The aim of this thesis was to identify metabolomic and proteomic changes in 1.5-3.5 year-old Standardbred horses subjected to two different high-intensity training programmes. Metabolomic differences between the training groups were only observed at 2 years of age, and were associated with energy production, amino acid metabolism, pH-buffering and vascular responses. Both the metabolomic and proteomic profile in Standardbred horses changed over time.

Lisa Johansson

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Metabolomic and proteomic changes in Standardbred horses in training

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Cover: Staro Gypsy King, one of the horses in the study
(photo: Johanna Berg-Johansson)

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Metabolomic and proteomic changes in Standardbred horses in training

Abstract

Metabolomic and proteomic changes in blood plasma were analysed in 16 young Standardbred horses from the age of 1.5 to 3.5 years. All horses had the same training programme from September as 1.5-year-olds until March as 2-year-olds, when high-intensity training was introduced and the horses were divided into two training groups, High and Low. Both groups followed the same training programme, but the Low group performed 30% shorter high-intensity training distances than the High group. The same speed was aimed for with both groups. In blood samples collected from age 1.5 to 3.5 years, insulin-like growth factor 1 (IGF-1) was analysed with an ELISA kit, metabolomic profile was analysed using targeted absolute quantitative mass spectrometry and proteomic profile was analysed using untargeted mass spectrometry.

There was no significant difference in IGF-1 between the training groups, but the expected ongoing IGF-1 decline was interrupted at a time which coincided with the onset of high-intensity training. Metabolomic differences between the training groups were only observed at 2 years of age, but concentrations of several metabolites changed significantly over time compared with at 1.5 years of age. Metabolites that differed significantly between the training groups and over time are associated with aerobic energy production and amino acid metabolism, and potentially also pH-buffering and vascular responses. The proteomics data did not reveal any significant differences between the training groups but the concentrations of 17 proteins related to energy metabolism, bone formation and circulatory functions changed significantly over time. In summary, both the metabolomic and proteomic profile in young horses in training changed over time, while the metabolic profile was also affected by training programme.

Keywords: Metabolomics, proteomics, IGF-1, equine, exercise, high-intensity training
Metabolomic and proteomic changes in Standardbred horses in training

Abstract

I min avhandling tittade vi på metabolomik och proteomik förändringar i blodplasma-prov från 16 travhästar från 1.5 års ålder till 3.5 års ålder. Alla hästar hade samma träningsprogram från september när de var 1.5 år till mars när de var 2 år. I mars introducerades hög-intensiv träning och hästarna delades in i två olika träningssamhällen, Hög och Låg. Båda gruppen fanns samma träningsprogram med skillnaden att träningsgruppen Låg hade 30% kortare hög-intensiv träningstid jämfört med träningsgrupp Hög. Båda gruppen var tävlades också i samma hastighet. Blodprover togs från 1,5 till 3,5 års ålder. Insulin-like growth factor 1 (IGF-1) analyserades med ett ELISA-kit. Metabolomikprofilen analyserades med en riktad absolut kvantitativ masspektrometri och proteomiken analyserades med en oriktad masspektrometri.

Inga signifikanta skillnader i IGF-1-koncentrationen hittades mellan träningsgrupperna men ett avbrott i den förväntade nedgången i IGF-1 observerades och den inträffade samtidigt som hög-intensiv träning introducerades. Skillnader i metabolomik-profilen mellan träningsgrupperna hittades bara vid 2 års ålder men skillnader över tid kunde hittas för alla ålder (2, 2.5 och 3.5) jämfört med 1.5 års ålder. Metaboliterna som skilde skillnaden för både träningsgrupper och över tid var associerade med aerob energiproduktion och aminosyrametabolismen, och kanske också med pH-buffering och kardiovaskulära förändringar. Proteomikanalysen visade inte på några signifikanta skillnader mellan träningsgrupperna men 17 proteiner skiljde sig skillnaden över tid och de var relaterade till energimetabolism, bentillväxt och cirkulationsfunktioner. Sammanfattningsvis visade studierna att både metabolomik- och proteomik-profilen förändrades över tid, men att skillnader mellan träningsgrupperna, bara kunde observeras i metabolomik-profilen.

