimes”; “regular” or “continuous”.

Table BB. Relevant items of standardized protocol during HIT training observations, with short description

Behaviour	F/C*	Description
Reactive ear play	C	Reaction of ears towards stimuli from the environment
Tense body	C	Whole body stiff, hind quarters underneath body, tail against hind quarters
Play with bit	C	Cheeks are loose, horse let bit move inside mouth
Tense jaws	C	Teeth occluded, tense jaw, no bit movement
Head bobbing/nodding	C	idem
Tail sweeping	C	Vigorously sweeping of tail
Location in relation to front beam	C	In front against safety band or far behind (markers on treadmill itself)
Jumping / rearing	F	Rearing and jumping in treadmill, legs sometimes over safety band
Stumble	F	Stumble over own feet
Snort	F	Snorting sound
Defecation	F	Defecation
Canter	F	Going too fast: to a more economical gait
Look back	F	Turns head to see trainer
Hang	F	Pull on safety rope (backwards)
Kick	F	Clear kick backwards
Whinny	F	Whinny

* Frequency or categorical recording

Since the number of intervals was unpredictable during period 3b for the intensified trained horses, all results were standardised towards the number of intervals per session. Due to logistic and time constraint the results of the data recorded in categories are not yet available.

Novel Horse assessments

Before testing, the horses were habituated to the arena twice in 2004 and six times in 2005. At the start of the test, the horse was fitted with a girth with the remote Heart Rate recording equipment. The horse was lead into an indoor arena (Figure dd) equipped with a Sony analogue Video recorder fixed at a pre-set location. Prior to release into the arena the video, stopwatches and Polar were started simultaneously. The horse was free to explore the arena for 5.0 minutes (pre-exposure phase) after which the Novel Horse was announced with a firm knock on the door and the door was

opened fully. The Novel Horse (NH) was presented at an angle of 90° in front of a beam separating both animals. The NH was holding loosely on a lead rope by one of the assistants. Both animals were allowed to interact freely over the beam (novel horse phase). After 5.0 minutes the door was closed and the horse was left in the arena for another 5.0 minutes (post-exposure phase). To eliminate potential sexual hormonal interference only geldings were used as NH. At each assessment point it was unpredictable for the horse, which NH would be presented, in principle the NH was only used twice. Each experimental year, all couples met the same novel horse on the same assessment point. Per couple of horses the NHT assessments were performed twice a week between 14:30 and 16:00 h. Reconstruction work at the facilities caused that it was not possible to use the same room for the NHT assessment in both years. In the second year, the location was more suitable for the experiment, with less variation of the olfactory information on the bedding in the arena and less disturbance.

The videos were coded later with the help of The Observer® software (Noldus, Wageningen, The Netherlands). Predefined behaviours (Table CC) were either scored as frequencies or as percentage of the total observed time. In the statistical analyses the three phases of this test (pre-exposure, novel horse exposure, and post-exposure) were considered three separate but linked assessments.

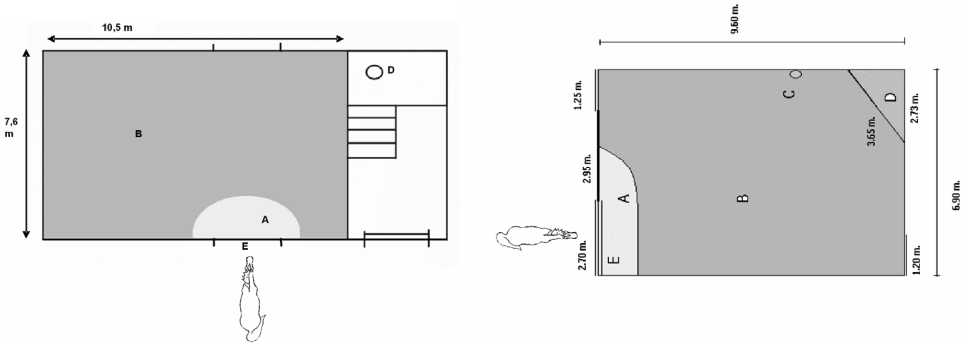

Figure dd. Ground plan of NHT testing arena in 2004 (left) and 2005 (right). Coding: A interaction area; B Test area; C water; D Camera on elevated platform; E, Slide door with beam for exposure to Novel Horse. In light grey: “within 1 horse length of Novel Horse”.

Table CC. Relevant items of standardized protocol of trotter during NHT observations, with short description

Behaviour	F/C*	Description
Foraging	C	Eat bedding
Exploration – standing	C	Explore arena while standing still, excluding bedding
Exploration – walking	C	Explore arena while walking around, excluding bedding
Stand alert	C	Stand still with ears pointed forward
Stand	C	Stand with head above withers but no particular attention
Startling	F	Sudden startling reaction with prompt action
Frisky	C	Jumping around, kicking out, rearing, “playful” running
Walk towards NH	C	Walk towards novel horse
Stand with NH	C	Stand besides novel horse without touching
Nose to nose	C	Nose to nose
Play	C	Play between trotter and novel horse
Allogroom	C	Allogrooming between trotter and novel horse
Leave NH	F	Walk away from novel horse
Scraping	F	Scraping ground
Rearing / jumping	F	Rearing / jumping only once
Snort	F	Snorting sound
Autogrooming	C	Autogrooming
Defecation	F	Defecation
Urinating	F	Urinating
Flehmen	F	Flehmen
Relaxing penis	F	Let penis hang down

* Frequency or categorical recording

Time Budget assessments

For the time budget assessments a remote video was adjusted high above each box of each horse of a couple. The observations were performed from 15:45-21:45 h twice a week including at least a day during which the intensified trained horse performed a HIT training in the morning. Around 18:00 h the horses received concentrates and around 19:30 h the horses received silage. All visits of all personnel in the stables were recorded with exact times in a notebook in 2004. In 2005, an additional camera was directed towards the corridor between the horses and a splitter, which recorded the output of all three cameras simultaneously on one tape. The videos were coded later with the help of The Observer® software (Noldus, Wageningen, The Netherlands). Predefined behaviours (Table DD) were either scored as frequencies or as percentage of the total observed time. Since the horses got increasingly more food and there was an individual difference in amount of food provided: the time spent on eating was, for each assessment, divided by the amount of concentrates / hay (in kg) given. This way the time spent per kg food could be calculated.

Table DD. Relevant items of standardized protocol during TB observations, with short description

Behaviour	F/C*	Description
Eat silage	C	Eat silage
Eat concentrates	C	Eat concentrates from feeding bowl
Eat faeces	C	Eat own faeces
Eat misc	C	Eat something but not possible to see what
Lick / gnaw	C	Lick or gnaw on objects in stable
Lick saltlick	C	Lick saltlick
Drink	C	Drink from automatic water bowl
Defecate	F	Defecate
Urinate	F	Urinate
Rest Lateral	C	Rest flat, head touches ground
Rest sternal	C	Rest
Rest stand	C	Stand head below withers, ears sideways
Stand alert	C	Stand still with ears pointed forward
Stand misc	C	Stand with head out of sight (due to high location camera)
Walk around	C	Non-stereotypic box walking
Exploration	C	Sniff on parts of stable (except bedding)
Restless	C	Restless behaviour, quick short explorations combined with stand alert
Roll	C	Rolling
Scraping	C	Scraping with hoof on ground
Cribbing	C	Cribbing or airsucking
Autogrooming	C	Autogrooming
Distraction	C	React on something/someone outside box

* Frequency or categorical recording

Heart Rate measurements

During the HIT and the NHT observations the horses were equipped with remote heart rate monitoring devices: Polar 810i® receivers with Polar® Equine T52H Horse Heart Rate Transmitter. Both the transmitter and the receiver were fitted to an elastic girth and attached to the horse according to the guidelines of Polar® and the contact was enhanced by Chemolan® Contact gel. The receiver was scheduled to measure RR intervals according to the directions of use of the Polar. The data were uploaded to a computer twice weekly. The errors in the recorded HR profile were corrected per interval according to the basal option offered in the program, provided that the number of corrections per interval was lower than 10%. In case a higher number of corrections were needed, the interval was considered not suitable to use.