Keywords: Metabolomik, proteomik, IGF-1, equine, träning, hög-intensiv träning
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II. Johansson, L., Ringmark, S., Bergquist, J., Skiöldebrand, E. and Jansson, A. A metabolomics perspective on two years of high-intensity training in horses. (Submitted)

III. Johansson, L., Ringmark, S., Bergquist, J., Skiöldebrand, E. and Jansson, A. A proteomics perspective on two years of high-intensity training in horses. (Manuscript)

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Abbreviations

3-IAA  Indoleacetic acid  
AA     Amino acid  
AABA   Alpha-aminobutyric acid  
AconAcid  Aconitic acid  
ADMA   Asymmetric dimethylarginine  
ALS    Acid -labile subunit  
Apo    Apolipoprotein  
ATP    Adenosine triphosphate  
AZGP1  Alpha-2-glycoprotein 1 zinc-binding  
b.Ala  Beta alanine  
BCS    Body condition score  
BLUP   Best linear unbiased prediction  
BPI    Bactericidal/permeability-increasing  
BPIFA2 BPI fold containing family A member 2  
bpm    Beats per minute  
C0     Carnitine  
C6     Hexanoylcarnitine  
CD5L   CD5 molecule like  
CE     Cholesteryl ester
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cer</td>
<td>Ceramide</td>
</tr>
<tr>
<td>CP</td>
<td>Crude protein</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of variation</td>
</tr>
<tr>
<td>DG</td>
<td>Diglyceride</td>
</tr>
<tr>
<td>DiCA</td>
<td>Dodecanedioic acid</td>
</tr>
<tr>
<td>ECM1</td>
<td>Extracellular matrix protein 1</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray ionisation</td>
</tr>
<tr>
<td>FA</td>
<td>Fatty acid</td>
</tr>
<tr>
<td>FDR</td>
<td>False discovery rate</td>
</tr>
<tr>
<td>FIA</td>
<td>Flow-injection analysis</td>
</tr>
<tr>
<td>GH</td>
<td>Growth hormone</td>
</tr>
<tr>
<td>HAD</td>
<td>3-hydroxyacyl-CoA dehydrogenases</td>
</tr>
<tr>
<td>HArg</td>
<td>Homoarginine</td>
</tr>
<tr>
<td>HexCer</td>
<td>Hexosylceramide</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin-like growth factor 1</td>
</tr>
<tr>
<td>IGFBP</td>
<td>Insulin-like growth factor binding protein</td>
</tr>
<tr>
<td>IS</td>
<td>Internal standard</td>
</tr>
<tr>
<td>JCHAIN</td>
<td>Joining chain of multimeric IgA and IgM</td>
</tr>
<tr>
<td>Leu</td>
<td>Leucine</td>
</tr>
<tr>
<td>ME</td>
<td>Metabolisable energy</td>
</tr>
<tr>
<td>Met.SO</td>
<td>Methionine-sulfoxide</td>
</tr>
<tr>
<td>MRM</td>
<td>Multiple reaction monitoring</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>MS/MS</td>
<td>Tandem mass spectrometry</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>PC</td>
<td>Phophatidylcholine</td>
</tr>
<tr>
<td>PUFA</td>
<td>Polyunsaturated fatty acids</td>
</tr>
<tr>
<td>SDMA</td>
<td>Symmetric dimethylarginine</td>
</tr>
<tr>
<td>SERPING1</td>
<td>Serpin family G member 1</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricarboxylic acid cycle</td>
</tr>
<tr>
<td>TG</td>
<td>Triglyceride</td>
</tr>
<tr>
<td>UPLC</td>
<td>Ultra-high high performance liquid chromatography</td>
</tr>
<tr>
<td>Val</td>
<td>Valine</td>
</tr>
</tbody>
</table>
1. Introduction

The horse has been an important animal for human civilisation since the Bronze Age, when it was first domesticated (Atsenova et al., 2022). Different breeds emerged over time and region to fulfil different roles as necessary, e.g. in agriculture, transportation or warfare. Interest in equestrian sports has increased since the 20th century and today the majority of all horses are kept as companion or sport horses. Equestrian sports are popular in many countries and harness racing is especially popular in the USA, France and Sweden. In Sweden, 3200 Standardbred trotters are born every year and 12,000 compete annually in harness racing. Standardbred trotters are bred to compete at a young age and up to 70% of trained horses will go on to start a preparation race at 2 years of age (Swedish Trotting Association, 2023), which means that it is common for training to start when they are 1 year old.