The parameters used were: mean Heart Rate, Mean RR interval, RmSSD, Low Frequency and High Frequency, with their specific properties described by Malik (191).

For the analyses of the HRV data collected during the HIT assessments, the data were split at intervals, which were characterised by a speed change. Since the number of intervals was unpredictable during period 3b for the intensified trained horses all results were standardised towards the number of intervals per session. For the analyses of the HRV data collected during the NHT assessments, the data were split according to the three phases: pre-exposure, novel horse and post-exposure.

Data analysis

All behavioural and heart rate data collected during period 2 were averaged and used as the control values for each horse. All assessments in period 3a, 3b and 4 were made relative to the control values of phase 2 (thus per individual). These data were averaged per week (in week 1 of period 4 there were no assessments due to the high number of biochemical and histological tests after Set 3). In the majority of cases, the data were not normally distributed, so it was decided to perform a logarithmic transformation on all data. These data were the input for the used Linear Mixed Model procedure of the statistical analysis in SAS® (version 9.0; SAS Inst., Inc., Cary, NC, USA). For the analysis “group” and “period” were the main variables. Log week minus Log (3) (the number of weeks per period) and year were covariates in order to compare the result of the training at the end of each period. (Pair and individual horse within the pair were taken as random factors, taking into account the relationship of measurements from the same pair and animal, respectively.) This way the individual horse functions as the experimental unit. Thus the general model was:

$$Y_{ij} = \mu + Group_i*Period_j + (Log(week) - Log(3))(Group_i*Period_j) + Year_k + Pair_l + Horse(Pair)_{lm} + E_{ijklm}$$

Least Square Means (LSM) for group and period were calculated and sliced for the direction of difference between the results. To determine whether there was a difference between control horses and intensified trained horses within periods the analysis was “sliced” for period. To determine whether there was a difference between periods within a group the analysis was “sliced” for group. For the statistical analyses a Bonferroni correction (219) was performed comparing three phases: $p< 0.017$ was considered significant, for all other analyses $p<0.05$ was considered significant.

Results

Differences between control versus intensified trained horses within and between periods

In Table EE the distribution of observations is presented. Overall, all horses increased in bodyweight (data not shown), however some had some stagnation in weight gain during the intensified training period.

Despite professional help of the Polar Company, electromagnetic fields of the treadmill at speeds above 4.5 m/sec caused severe interference disturbances in the heart rate frequency and R-R interval measurement in the Polar 810i receiver. About 60% of the measurements on the treadmill were affected so the heart rate measurements during training cannot be presented in this paper.

Table EE. Number of sessions suitable for analyses per assessment and per group.

Test	Period	Basic	Intensified	Highly intensified	Detraining
HIT	Intensified	4-7	8-10	9-10	5-7
	Control	4-6	5-6	5-7	5-7
NHT	Intensified	2 (’04) – 6 (’05)	6	7-8	6
	Control	2 (’04) – 6 (’05)	6	7-8	6
TB	Intensified	3 (‘04) – 6 (’05)	6 - 7	7	3 - 4
	Control	3 (‘04) – 6 (’05)	5 - 7	5 – 6	3 - 4

General results of the assessments

- Measurements during training: HIT assessments

Observations of the horses during handling before exercise like entering the building with the treadmill, but also behaviour during preparation, approach of the treadmill, entering the treadmill as well as leaving the treadmill, did not show any differences between the two groups.

In the LMM model only the frequency observations during the HIT training could currently be included. Intensified trained horses showed clear individual responses in relation to the different periods. For instance some horses showed a strong increase in “tail sweeping”, “stumble frequency” or “changing to canter” frequency during the intensified and/or highly intensified training period in relation to the baseline frequency, while others did not show any increase.

- Measurements during Novel Horse Test

All horses seem to like the Novel Horse environment after the habituation sessions: after entering the test arena, they all showed either foraging behaviour or some

vigorous locomotion patterns associated with rebound effects of horses released in a field/paddock after confinement (330). Since all horses in all sessions at least came and “nose-nosed” with the Novel horse confirmed that the chosen stimulus (an unknown gelding) was an interesting one for all horses in all periods. Unfortunately, it was not always possible to distribute the Novel Horses at random. During the experiment in 2004 one of the novel horses had to be euthanized due to age-related health problems. One horse from the control group had to be excluded from the heart rate analyses due to an Atrio-Ventriculair block, since this disturbed the HRV data during the NHT too much.

The heart rate variables measured during the Novel Horse test did not give a clear picture of the relative influence of the parasympathetic or sympathetic influences on the heart rate of the tested horses.

- Measurements during time budget observations

The time budget observations of 6 hours required precise recording of possible arousing stimuli. Since the manual recording was suboptimal during 2004, in 2005 a third camera was installed which registered every movement in the stable corridor.

Differences between groups per training period

When the groups were compared to each other by means of the LMM, there were interesting differences between the groups (table FF).

Table FF. Significant differences between intensively trained and control horses in different periods of training.

Intensified (period 3a)			**F**	**P**	**D***	**Trends****
HIT		Frequency of defecation	15.76	0.0002	⇓	⇓⇓⇓⇓
NHT	Pre exposure	Duration of foraging	10.61	0.002	⇑	⇑⇑–
	Pre exposure	Duration of exploration while standing	4.32	0.04	⇓	⇓⇓⇓
TB		Duration resting	6.98	0.01	⇓	⇓⇑⇓
Highly intensified (period 3b)						
HIT		Frequency of defecation	10.82	0.002	⇓	⇓⇓⇓⇓
NHT	Pre exposure	Frequency of flehmen	6.59	0.01	⇓	⇓⇓⇓⇓
	Novel horse	Frequency of snorting	5.11	0.03	⇑	⇑⇑⇑⇑
	Post exposure	LF	4.56	0.04	⇓	⇓⇓⇓⇓
TB		Duration exploration	6.38	0.01	⇑	⇑⇑⇑⇑
Detraining (period 4)						
HIT		Frequency of Defecation	16.29	0.0001	⇓	⇓⇓⇓⇓
		Frequency of Snorting	7.78	0.007	⇑	⇑⇑⇑⇑
NHT	Pre exposure	Frequency of snorting	4.69	0.04	⇑	⇑⇑⇑⇑
	Pre exposure	Frequency of flehmen	7.02	0.01	⇓	⇓⇓⇓⇓
	Novel horse	Duration of play with Novel horse	10.39	0.002	⇓	⇓⇓⇓⇓

*D = direction of difference of the intensified trained horses compared to the control horses
**Trends = the direction of difference of the intensified trained horses compared to the control horses in the other training periods. In bold and slightly larger are the significant differences. The first arrow represents period 3a, the second period 3b and the third period 4.
P = significant value.
F = Wald's F statistic

- Group differences during the intensified training

During period 3a, the intensified trained horses rested significantly less in their stable compared to the control horses (LSM (log of relative duration) C-highly intensified period = -0.14; IT-highly intensified period = 0.04). They might have been hungry and searching for food since in the pre-exposure phase of the NHT, the intensified trained horses significantly foraged more (LSM (log of relative duration foraging) C-intensified = 1.37 vs. IT- intensified = 1.69). Maybe as a consequence, they explored less the arena than the control horses: LSM (log of relative duration exploration arena while standing) C-intensified = 1.31 vs. IT-intensified = 1.13.

There was a significant difference between the intensified trained horses and the control horses in defecating in each training period (table FF). In each training period the intensified trained horses defecated less than the control horses: (LSM (log of relative defecation frequency) C-intensified = 0.41 vs. IT-intensified = -0.21; C-highly intensified = 0.48 vs. IT-highly intensified = -0.035; C-detraining = 0.52 vs. IT-detraining = -0.13).

- Group differences during the highly intensified training

It seemed that the intensified trained horses lost more interest in the arena: there was a non-significant tendency to forage more and explore less after entering the arena compared to the control horses. In addition, the intensified trained horses performed flehmen significantly less during the pre-exposure phase of the NHT assessment compared to the control horses. LSM (log of relative flehmen frequency): C-highly intensified = -0.219 vs. IT- highly intensified = 0.02. (During flehmen the horses actively guide air over their vomero-nasal organs to have a better assessment of strange smells [28], [13]). During this period, there was a significant higher exploration of the home stable: LSM (log of relative duration exploring box) C-intensified = 0.025 vs. IT-intensified = -0.18.

The frequency of snorting, usually either a symptom of arousal or just nasal discharge [28], was significantly higher when the novel horse was present (LSM (log of relative snorting frequency) C-highly intensified = -0.03 vs. IT-highly intensified = 0.20). The only significantly changed Heart Rate Variation parameter was the LF. During the post exposure part of the NHT the LH was significantly lower for the intensified trained horses: LSM (log of relative LF) C-highly intensified = 0.09 vs. IT-highly intensified = -0.22.

- Group differences during the detraining

Some parameters showed the same deviation as during the highly intensified training period: frequency of flehmen stayed lower for the intensified trained horses LSM (log of relative flehmen frequency): C-detraining = -0.218 vs. IT-detraining = 0.02. Snorting

stayed higher (LSM (log of relative snorting frequency(nht)) C-detraining = 0.03 vs. IT-detraining = 0.31), this time during the pre-exposure. During the HIT there was also a significant increase in snorting frequency: (LSM (log of relative snorting frequency(hit)) C-detraining = -0.55 vs. IT-detraining = 0.01).

The relative duration of play with the Novel Horse, had a tendency to be shorter for the intensified trained horses during all periods, but it became apparent during the detraining period: (LSM (log of relative duration play) C-detraining = 1.19 vs. IT-detraining = 0.65). The control horses increased their play time (HI / 0.77 to DT / 1.19) during the detraining, while the intensified trained horses spent the same time playing (HI / 0.69 to DT / 0.64).

Training effect within groups

During the HIT and the TB assessment there was hardly any significant difference between the periods. The results are shown in Table GG.

Table GG. Differences between the training periods within a group (only the significant items are shown).

Comparison*	Group	Assessment	Phase	Parameter	Estimate^	t	P
I vs. HI	C	NHT	NH	Duration Walk to NH	0.175	2.78	0.007
	C	NHT	NH	Frequency Mean HR	0.07	3.02	0.004
	C	NHT	NH	Mean RR Interval	-0.04	-2.94	0.005
	IT	NHT	Post	# transitions	0.16	2.53	0.013
HI vs. DT	C	NHT	Pre	Duration Stand explore	0.32	2.66	0.009
	C	NHT	NH	Duration Nose to nose	0.27	2.99	0.004
	IT	HIT		Frequency Canter	0.33	2.53	0.013
I vs. DT	C	NHT	Pre	Duration Forage	-0.219	-2.7	0.008
	C	NHT	NH	Duration Walk to NH	0.62	2.58	0.012
	C	NHT	NH	Duration Play	-0.54	-3.21	0.002
	C	NHT	NH	Frequency Leave NH	0.148	2.74	0.008
	C	NHT	NH	Frequency Mean HR	0.07	2.95	0.004
	C	NHT	NH	Mean RR Interval	-0.04	-2.96	0.004
	IT	NHT	Pre	Duration Walk explore	-0.18	-2.59	0.012

^indicates difference between the LSM's of the indicated training phases
NB the estimate is contra indicative: when the estimate is positive the second item of the equation is smaller/lower compared to the first item.
*I = intensified training; HI = highly intensified training; DT = detraining
pre = pre exposure; NH = novel horse; post = post exposure
T = T LMM test statistic
P = the significant values

- Training effect between intensified training and highly intensified training
The control horses seemed to become more interested in the Novel Horses when comparing the highly intensified training period with the intensified training period (where the training regime did not change for this group). They take significant shorter time to walk to the NH, tend to have shorter nose-nose (greeting [28]) contact and tend to leave the NH less frequently. This presumed increased contact seemed to have positive physiological feedback: during the contact with the NH the control horses' mean heart rate lowered, while the mean RR interval increased.

The intensified trained horses had significantly less transitions when the novel horse left, maybe the intensified trained horses were to tired to be frustrated when the novel horse left.
During the HIT they tended to canter more often and defecated less on the treadmill.

- Training effect between highly intensified training and detraining
The control horses tend to forage more and explore less in the arena before the NH arrived in the detraining compared to the highly intensive training. When the NH arrived the control horses tended to play longer but had significantly shorter nose-nose contact.

The intensified trained horses cantered significantly less on the treadmill during the detraining compared to the highly intensified training.

- Training effect between intensified training and detraining
The trend that the control horses seemed to "know" the arena continued: when in the pre exposure phase, they predominantly foraged and flehmed less and less clearly anticipated on the novel horse. But when the novel horse was there, they walked faster to the NH, played longer and left them less often. Also there was a positive physiological feedback with a significant lower heart rate as well as a higher mean RR interval.

For the intensified trained horses the detraining had only a limited effect when comparing the intensified training period with the detraining period: they explored more in the arena but less in their home stable.

One interesting general observation is worth mentioning: some horses showed a large number of different behaviours just before the door opened which allowed them interaction with the Novel Horse. So, several horses anticipated strongly in a positive way (ears pointed forwards, focus on door, within 1 horse length of the door, directly nose to nose to the Novel Horse etc) the last minute before the test horse was exposed to the Novel Horse.

Discussion

This is the first systematic study in relation to equine overreaching/ overtraining in which the behaviour of the horses was systematically analysed by means of three different assessments. The main finding was that it is possible to assess the mental aspects of the horses and to distinguish intensively trained horses from normal trained control horses on several aspects representing meaningful parameters. Also significant training effects within both groups could be detected. Based on all available data in the experiment, it could be concluded that the intensified trained horses were, at least, overreached. They showed a loss of performance combined with neuro-endocrine disturbances, which was not fully recovered after 4 weeks of detraining (E. de Graaf-Roelfsema, Chapter 5 & 8).

Since behavioural data usually are more variable and more prone to external influences, it is of utmost importance that the assessments are carried out in a concise and undisturbed way (187). This is especially true when working with low numbers of animals. In the current study, unfortunately, several external factors (for instance the fact we had to use two NHT test arena's one in each year) caused an unknown amount of extra variation, possibly leading to suboptimal results. Nevertheless, several interesting findings can be reported. The three developed assessment tests seem to measure relevant items. The structural standardized protocol which was used to observe before, during and after the actual High Intensity Training (HIT) gave information on the (un)willingness of the horse to perform. Some items (snorting, defecating) analysed can possibly be related to "tension" as in a human POMS.

The information gathered from the 6-hour Time Budget hours gave information on the resting and feeding behaviour, but also could give potential information on reactivity (tension – reactivity - fatigue). The information from the specially designed Novel Horse test provided data on tension - anxiety, but also on anticipation towards an announced positive stimulus, foraging behaviour and general reactivity. This assessment seemed to work well since all horses during all tests went to the Novel horse at least once. It is debatable whether the use of a horse as stimulus does influence the behaviour of the test horse. Influences cannot be excluded, but by proper selection of the novel horses (geldings), good organisation and experimental set up and using the same novel horse for all tests confounding factors can be minimized. Hence, in the future it is planned to assess the influence of the individual NH's on the results of this study.