1.1 Training effects in sport horses

The aim of training sport horses is to increase performance capacity and to prevent injuries by gradually increasing loads so that tissues adapt. Different tissues adapt differently, one adaptation that takes place in muscle is increased activity of the enzymes involved in aerobic metabolism. For example, after only one week of high-intensity training, production of citrate synthase (an enzyme involved in the tricarboxylic acid (TCA) cycle that produces adenosine triphosphate (ATP) under aerobic conditions) increases by 27%, while after 5 weeks of training it increases by 47% (Essen-Gustavsson et al., 1989). Another enzyme which increases following the onset of training is 3-hydroxyacyl-CoA dehydrogenase (HAD) which is involved in beta-oxidation of fatty acids (Essen-Gustavsson & Lindholm,
A 19% increase in muscle buffer capacity after high-intensity training for 34 weeks has also been reported (McGowan et al., 2002).

The cardiovascular system of horses in training also adapts by increasing the capacity to transport oxygen throughout the body, through increases in red blood cells and blood volume (Evans, 1985). This is in accordance with results previously published (Ringmark et al., 2015). Haematocrit concentrations in blood were found to increase for both the high and low training groups, and recovery heart rate and resting heart rate were lower in the high training group (Ringmark et al., 2015).

An increasing training load leads to continuous adaptations by the musculoskeletal and cardiovascular systems, but if the training load is too high it can lead to injuries to the musculoskeletal system. The most common reason for interruption of training in Thoroughbred and Standardbred horses is lameness (Bailey et al., 1999; Dyson et al., 2008; Vigre et al., 2002).

1.2 Growth in sport horses

Standardbred horses start training at a young age, while they are still growing, but the growth rate of these young horses starts to plateau at 1.5 years of age (Figure 1). While the young horse is growing, its energy and protein requirements exceed those of the adult horse, but the requirements decrease to the levels in adults at around 3 years of age (National Research Council, 2007). One of the most important hormones for growth is growth hormone (GH) which is stimulated by physical activity, stress and protein-rich food.
1.2.1 Insulin-like growth factor 1

Growth hormone (GH) is produced in the anterior pituitary gland and is secreted in a pulsatile manner (de Graaf-Roelfsema et al., 2007; Yakar et al., 2018) (Figure 2). It has a half-life of only about 20 min (Faria et al., 1989). Growth hormone stimulates production of the hormone insulin-like growth factor 1 (IGF-1), which mediates most of the actions of GH (Kraemer & Ratamess, 2005). Growth hormone and IGF-1 stimulate growth in a large range of different tissues, such as cartilage, bone, skeletal muscle, fat, liver, kidney etc. (Ballesteros et al., 2000; Verwilghen et al., 2009). IGF-1 is mainly produced in the liver, but can also be produced by other tissues (Kraemer & Ratamess, 2005). On secretion into the circulation, about 75% of all IGF-1 binds to IGF-binding proteins (IGFBP) and acid-labile subunits (ALS) in a ternary complex, which increases IGF-1 half-life to ~16 hours (Yakar & Isaksson, 2016). Approximately 20% of IGF-1 is only bound to IGFBP in a binary complex and has a half-life of ~90 minutes, while around 5% of IGF-1 circulates freely, with a very short half-life of ~10 minutes (Yakar & Isaksson, 2016; Yakar et al., 2018).