Defecating was in all training phases significantly lower for the intensified trained horses compared to the control horses. The reason is not completely clear, but it might be suggested that a higher energy demand during the intensified training period might improve absorption of food by the gut, decreasing the amount of faeces. In addition, the intensified trained horses also foraged more during the NHT assessments in the

intensive training period. It could be novelty to be able to forage, since in 2004 the horses were housed on wood shavings only while in 2005 they received a limited amount of straw over their bedding. Based on 24 hour observations of 2 horses in 2004 it was concluded that they were foraging on their bedding between 19 - 20% of their time (additional to their normal feeding behaviour). Therefore, it was decided to give all horses in 2005 one sheet of fresh straw each day around midday in order to increase their welfare by providing material to forage and preventing intake of bedding. In almost all cases the horses ate all the straw before the observations started. However, if novelty was the reason for more foraging, the control horses would have foraged more also. Alternatively, the intensified trained horses needed more energy for the intensified training and were therefore hungry leading to more intense foraging, since they could not do so in their stable: in 2004 they did not get any additional straw, while in 2005 all the horses had always finished their additional straw before the NHT assessment took place. This can be confirmed by the tendency that they spent less time eating silage (eating faster?) in their stable during the highly intensified training compared to the intensified training ((estimate = 0.091; t = 2.23; p = 0.03)

In contrast, since there was no difference in foraging time (per kg) nor in time consuming concentrates (per kg) between the groups, hardly ever any food was left before the next feeding session and most horses increased weight (325) the energy intake of the horses seemed sufficient. In most studies related to equine overreaching/overtraining one of the main symptoms was that the animals lost weight (33, 127, 319). This was not the case in this study. A difference can be that the amount of concentrates was adapted to the amount of work during the study.

A clear training effect was that the intensified trained horses cantered more in the highly intensified training period. Some intensified trained horses seemed to have difficulties to keep balanced during the trot and therefore changed to the less energy-demanding canter. Alternatively, they discovered that starting to canter lowered their total amount of work. Since during detraining the frequency of cantering significantly decreased (Table GG) and there was less cantering during SET 4 (E. de Graaf-Roelfsema, personal communication), they indeed had to work very hard (both mentally and physically) during the highly intensified training period. So it is very plausible, it was a combination of both.

A training effect was found pre-dominantly within the control animals. This was expected for the comparison with the detraining data, but the training also had an effect when comparing the highly intensified period with the intensified period. Possibly these relative young horses were still improving their training skills. On the other hand it can also mean that detraining time for the intensified trained horses was too short to measure a real difference as is suggested in an other study by Golland et al (115).

Overall, one could say that during the highly intensive training period there is an increasing internal conflict (as shown by longer exploration in own stable, but less exploration in NHT test area, more cantering during the HIT) combined with less attention to the environment (shown by less exploration in NHT test area, less flehmen, less play with novel horse). Also the intensified trained horses performed flehmen significantly less during the pre exposure of the NHT assessment compared to the control horses. The fact that the control horses performed flehmen at the same point in training must have meant that there must have been interesting odours. During the HI period there was still a significant higher exploration of the home stable: although it is unlikely they were hungry, it could indicate a general frustration. One of the intensified trained horses had a tendency to start to crib, which could be an indication of frustration as well (207). This frustration cannot be seen apart from the fact that the horses were housed in a relative impoverished environment (almost no physical social contact, limited free locomotion, limited control over feeding and foraging needs). This was partly done to simulate some kind of equine equivalent of the human social and environmental stressors (33, 180, 265). Apparently, indeed it seemed to work to induce more conflicting behaviours in the intensively trained animals.

Whether these measured changes can be attributed to overreaching/overtraining has to be studied further in combination with the other physiological data. In this study the Heart Rate data did not give very good results and needs reworking. One of the reasons could be the lower basal HR of horses. Therefore, a different HF and LF range would be more suitable (now 0.04-0.15 Hz and 0.15-0.4 Hz, respectively), some authors use for this reason HF of 0.01-0.15 Hz, and LF of 0.15-0.5 Hz (266).

When looking at the training effects there seemed to be a correlation (within the control horses) between increasing interaction with the novel horse and lower heart rates that points to relaxation. Presumed increased contact seemed to have positive physiological feedback. During contact with the NH, the control horses mean heart rate lowered, while the mean RR interval increased indicating that the sympathetic influence diminished. The horses “enjoy” increasingly better the possibility to interact with the NH and are more relaxed. This is in line with the relaxing capabilities of allogrooming, which those horses experience with most Novel Horses (92, 205).

It is clear that distinct behaviours like defecation, flehmen, resting, and exploring can be used as indicators of certain mental states of groups of animals. On the other hand, several behaviours represented an individual reaction on the intensive training, or meeting with a novel horse. This type of data were rather scattered throughout the data set. It could be advised to perform matching of the horses for couples on a mental basis in order to minimize variation. One possible option would be to perform a “horsonality” test before pairing the animals (347, 348, 350).

Another interesting finding was that all horses anticipated positively, but individually very characteristic, towards the knock on the door announcing the novel horse. Some horses showed very frisky behaviour already from the moment they heard the “hoof steps” in the corridor, others stayed close to the door. This could be used to assess the animal’s mood by adapting the NHT slightly. Or a separate anticipation test could be developed to measure reward sensitivity (302, 326). Theoretically, strong anticipating horses will show more reward sensitivity towards meeting the Novel Horse, than less anticipating horses. However, as Spruijt et al (302) suggested, alternatively it can also mean that strongly anticipating horses assess their housing and managing conditions as more critical to fulfil their behavioural needs, compared to the other horses. A start can be made by analysing the anticipation behaviour of the animals in this study in more detail. After a period of detraining, not all elements had returned to baseline values, which indicated that the intensified trained horses were not fully recovered yet.

Other future plans are to validate and test the sensitivity of the outcome of these results, to analyse in more detail, which elements can contribute to an equine POMS and to combine these data with the other physiological data of this study.

Conclusion

The two groups could be distinguished on basis of behavioural data collected standardised and systematically and the basis of the development of an equine POMS is laid with this study. Are there indications found for the existence of mental stress or “psychological overload” in the intensified trained horses in the current study? There is a strong suggestion, because possible indicators of internal conflict were found and the low environmental responsiveness is well comparable to changes in mood states associated with overtraining in humans.

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Chapter 11

General Discussion

Endocrinological and behavioural aspects of exercise stress

The aim of this thesis was to study the influence of exercise stress on behaviour and hormonal status in horses in order to unravel some pathophysiological mechanisms of poor performance. The ultimate goal of the present study was to induce a first stage of the overtraining syndrome (OTS). The difficulty of the study was that the conclusion whether inducing overtraining took place or not could only be drawn afterwards. In this chapter, the main findings of this thesis will be discussed followed by an answer to the question whether overtraining succeeded or not. At the end the experimental design, practical applications of the results for common practice and the possibility of the horse, as a model for human beings, will be briefly discussed.

Glucose metabolism and exercise stress

The amount of carbohydrates and fat available for muscle critically determine how much work a horse can do, whereby carbohydrates are the main energy substrate for muscle during anaerobic workloads. During acute intensive exercise catecholamine levels increase, thereby inhibiting insulin release directly followed by increasing blood glucose levels after an initial drop. Hepatic glucose output increases, facilitating the working muscle with carbohydrates via an insulin independent mechanism (108, 264). Directly after exercise, catecholamine levels drop and insulin concentrations rise rapidly facilitating the recovering muscle to restore glycogen reserves via an insulin dependent pathway. The standardized exercise test (SET) used during the present study was performed at partly anaerobic energy expenditure, as shown by the increasing (heparinized) blood lactate levels measured during the SET and the decreased glycogen content in vastus lateralis and pectoralis descendens muscle of the horses (325).