Because IGF-1 has a longer half-life than GH and is more stable throughout the day, analysis of IGF-1 instead of GH could be more relevant

Figure 1. Changes over time in the growth rate of young horses. Source: (Jansson, 2013).
when monitoring long-term growth stimuli responses. A few previous studies have examined the effects of training on plasma IGF-1 levels in horses, but with conflicting results. For example, Noble et al. (2007) observed no differences in IGF-1 levels after a nine-week training programme with moderate to high training intensity, whereas a study by Jackson et al. (2003) comparing groups of young horses in light or intensive training over 20 weeks found that the group with the lightest training (only walk) had higher IGF-1 levels. However, neither of those studies controlled for nutrient intake and energy balance, which have been shown to have significant effects on plasma concentrations of IGF-1 (Salazar-Ortiz et al., 2014; Sticker et al., 1996). Age and sex are other factors known to influence IGF-1 levels. A study by Fortier et al. (2005) analysing blood samples collected from 100 thoroughbreds between 9 days and 2 years of age showed that IGF-1 concentrations are highest at the onset of puberty (about day 225) and decrease with age. Another study analysing blood samples collected on three different continents from 1880 Thoroughbreds aged 1-29 years observed a decrease in IGF-1 levels with age and higher concentrations in stallions than in mares and geldings (Noble et al., 2007).

![Figure 2. Growth hormone (GH) and insulin-like growth factor 1 (IGF-1) synthesis](image)

*Figure 2. Growth hormone (GH) and insulin-like growth factor 1 (IGF-1) synthesis*
1.3 Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) is a commonly used laboratory method for measuring the amount of a protein, e.g. IGF-1, present in a sample. ELISA works by binding the protein directly to a microtitre plate or to specific antibodies in a coating on the plate, after which an antibody specific to the protein, bound with a conjugated enzyme, is added and binds to the protein (Figure 3). A substrate for the enzyme is added and the colour change is measured, since it reflects the amount of protein present in the sample (Figure 3).

![Image of ELISA steps](image)

*Figure 3. Steps involved in measuring protein concentration in a sample by enzyme-linked immunosorbent assay (ELISA).*

1.4 Mass spectrometry

Mass spectrometry (MS) is a very useful analytical technique that is employed to identify, characterise and quantify different atoms or molecules in a sample by measuring the mass-to-charge ratio of ions (Domon & Aebersold, 2006). The most basic MS device is composed of three different components: an ion source, a mass analyser and a detector (Zhou *et al.*, 2012). The ion source ionises the molecules in the sample. The molecules are then transferred by a magnetic or electric field to the mass analyser, which separates the ions according to their mass-to-charge ratio. The detector detects the charge or current of an ion when it passes by, or hits a surface.
There are several different ion sources and types of mass analyser, all of which have advantages and disadvantages, and the best option to use depends on the sample (Domon & Aebersold, 2006). It is also quite common to couple two or more mass analysers, in tandem mass spectrometry (MS/MS). Another quite common method is MS coupled to liquid chromatography (LC), which separates the molecules in the sample prior to introduction into the ion source.

The MS approach used can be targeted or untargeted. With a targeted approach, the focus is on predetermined molecules and the sample can be prepared to optimise conditions for detection of those molecules. With an untargeted approach, the aim is to identify as many molecules as possible in a sample, although molecules that are present in low concentrations can be difficult to detect (Zhou et al., 2012).

1.4.1 Metabolomics

Metabolomics is the study of small molecules (metabolites) involved in cell metabolism as substrate, intermediates or products (Zhou et al., 2012). Metabolomics studies on the effects of exercise on humans have been performed for decades (Khoramipour et al., 2022), but are still not common in animals. Among livestock animals, the horse is the least studied with regard to metabolomic analyses (Goldansaz et al., 2017). Exercise studies in horses are even fewer (Klein et al., 2021), despite horses being commonly used in competitive sports. Of the metabolomic exercise studies done on horse only one have looked at training effects over time (Table 1).
<table>
<thead>
<tr>
<th>Year</th>
<th>Breed</th>
<th>Type of sample</th>
<th>Duration</th>
<th>No. of Animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bazzano et al.</td>
<td>2020</td>
<td>Standardbred</td>
<td>saliva, serum</td>
<td>1 exercise test</td>
</tr>
<tr>
<td>Jang et al.</td>
<td>2017</td>
<td>Thoroughbred</td>
<td>plasma, muscle, urine</td>
<td>1 exercise bout</td>
</tr>
<tr>
<td>Klein et al.</td>
<td>2020</td>
<td>Standardbred</td>
<td>muscle</td>
<td>12 week</td>
</tr>
<tr>
<td>Le Moye et al.</td>
<td>2019</td>
<td>Arabian</td>
<td>plasma</td>
<td>1 Endurance race</td>
</tr>
<tr>
<td>Le Moye et al.</td>
<td>2014</td>
<td>Arabian</td>
<td>plasma</td>
<td>1 Endurance race</td>
</tr>
<tr>
<td>Luck et al.</td>
<td>2015</td>
<td>Arabian</td>
<td>plasma</td>
<td>1 Endurance race</td>
</tr>
<tr>
<td>Mach et al.</td>
<td>2017</td>
<td>Arabian</td>
<td>plasma</td>
<td>1 Endurance race</td>
</tr>
<tr>
<td>Ohmura et al.</td>
<td>2021</td>
<td>Thoroughbred</td>
<td>muscle</td>
<td>1 exercise bout</td>
</tr>
<tr>
<td>Ueda et al.</td>
<td>2019</td>
<td>Thoroughbred</td>
<td>plasma</td>
<td>1 race</td>
</tr>
<tr>
<td>Wang et al.</td>
<td>2022</td>
<td>Yili</td>
<td>plasma</td>
<td>1 race</td>
</tr>
</tbody>
</table>
1.4.2 Proteomics