Training induced higher blood glucose levels during acute exercise and a less pronounced rebound hyperinsulinaemia compared to the untrained situation. Higher blood glucose levels can be caused by increased hepatic secretion and/or a decrease in the clearance/use by peripheral tissues. The latter can be caused by decreased dependency on glucose metabolism of skeletal muscle (for example through an increased fatty acid oxidation rate) or decreased insulin sensitivity. It can be hypothesized, that the training employed in the present study had increased lipolysis and consequently fatty acid (FA) oxidation. Increased circulating FA levels have shown to inhibit muscular glucose uptake in exercising men (128). Decrements in insulin sensitivity can be excluded, because the euglycaemic hyperinsulinaemic clamp (EHC) did not reveal decreased glucose metabolism (Ch. 8). In addition, it has been shown

that there is no need for insulin to increase glucose uptake in contracting muscles (264). Altogether, this makes it plausible, that the liver was able to increase the glucose secretion, which spares the amount of muscle glycogen used during exercise. Indeed, the glycogen content in the vastus lateralis muscle was not decreased after acute exercise after the training period of 18 weeks (325). Although blood glucose levels rose to higher levels than in the untrained situation, a less pronounced hyperinsulinaemic response was found directly after exercise in the trained situation. Therefore, it can be concluded that the rise in insulin after exercise is not only related to the amount of blood glucose. A reduction in plasma catecholamine levels as a consequence of aerobic training has been described for horses and might explain the reduced hyperinsulinaemic response post exercise (15).

After a period of intensive training, a trend was visible for lower plasma glucose levels during the SET and a more pronounced hyperinsulinaemic response after exercise, resembling the results found during the exercise test performed by the untrained horses. Decreased plasma glucose levels might be caused by decreased hepatic glucose output due to altered decreased catecholamine release and/or increased clearance peripherally. Sympathetic withdrawal (resulting in parasympathetic dominance) in combination with hypoglycaemia has been described for athletes suffering the overtraining syndrome. However, the exaggerated hyperinsulinaemia post exercise fits more to an increased stress response and therefore sympathetic dominance. Decreased hepatic output could point towards glycogen depletion, but muscle biopsies of these horses did not show any glycogen depletion (325) and therefore decreased hepatic output would be less a likely cause. On the other hand, the EHC results showed an increase in glucose metabolism and insulin sensitivity during intensified training as compared with detraining. However in agreement with the previous findings, there was dissociation between glucose metabolism and insulin sensitivity. The question rises whether the latter phenomenon could be the result of increased catecholamine levels as well. From literature it can be concluded that epinephrine seems to have two opposing effects on glucose metabolism in skeletal muscle: by increasing delivery, glucose uptake is facilitated, and by impairing cellular uptake itself, net glucose extraction from the circulation may be diminished (70, 168, 220, 284). Nevertheless, studies on mental stress and insulin sensitivity using the EHC found improvement of glucose metabolism under the influence of hyperinsulinaemia directly after stress tests during the EHC implicating that mental stress improves insulin sensitivity (220, 284).

Might our horses have been mentally stressed as well? Although exercise was used as the stressor, adaptations in muscles were not apparent for the intensified trained group compared to the control group. In contrast, mental aspects like behaviour and hormone levels were indeed significantly changed for the intensified trained group.

This could mean that stress-related disorders, including a state of overreaching or overtraining, originate in the brain and precede alterations in peripheral organs like muscles. Or it could mean that not the training load per se, but the stress of daily exercise induced more mental stress than physical stress. This agrees with the results of the electromyography (EMG) and muscle biopsy studies indicating that the intensified training protocol did not increase stress levels above the maximal threshold for skeletal muscle (325, 329); except for the difference in expression of seven proteins in muscle of intensified trained horses compared to control horses as assessed by proteomic analyses which might indicate that other proteins or signal transduction pathways are involved (329). Furthermore, it cannot be excluded that another stress hormone might play a role in improving insulin sensitivity. From the results reported in chapter 6, it turned out that administration of a single dose of cortisol increased glucose metabolism and enhanced peripheral insulin sensitivity. The mechanism for this unexpected finding is that endogenous cortisol is able to increase plasma glucose levels by inducing a state of hepatic insulin resistance leading to increased endogenous hepatic gluconeogenesis. Combining this with increased delivery to peripheral tissues of glucose due to catecholamines this might optimize glucose metabolism during acute exercise. Further research is needed to support this hypothesis regarding the possible anabolic function of cortisol during exercise.

At the end of detraining, glucose metabolism and insulin sensitivity were back to values comparable to the control group, but their correlation was still lost. Apparently, the horses were not fully recovered yet.

Growth hormone and exercise stress

Growth hormone (GH) administration can induce insulin resistance as was shown in chapter 4. However, some recent studies reported an improvement of insulin sensitivity after long-term GH treatment at a low dose mainly mediated via increased levels of free IGF-1 in man (367, 368). The insulin antagonistic effects of GH seem to be a dose-dependent phenomenon and a positive direct relation between changes in free IGF-1 levels and glucose metabolism (revealed from EHC studies) was demonstrated. The increase in GH pulse frequency found in the intensified trained horses did not (simultaneously) increase plasma IGF-1 concentrations. However, an effect of the changes in GH pulsatility on insulin sensitivity cannot be excluded. Interestingly, the prolonged effect of long-term GH administration on insulin sensitivity found in Chapter 5 has also been described after long-term GH administration at a low dose in man (367). The prolongation of the GH effect is probably not dose-dependent. Normalization of the GH pulsatility parameters will therefore not exclude an ongoing effect of the altered

pulsatility. It may only indicate that the trigger for induction of the change in GH pulsatility pattern has disappeared.

The horses were growing during the experiment as shown by the significant increase in bodyweight and height at the withers (Chapter 2). GH is the main regulator of linear growth (123). It is therefore not surprising that the young horses secreted more GH by increasing their pulse amplitudes compared to the “older” horses as indicated in Ch. 5. Extrapolation of the basic GH profile data to older horses seems therefore not directly appropriate. However, the changes in GH pulsatility found after intensified training are expected to be universal, because horses were their own control, the control couple partner was age-matched and in addition, the period including the 3 GH profiles only took 10 weeks of the total 32 experimental weeks.

The variability of GH half-life is enormous in literature as well as in this thesis. In the GH studies described in chapter 4 and 5, endogenous half-life ranged from 1.2 to 24.8 minutes. It can be concluded that GH half-life is variable within and between horses and varies under different circumstances. Only GH half-lifes calculated under the same specified circumstances in the same species could be worth comparing. However, an indication for a range can be derived from this thesis for equine GH half-life, as well as some factors influencing GH half-life providing a sound basis for future studies.

Behaviour and exercise stress

Changes in mood state are considered a useful indicator of overreaching/overtraining in man. Objective markers to indicate psychological disturbances in horses are not readily available and therefore developed or adapted for the current experiment. A Profile of Mood State (POMS) for horses would be ideal and an attempt is made in this thesis to acquire background information about the horses' behaviourally responses to chronic stress, in order to provide information for a POMS for horse owners. Like in humans, the individual variation in behavioural adaptations to the intensified training was enormous, but all five intensified trained horses showed obvious changes in their behaviour c.q. mood state. Due to the individual variation, translating the results found in this study to the individual horse was difficult. The most important finding upon intensified training was a decrease in the horse’s interaction with its (every time new) social and physical environment: less exploration in NH test arena, less playful interactions with the novel horse (NH), less flehmen, and less transitions as the NH leaves. These behavioural changes were still apparent at the end of the recovery phase as compared to the control group. The control group showed symptoms of habituation to the test area: the novel horse is still interesting and they go to it faster, play longer,

leave less often, but the test area itself is less exciting during the recovery phase compared to the intensified period. It appeared that the standardized test was the most useful for comparison of all horses. The observations of normal behaviour in the stable and during the training showed clear individual changes, but no group effects in the analysed data up till now. In conclusion, for experimental behavioural studies it seems that response-to-test situations arouse more equal responses than observation of behaviour under normal circumstances. For studying individual horses, observation of behaviour under normal circumstances provides more than enough information to decide whether mood disturbances play a role.

What can be learned from the experimental design of the present study? From theoretical ideas to a practical solution.

One of the major prerequisites for the selection of the experimental animals was that they had not performed any training before the start of the experiment in order to exclude any chance on prior overtraining problems beforehand. Standardbreds start their training at the age of 1.5-2 years, so it is difficult to find untrained Standardbred horses older than 2 years of age. Hence, the horses used in the current study were very young, which gave some problems. The basic endurance training of four weeks did not prepare the horses well enough for the incremental exercise test needed for determination of the individual maximal heart rate. The horses could not cope with the speeds necessary for reaching the maximal heart rate. Therefore, the second incremental exercise test combined with values from literature served as a basis. The initial training schedule was made for adult horses and although it was changed for the younger horses in advance, it still appeared to give the horses a hard time and needed to be changed during the experiment. For this reason, the adaptation period differed for the groups between year 1 and year 2.

The choice for Standardbreds was confounded on the knowledge that this breed is easy to manage on a high-speed treadmill. However, they were allowed only to trot during the experiment. In general, when a horse is allowed to choose gaits, the horse has a tendency to voluntarily utilize the gait, which is energetically the most efficient at a given speed. Net energy cost varies minimally over a great variety of speeds, because as speed increases horses change gait to an energetically more sufficient one. When the horse is forced to maintain a certain gait for an extended period of time and at speeds that are not energetically optimal for the gait in question performance may be altered (251). For instance, a Hackney trotting at speeds of 8.5 m/s on a treadmill had an increase of 107% over the minimum cost. Although the Standardbreds are bred to trot on higher speeds, they cannot do it more economically than other

breeds (360). Some intensified trained horses changed their gait during the intensified training period probably because they could not cope with the high-energy demands of the energetically inefficient gait at the given speed or due to loss of coordination because of central fatigue. As shown by Rietjens et al (266) in human athletes, central fatigue is the earliest manifestation of overreaching and precedes peripheral fatigue.

The detraining period was included in the experiment for two reasons. Firstly, some overtrained human athletes show severe symptoms of the OTS after a period of rest (Keizer, personal communication). Secondly, because recovery from overreaching occurs normally within 2-3 weeks of rest and recovery of the OTS takes much longer, a detraining period of 4 weeks was estimated to be long enough to distinguish between overreaching and overtraining. Reviewing the experimental setup of the current study retrospectively, a third reason can be added. For the endocrinological studies obvious trends were visible between the control and the intensified trained group at period 3. However, the significant differences occurred in period 4 after a period of relative rest, underlining the importance of the trends seen in period 3. Without the data of period 4, these trends would not have been noticed.

At the end of a scientific experiment the question raises whether it revealed tools for common practice. Performing a euglycaemic hyperinsulinaemic clamp or collecting nocturnal GH samples is not a practical tool for diagnosing overtraining in practice. However, decreases in performance as well as changes in behaviour can be objectively determined by an owner provided that regular notes are written down about, for instance, performance improvement. Because there is a large individual variation in behavioural responses, it is very important to gather basic knowledge of the normal behaviour of ones own horse. Regular observation and recording of the behaviour of the horse could help to detect mood disturbances in an early stage. Observations should take place on predetermined fixed times, for instance, during the training, around feeding time, after the training in the field or in the stable in order to make comparisons possible. Changes in normal patterns of longer duration could indicate mood disturbances. Keeping records of the horse's behaviour is essential since the changes in mood state occur gradually and are therefore easily missed in a day-to-day meeting situation. In Figure 1, a first attempt is made to categorize behavioural changes, which could indicate mood disturbances.

Overtraining due to exercise stress?

The ultimate goal of the experiment was to induce early overtraining. Since there are no distinctive parameters for diagnosing overtraining in the literature, it was difficult to decide which training load was heavy enough to induce chronic exercise stress on the

one hand and preventing injury on the other hand. Retrospectively, not the training load appeared to be the most important, but the crucial factor was deletion of the resting days. In addition, extra stress was added to the experiment by making the training unpredictable.

Figure 1. Behavioural parameters possibly indicating mood disturbances

Observation during training	**Observation in stable before and after training**	**Observation entering field/paddock and other horses**
		General after release:
- entering riding arena and tacking up easy or not	- time resting	- foraging time
- willingness to perform (cooperation in entering treadmill)	- standing alert	- time spent standing alert
- defecating frequency	- walking around	- duration of friskiness
- freq. change to canter	- abnormal behaviour / onset stereotypy	- frequency of defecating
- stumble frequency	- time eating hay	- (over) urinating
- snorting frequency	- time eating concentrates	- Frequency startle reaction
- frequency vigorous tail sweeping	- frequency eating faeces	
- ear play (fixed or alert to environment)	- time foraging / eating uneatable bedding	Reaction changes towards arena with new (olfactory) stimuli:
- play with bit	- anticipation towards other horses/individuals	- time exploration arena
- body tension, during and after training	- anticipation on food	- frequency defecating
- jaw tension during training		- frequency flehmen
- pulling rope		- frequency
		- neigh
		- frequency urinating
		Reaction in relation with a horse:
		- frequency stand alert
		- Anticipation towards / on CS* to announcement NH presence
		- Latency time to walk to NH
		- total time spent with or close to NH
		- frequency nose contact
		- duration play with NH
		- frequency of rearing
		- frequency walk away from NH
		- duration relaxation penis

In non Italic: analysed parameters
In Italic: not yet fully analysed but initial analyses show potentially promising parameter
*CS = conditioned stimulus

The mental and physical stress implied by this approach seemed to be enough to induce symptoms associated with overreaching/early overtraining. Under these

circumstances the horse could serve as a model for humans for studying overtraining or possibly other stress-related pathophysiologic mechanisms since patterns of inducing overtraining seemed to be similar to humans.

Overtraining occurs as an accumulation of training and/or non-training stress results in long-term decrement in performance capacity with related physiological and/or psychological signs and symptoms of overtraining. Restoration of performance capacity may take at least several weeks or months (125). The period of rest needed for recovery is crucial to distinguish between overtraining and overreaching. However, the exact time span needed for recovery of overreaching or overtraining is unknown. Periods reported for overreaching are a couple of days to a couple of weeks (2,3,6) and for overtraining a couple of weeks to a couple of months or even years (125, 179, 266). In the current study, the intensified-trained horses showed obvious signs of loss of performance combined with symptoms of psychological nature (behavioural changes), as well as pathophysiological nature (endocrinological changes), which did not fully disappear after four weeks of detraining. Therefore, it can be concluded that the horses were at least long-term overreached or early overtrained, depending on the definition used, and that the experimental set up used was able to induce early overtraining in young Standardbred horses. In addition, since no alterations were found on musculair level, it can be concluded that the applied stress involved alterations in the central neuro-endocrine system preceding the peripheral systems indicating that physical stress starts with alterations in the central neuro-endocrine system or that the horses endured more mental stress than physical stress. Future research with reference to pathophysiological mechanisms underlying stress-related disorders should aim at alterations at central neuro-endocrine levels.

Conclusion

In conclusion, the results of the current experiment show that the used training protocol for inducing overtraining in horses was valid. Overtraining, like in human athletes, is associated with a neuro-endocrine imbalance and behavioural symptoms of internal conflict in Standardbreds. For the first time, deregulation of insulin related glucose metabolism as well as impaired coordinate control of GH secretion could be related to pathophysiological mechanisms underlying overtraining.