Proteomics is the study of proteins, which play a major role in maintaining the health and function of all living organisms. Proteins such as antibodies, hormones, enzymes and many others are involved in a wide range of physiological processes. Most of these proteins can be isolated from different tissues (e.g. liver, skeletal muscle) (Jiang et al., 2020). However, taking samples from living tissues is invasive and is not practical in e.g. long-term monitoring of training effects, so blood samples are more commonly used. The proteome has been well studied in humans (Anderson & Anderson, 2002), but not as thoroughly in other species, including the horse (Miller et al., 2004). Only a few studies have focused on exercise proteomics in horses (Bouwman et al., 2010; Ichibangase & Imai, 2009; Scoppetta et al., 2012) (Table 2).

Table 2. Summary of previous proteomic studies in horses

<table>
<thead>
<tr>
<th>Year</th>
<th>Breed</th>
<th>Type of sample</th>
<th>Duration</th>
<th>No. of Animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bouwman et al. 2010</td>
<td>Standardbred</td>
<td>muscle</td>
<td>24 weeks</td>
<td>16</td>
</tr>
<tr>
<td>Ichibangase &amp; Imai 2009</td>
<td>Thoroughbred</td>
<td>muscle</td>
<td>15 weeks</td>
<td>4</td>
</tr>
<tr>
<td>Scoppetta et al. 2012</td>
<td>Anglo-Arabian</td>
<td>plasma</td>
<td>1 Endurance race</td>
<td>8</td>
</tr>
</tbody>
</table>
2. Aims of the thesis

The overall aim of this thesis was to compare metabolomics and proteomic changes in blood plasma sampled from 16 Standardbred horses kept under standardised conditions and divided into two training groups from the start of training at 1.5 years of age until 3.5 years of age.

Specific objectives were to:

- Determine the concentration of plasma IGF-1 (using ELISA) in response to high-intensity training for two years (Paper I)

- Compare IGF-1 concentrations in horses subjected to two different training programmes for two years (Paper I)

- Explore differences in metabolomic profile in horses kept under standardised conditions and subjected to two different training programmes for two years (Paper II)

- Determine changes in metabolomic profile in horses over the two-year training period (Paper II)

- Compare the proteomic profile in horses kept under standardised conditions and subjected to two different training programmes for two years (Paper III)

- Determine how the proteomic profile changes over time during the two-year training period (Paper III)
3. Material and Methods

This thesis is based on data obtained from 16 Standardbred horses in experiments described in a previous PhD thesis (Ringmark, 2014), where samples were collected and physiological measurements were performed on the horses from the age of 1.5 years to 3.5 years. The horses were divided into two different training groups and fed a forage-only diet. The study was performed at the National Centre for Trotting Education, Wången, Sweden, between September 2010 and December 2012.