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Nederlandse samenvatting

Stressgerelateerde ziekten, zoals burn-out, posttraumatische stress-stoornis (PTSS) en overtraining syndroom, komen steeds meer voor of worden vaker onderkend bij de mens. Bij het paard bestaat de indruk dat stressgerelateerde ziekten meer voorkomen dan nu gediagnosticeerd worden. Het overtraining syndroom wordt vaak alleen geassocieerd met paarden in de topsport. Echter, ook andere paarden kunnen dagelijks stress ondervinden door de manier waarop ze gehouden worden of door de manier van training. Deze en andere stressfactoren kunnen leiden tot een overmaat aan stress wat uiteindelijk kan resulteren in ziekten zoals het overtraining syndroom of een burn-out.

Stress wordt vaak geassocieerd met negatieve gevolgen, maar vanuit een biologisch oogpunt is stress bedoeld als een positieve reactie. Tijdens stress worden de zintuigen op scherp gezet, waardoor men beter kan presteren. Een beetje stress is dan ook nodig voor een optimale prestatie of het verbeteren van een prestatie. Van dit principe (het “overload” principe) wordt gebruik gemaakt voor het verbeteren van sportieve prestaties. Tijdens een training wordt het lichaam belast (d.w.z. het ondervindt stress), energiebronnen worden aangesproken en de balans van het lichaam wordt als het ware verstoord. Direct na de training zal het lichaam proberen de balans te herstellen. Het doet dit door zelfs iets meer te herstellen dan de uitgangspositie, zodat een zelfde mate van training in het vervolg het lichaam minder uit balans brengt. Dit herstel noemen we de supercompensatie. Indien de daaropvolgende trainingen plaatsvinden op momenten dat de supercompensatie optimaal is, zal het lichaam steeds beter bestand zijn tegen de trainingen en zal er vooruitgang in prestatie geboekt worden. Indien begonnen wordt met trainen voordat het lichaam hersteld is, zal de prestatie na verloop van tijd verminderen.

Zowel tijdens de training als tijdens het herstel na de training zijn stresshormonen nodig. Langdurige activiteit van stresshormonen leidt echter tot de symptomen van stressgerelateerde ziekten, zoals verminderde prestaties, vermoeidheid, en depressiviteit. Overtraining wordt gedefinieerd als een vermindering van de prestatie ondanks gelijk gebleven of toegenomen training. Er zijn meer dan 200 symptomen beschreven voor overtraining, maar geen enkel symptoom is specifiek voor het ziektebeeld. Dit betekent dat overtraining (of een burn-out) nog steeds niet met zekerheid vastgesteld kan worden in een vroeg stadium. Bij de mens wordt een

enquête gebruikt om de diagnose overtraining te stellen bij zieke atleten. Deze enquête (POMS; Profile of Mood State) brengt veranderingen in gedrag en gemoedstoestand in kaart, die tot nu toe de meest betrouwbare indicatoren voor overtraining lijken te zijn. Helaas is deze methode bij het paard niet toepasbaar en blijft het voor de mens een enigszins subjectieve bepalingsmethode. Er is dan ook dringend behoefte aan een diagnostische parameter om stressgerelateerde ziekten in een vroeg stadium te kunnen vast stellen. Het herstel in een vroeg stadium is vaak goed mogelijk in tegenstelling tot langdurig bestaande stressgerelateerde ziekten, waarvan het maar de vraag is of herstel mogelijk is.

Als oorzaak voor de stressgerelateerde ziekten wordt gedacht aan een neuro-endocriene onbalans, d.w.z. dat de regulatie van hormonen in de hersenen verstoord geraakt is. Er bestaan aanwijzingen dat de verstoring van de regulatie van de stresshormonen kan overgaan in een uitputting van deze stresshormonen onder invloed van zeer langdurige continue stress met desastreuze gevolgen. Onderzoek naar de verstoorde regulatie van deze hormonen zou misschien een diagnostische parameter kunnen opleveren om overtraining in een vroeg stadium te kunnen aantonen.

Het onderzoek in dit proefschrift is gericht op het bestuderen van de regulatie van groeihormoon en de suikerstofwisseling tijdens training en overtraining bij paarden. Voor dit onderzoek werden de paarden getraind volgens een speciaal protocol, ontworpen voor het induceren van vroegtijdige overtraining. Om een indruk te krijgen of de paarden leden aan vroegtijdige overtraining werden op gezette tijden inspanningstesten en gedragsobservaties tijdens training, op stal en tijdens contact met een ander paard uitgevoerd (**Hoofdstuk 1**).

In **hoofdstuk 2** wordt een uitgebreide beschrijving van het trainingsprotocol gegeven evenals een beschrijving van alle testen die uitgevoerd werden in het kader van dit proefschrift, maar ook in het kader van andere proefschriften en studentenonderzoeken. Het experiment bestond uit vier perioden: een gewenningsperiode (4 weken), een trainingsperiode (18 weken), een intensieve trainingsperiode (6 weken) en een herstelperiode (4 weken). Aan het begin van de intensieve trainingsperiode werden de 10 paarden (allen dravers) eerlijk verdeeld over een controlegroep en een testgroep. Tijdens de intensieve trainingsperiode trainde de controlegroep 4 dagen per week en de zwaarte van de training was vergelijkbaar met de training gedurende de trainingsperiode. De testgroep trainde de eerste 3 weken van de intensieve trainingsperiode zes dagen per week en de laatste drie weken 7 dagen per week. De training werd steeds langer en zwaarder in deze periode. De

herstelperiode was bedoeld om te zien of eventuele veranderingen gemeten tijdens de intensieve trainingsperiode zouden kunnen herstellen binnen vier weken of niet. Bovendien was bekend dat sommige humane atleten pas symptomen van overtraining lieten zien na een korte rustperiode en niet tijdens de intensieve inspanningsperiode. Eventuele problemen zouden dus ook pas tijdens de herstelfase kunnen gaan optreden.

In dit hoofdstuk worden ook de resultaten van de inspanningstesten beschreven. De intensief getrainde dravers konden de inspanningstest aan het einde van de intensieve trainingsperiode niet volhouden in tegenstelling tot de controlegroep. Eén draver staakte daadwerkelijk, drie andere dravers konden het draven niet volhouden en gingen in galop. Na de herstelperiode waren de testdravers nog steeds niet in staat de inspanningstest goed te volbrengen, wat wijst op een verminderd presteren ondanks intensievere training welke niet herstelt na 4 weken relatieve rust.

Hoofdstuk 3 geeft een overzicht van de al bestaande literatuur over veranderingen in de regulatie van de hypofyse-bijnier-as (regelt afgifte van cortisol) en de groeihormoonas bij paarden en mensen gemeten na acute inspanning, training en (indien bekend) overtraining.

In **hoofdstuk 4** wordt beschreven hoe groeihormoonafgifte gekwantificeerd kan worden. Groeihormoon (GH) wordt geproduceerd en afgegeven door de hersenen. De afgifte van groeihormoon wordt vooral door twee hormonen bepaald. Het groeihormoon-releasing hormoon, dit hormoon activeert de afgifte van groeihormoon en somatostatine, dit hormoon remt de afgifte van groeihormoon. De afgifte van de hoeveelheid groeihormoon in het bloed kan onder invloed van deze twee hormonen sterk en snel wisselen. We noemen dit een pulsatiele afgifte. Dit heeft tot gevolg dat een eenmalige bepaling van de groeihormoonconcentratie in het bloed niet zo zinvol is. Om iets te kunnen zeggen over de groeihormoonafgifte moet regelmatig (bijv. iedere 5 tot 20 minuten) en gedurende langere tijd (bijv. 8 tot 24 uur) bloed afgenomen worden, zodat er concentratiecurves gemaakt kunnen worden. Twee mathematische computerprogramma's speciaal ontworpen voor het analyseren van hormoonprofielen worden in dit hoofdstuk beschreven en voor het eerst met succes toegepast op groeihormoonprofielen bij het paard.

In **hoofdstuk 5** wordt met dezelfde programmatuur gekeken naar de groeihormoonprofielen van de paarden na perioden van training, intensieve training en herstel. De groeihormoonafgifte veranderde duidelijk na een periode van intensieve training bij de testgroep naar een onregelmatiger patroon met meer pieken, kleinere

pieken en langere groeihormoon halfwaardetijd. Na de herstelperiode waren de veranderingen nog steeds meetbaar, zij het in mindere mate. Groeihormoon speelt vooral een rol direct na inspanning tijdens de herstelfase, dus bij het herstellen van de lichamelijke balans. Mogelijk is de toename in groeihormoonpieken voordeliger voor het herstellen van de lichamelijke balans bij de overtrainde dieren.

De **hoofdstukken 6, 7, 8** en **9** gaan over de regulatie van de suikerstofwisseling. In het lichaam wordt de opname van suiker (glucose) in de cellen voornamelijk geregeld door insuline, een hormoon geproduceerd door de alvleesklier. Tijdens inspanning wordt er veel glucose verbruikt door met name de spieren. Een deel van de benodigde glucose ligt opgeslagen in de spieren en een ander deel wordt aangemaakt door de lever en via de bloedbaan richting spieren getransporteerd. Direct na inspanning worden de voorraden in de spier weer op peil gebracht. Dit kan bij het paard na langdurige inspanning wel 72 uur in beslag nemen. Het is bekend dat de gevoeligheid van de cellen voor insuline kan verminderen (cellen worden resistenter ten aanzien van insuline) door o.a. hoge concentraties van het stresshormoon cortisol en hoge groeihormoonconcentraties. Het is ook bekend dat acute inspanning en mogelijk training juist de gevoeligheid van de cellen voor insuline verbetert. De invloed van de verschillende factoren op de insuline gevoeligheid werden eerst afzonderlijk bepaald (**Hoofdstuk 6 en 7**). Samengevat hadden een training van 18 weken en een eenmalige toediening van groeihormoon geen effect op de insuline gevoeligheid van de perifere weefsels. Eénmalige toediening van cortisol gaf juist een toename van de insuline gevoeligheid van de perifere weefsels en langdurige toediening van groeihormoon gaf een duidelijke afname te zien.

In **hoofdstuk 8** wordt de insuline gevoeligheid van de perifere weefsels gemeten bij de paarden na een periode van training, intensieve training en herstel. De uitkomst van deze studie was moeilijk te voorspellen, omdat mogelijkerwijs alle bovengenoemde factoren tegelijkertijd de insuline gevoeligheid zouden kunnen beïnvloeden. Bij de overtrainde paarden trad er na de intensieve trainingsperiode een verandering op in de regulatie van de glucose gestimuleerde opname door insuline, wat zich uitte in een verminderde correlatie. De herstelfase laat pas duidelijk zien wat er gebeurd is: gedurende de intensieve trainingsperiode is de hoeveelheid verbruikte glucose toegenomen en daarnaast is ook de hoeveelheid verbruikte glucose per eenheid insuline toegenomen ten opzichte van de herstelperiode. Kennelijk is de verhouding tussen deze toenames niet voor alle paarden gelijk tijdens de intensieve trainingsperiode. In de herstel periode is deze correlatie nog steeds afwezig.

Hoofdstuk 9 beschrijft de verandering in glucose en insuline concentraties in het bloed tijdens de inspanningstesten. Het is belangrijk voor een optimale prestatie dat de aanvoer van glucose naar de spieren zolang mogelijk gewaarborgd blijft tijdens inspanning. Na de trainingsperiode was een duidelijke verhoging van de glucose concentratie in het bloed tijdens inspanning zichtbaar in vergelijking met de gewenningsperiode. Kennelijk heeft training een positieve invloed op de mobilisatie van energie tijdens inspanning. Bij de testgroep zien we na de intensieve trainingsperiode in vergelijking met de trainingsperiode juist lagere glucose concentraties tijdens inspanning. Het lijkt er dan ook op dat de testpaarden na de intensieve training meer moeite hebben met het mobiliseren van energie vanuit de lever. Dit kan komen door een hormonale disregulatie of door een uitputting van energievoorraden.

Hoofdstuk 10 gaat over het gedrag van de paarden. Speciaal voor deze studie zijn drie testen opgezet die het gedrag en de gemoedstoestand van de paarden in beeld moeten kunnen brengen; de humane POMS is als het ware vertaald naar paardengedragingen passend bij verschillende gemoedstoestanden. Op basis van deze testen werden er duidelijke verschillen gevonden tussen de controlepaarden en de testpaarden tijdens de intensieve trainingsperiode. De testpaarden vertoonden heel duidelijk minder interesse in hun omgeving en in ander paarden, ze waren meer in zichzelf gekeerd. Ook hier waren de mentale veranderingen nog meetbaar na de herstel periode, zij het in mindere mate.

In het laatste hoofdstuk (**Hoofdstuk 11**) wordt de samenhang van de verschillende resultaten gepresenteerd en bediscussieerd. Twee belangrijke conclusies kunnen worden getrokken.
De eerste conclusie is dat het induceren van vroegtijdige overtraining bij de paarden met het beschreven protocol gerealiseerd is. De paarden voldeden aan de criteria van overtraining zoals gesteld bij de mens. Prestatievermindering ondanks toegenomen training, verandering in gedrag/gemoedstoestand en onvoldoende herstel na vier weken relatieve rust.
De tweede conclusie is dat een overmaat aan fysieke stress (vroegtijdige overtraining) in eerste instantie leidt tot verandering op hormonaal en mentaal niveau in tegenstelling tot veranderingen op spierniveau wat men mogelijk zou verwachten omdat gebruik gemaakt is van stress door fysieke inspanning.
Toekomstig onderzoek naar de oorzaken van stressgerelateerde aandoeningen zou zich moeten richten op neuro-endocriene veranderingen. Het paard zou daarbij uitstekend kunnen dienen als modeldier voor vergelijkbaar onderzoek bij de mens.

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Gerda (500), Sara (501), Fie (502), Pronto (503), Sipke (504), Saturday (25), Sunday (26)
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Utah, Url, Ucific, U47, Utopia, Udo, Charis, Vota, Idzard, Morris, Silver, Kevin, Vincent, Victor

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Ellen de Graaf-Roelfsema, september 2007

Curriculum Vitae

Ellen Roelfsema was born on January 27, 1974 in Sleeuwijk, the Netherlands. She graduated in 1992 at the ‘Altena College’ in Sleeuwijk. In that same year she started her studies of veterinary medicine at Utrecht University and graduated ‘with honours’ in April 1999. She started working as veterinarian at several small animal veterinary practices. In February 2000 she started working at the Department of Equine Sciences, Faculty of Veterinary Medicine, Utrecht University. Two years later she started this PhD study. In September 2006 she was registered as Diplomate in Equine Internal Medicine by the Royal Dutch Veterinary Association.

Ellen Roelfsema werd op 27 januari 1974 geboren te Sleeuwijk (Nederland). In 1992 behaalde zij haar VWO diploma aan het ‘Altena College’ te Sleeuwijk. In datzelfde jaar begon zij aan de studie diergeneeskunde aan de Universiteit Utrecht en in april 1999 behaalde zij het dierenartsexamen ‘met genoegen’. Zij werkte vervolgens als dierenarts in meerdere gezelschapsdierenpraktijken. In februari 2000 trad zij in dienst bij het Departement Gezondheidszorg Paard van de Faculteit Diergeneeskunde, Universiteit Utrecht. Twee jaar later begon zij met dit promotieonderzoek. In september 2006 werd zij geregistreerd als Specialist Inwendige Ziekten van het Paard door de Koninklijke Nederlandse Maatschappij voor Diergeneeskunde.

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