3.1 Horses and management

Sixteen Standardbred stallion yearlings (age at the start of the study 464 ± 31 days), with mainly an American pedigree, from four different Swedish breeders were included in the study. All horses were castrated in late December 2010 or early January 2011. They were housed in individual boxes (~9m²) for approximately 16 h per day from Monday to Thursday/Friday, while they spent the rest of the time together in a paddock (~20,000 m²) with access to shelter. The diet of all horses consisted of *ad libitum* access to haylage with known energy and nutrient content, which was supplemented with pelleted lucerne (Krafft AB, Malmö, Sweden), a commercial vitamin and mineral supplement (Krafft AB, Malmö, Sweden) and table salt (NaCl) to meet the nutrient requirements for their age and training intensity (National Research Council, 2007).
3.1.1 Training

The horses were trained by students at Wången Trotting Education Centre, under the supervision of professional trainers. From the start of the study as 1.5-year-olds until the middle of March as 2-year-olds, all horses were subjected to the same training programme. This started with breaking in September 2010 and progressed to trotting with a cart four times per week. Speed was gradually increased up to 5.6 m/s and distance trotted to 5-7 km. In the middle of March as 2-year-olds, the horses were divided into two different training groups that were balanced with regard to breeder and parameters known to affect performance, such as genetic potential (sire and mean pedigree index estimated with the Best Linear Unbiased Prediction (BLUP) method), percentage of French ancestry, inbreeding coefficient, age in days, height at withers, proportion of type IIA/type IIB muscle fibres, abnormal radiographic findings and conformation (Ringmark et al., 2015).

The two training programmes were designed by professional trainers and consisted of high-intensity training sessions (i.e. heart rate >180 bpm, measured using a Polar CS600X device, Polar Electro, Finland) two times per week, as heat training, interval training or uphill interval training (Table 3), plus 1-2 jogging sessions. One group was allocated to a control training programme (named ‘C-group’ in Paper I, ‘High group’ in Papers II and III and this thesis) and the other group to a reduced training programme (named ‘R-group’ in Paper I, and ‘Low group’ in Papers II and III and this thesis). The high-intensity training distance for horses in the Low group was 30% shorter than for horses in the High group. For example in interval training, the High group horses performed six repetitions, while the Low group performed only four. The same speed was aimed for with both groups.

Table 3. Composition of weekly high-intensity training sessions (heart rate >180 bpm) at different ages for 16 Standardbred horses divided into two training groups, High and Low

<table>
<thead>
<tr>
<th>Training type</th>
<th>Age</th>
<th>High</th>
<th>Low</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 years old</td>
<td>3 years old</td>
<td>2 years old</td>
</tr>
<tr>
<td>Heat</td>
<td>1-2 x 1600 m</td>
<td>2-3 x 1600 m</td>
<td>1-2 x 1100 m</td>
</tr>
<tr>
<td>Interval</td>
<td>6 x 500-700 m</td>
<td>6 x 700 m</td>
<td>4 x 500-700 m</td>
</tr>
<tr>
<td>Uphill interval</td>
<td>6 x 600 m</td>
<td></td>
<td>4 x 600 m</td>
</tr>
</tbody>
</table>
3.2 Blood sample collection

Blood samples were collected from the horses approximately every eight weeks throughout the study. In Paper I, data from six blood sampling occasions (November 2010, March 2011, May 2011, December 2011, May 2012, December 2012) were used for IGF-1 analyses. In Papers II and III, blood samples from four sampling occasions (December 2010, July 2011, December 2011, December 2012) were used for metabolomics and proteomic analyses (Figure 4). All blood samples were collected early in the morning (05:00-06:00 h) in each horse’s stall before any activity had started in the stable. In all cases, samples were drawn from the jugular vein into lithium heparin tubes (10 mL), using the vacutainer technique. Directly after collection, the blood samples were centrifuged at room temperature (10 min, 2,700 rpm, 920×g) and the plasma was frozen (-20 °C) for later analysis.

Figure 4. Time line showing blood samples used in Paper I (red dots) and blood samples used in Papers II and III (blue dots).
3.3 IGF-1 analysis by ELISA

To measure the concentration of IGF-1 in the plasma samples analysed in Paper I, ELISA kits from Immunodiagnostic Systems (Boldon, UK) were used. This kit is designed for human plasma, but humans and horses have 100% homology in the amino acid sequence of the IGF-1 protein (Otte et al., 1996) and the kit has been validated for horse plasma (Baskerville et al., 2017). The analysis was performed according to the manufacturer’s instructions. The samples were run in duplicate. All samples from the same horse were run on the same plate and horses from both training groups were included on all plates. The intra-assay coefficient of variation (CV) for the four ELISA plates used in Paper I was 4, 5, 5 and 7 %, respectively, and the inter-assay CV was 8%. The detection range was 10-1200 ng/mL.

3.4 Metabolomics

Targeted absolute quantitative analysis of metabolites was performed at the Mass Spectrometry Based Metabolomics Facility in Uppsala, Sweden. The kit MxP® Quant 500 (Biocrates, Innsbruck, Austria) was used, following the manufacturer’s recommended protocols. Further details of sample preparation can be found in Paper II. Sample analysis involved a combination of tandem mass spectrometry (MS/MS), flow-injection analysis (FIA) and ultra-high performance liquid chromatography (UPLC). For accurate quantification, a chemically homogenised and isotope-labelled internal standard (IS) mixture was used. Data were recorded using Analyst Mass Link software and transferred to MetIDQ software (version Oxygen-DB110-3005), which was used for further data processing. Metabolites were identified using isotopically labelled IS and multiple reaction monitoring (MRM) under optimised MS conditions, as provided by Biocrates. Concentrations of metabolites were quantified using a seven-point calibration curve, depending on the metabolite class.
3.5 Proteomics

Untargeted analysis of proteomes was performed at the Mass Spectrometry Based Proteomics Facility in Uppsala, Sweden. Full details of how the samples were prepared can be found in Paper III. In brief, proteins were digested with trypsin and each sample was injected into the LC-MS/MS system. Peptides were separated in reverse-phase on a C18-column with 90 min gradient and electrosprayed on-line to a Q-Exactive Plus mass spectrometer (ThermoFisher Scientific, Massachusetts, USA). MaxQuant (v.1.5.1.2.) was used for qualitative and quantitative database searches, with the criteria: taxonomy: Equus caballus, enzyme: trypsin, fixed modification: carbamidomethyl, variable modifications: oxidation and for identification of protein at least two matching peptides. The results obtained for all samples were combined to give a total label-free quantification value for each sample.

3.6 Statistical analyses

Detailed descriptions of the statistical analyses performed in Papers I-III can be found in the respective paper. In brief, the statistical analyses were performed using SAS 9.4 and R v4.0.3 (Paper I), R v4.1.2 (Paper II) and Microsoft Excel v16.0.5408.1001 (Paper III). Differences were considered statistically significant at $p<0.05$. In Papers II and III, the $p$-values were adjusted for multiple testing with the false discovery rate (FDR) method (Benjamini & Hochberg, 1995). In Paper I, a mixed model was used for the analyses of IGF-1. In Paper II, a linear model was used for analysis of differences between training groups and of changes over time. In Paper III, either a two-tailed student’s t-test or a two-tailed Welsh’s t-test was used for analysis of differences between the training groups, depending on whether the variance was equal or unequal. For analysis of changes over time, a two-tailed paired t-test was used.

Correlation analyses on acid-labile subunit (ALS) and IGF-1 values were performed in R v4.1.2 with the cor.test function, in two-sided tests using Pearson’s correlation coefficient ($r$).
4. Main results

4.1 IGF-1

There was no significant difference in IGF-1 concentrations between the training groups in Paper I, so the data from the two training groups were pooled. There was a significant decrease in IGF-1 concentrations over time ($p<0.0001$), with the exception of samples taken in May 2011 where IGF-1 concentration were not different from the starting concentration in November 2010 (Figure 5). This temporary interruption in decline in IGF-1 plasma concentrations coincided with the introduction of high-intensity training, which started in the middle of March. In the proteomics analyses in Paper III, it was found that ALS, which forms complexes with IGF-1, showed a tendency ($p=0.08$) to be significantly lower at 3.5 years of age compared with 1.5 years of age (Figure 5). At 3.5 years of age, ALS concentration in plasma was positively correlated ($r=0.78$, $p=0.002$) with IGF-1 concentration.
Figure 5. Changes over time in the concentrations (least-squares mean ± standard error) of insulin-like growth factor 1 (IGF-1, dark green) and acid-labile subunit (ALS, green) in blood plasma samples from 16 Standardbred horses in training (high-intensity training was introduced in March 2011). Concentrations represented by hollow marker are significantly different ($p<0.05$) from the first observation in November 2010 or December 2010.

4.2 Metabolomics

4.2.1 Differences between training groups

In Paper II, MS analysis was performed on 820 metabolites. Comparison of metabolite concentration, sums of metabolite concentrations and metabolite ratios obtained showed significant differences in the concentrations of 212 metabolites between the two groups at 2 years of age, but no significant differences were found at any other age. Of these 212 metabolites, the measured concentrations of 161 were lower in the High group of horses compared with the Low group, while the concentrations of the remaining 51 were higher in the High group (Figure 6). The major groups of metabolites that were present in significantly different concentrations in the two training groups included amino acids and related molecules (16 differed significantly) and triglycerides (79 differed significantly).
Figure 6. Volcano plot of metabolites present in higher (red), lower (blue) and unchanged (black) concentrations in plasma samples (taken at 2 years of age) from 16 Standardbred horses in high-intensity training (High group) than in samples from horses in lower-intensity training (Low group). Upper left quadrant contains adjusted $p$-values $<0.05$ and fold change $<-0.5$, upper right quadrant adjusted $p$-values $<0.05$ and fold change $>0.5$. Abbreviations: aconitic acid (AconAcid), cholesteryl ester (CE), methionine-sulfoxide (Met.SO), ceramides (Cer), polyunsaturated fatty acids (PUFA), amino acids (AA), triglyceride (TG). Source: Paper II.

4.2.2 Changes over time

Analyses of changes over time (i.e. with age) revealed that multiple metabolite concentrations, sums of metabolite concentrations and metabolite ratios in the horses differed significantly at 2, 2.5 and 3.5 years of age compared with 1.5 years. At 2 years of age, 133 metabolites had higher values and 129 had lower values than at 1.5 years (Figure 7A). At 2.5 years of age, 176 metabolites had higher values and 218 had lower values than at 1.5 years (Figure 7B). Finally, at 3.5 years of age, 143 metabolites had higher values and 314 had lower values than at 1.5 years (Figure 7C). The group of metabolites that were present in significantly higher concentrations and changed the most (most significant) were amino acids and related molecules and triglycerides. The metabolites that were significantly lower and changed the most were different lipids, indole and some amino acid related molecules.
Figure 7. Volcano plot of plasma metabolites present in higher (red), lower (blue) and unchanged (black) values at (A) 2 years of age, (B) 2.5 years of age and (C) 3.5 years of age compared with 1.5 years of age, for 16 Standardbred horses in training. Upper left quadrant contains adjusted p-values <0.05 and fold change < -0.5, upper right quadrant adjusted p-values <0.05 and fold change >0.5. Note different scale on the y-axis in panel A (0-10) compared with B and C (0-40). Abbreviations: aconitic acid (AconAcid), amino acids (AA), alpha-aminobutyric acid asymmetric (AABA), beta alanine (b.Ala), carnitine (C0), ceramides (Cer), cholesteryl ester (CE), diglyceride (DG), dimethylarginine (ADMA), dodecanedioic acid (DiCA), fatty acid (FA), hexosylceramides (HexCer), homoarginine (HArg), leucine (Leu), phosphatidylcholines (PC), symmetric dimethylarginine (SDMA), triglyceride (TG), valine (Val). Source: Paper II.
4.3 Proteomics

In the untargeted proteomic analyses in Paper III, 252 proteins were identified, but no significant differences were found between the two training groups.

4.3.1 Changes over time

Analyses of changes over time revealed that the levels of a total of 17 proteins changed significantly across the three ages studied (2, 2.5 and 3.5 years). At 2 years of age one protein was higher and one was lower compared to 1.5 years of age. At 2.5 years of age two protein was higher compared to 1.5 years of age and at 3.5 years of age nine proteins was higher and four proteins was lower compared to 1.5 years of age (Figure 8).
Figure 8. False discovery rate (FDR)-adjusted significantly different plasma proteins in 16 Standardbred horses in training at 2 years of age (blue), 2.5 years of age (yellow) and 3.5 years of age (green), compared with 1.5 years of age (grey). The y-axis shows label-free quantification (LFQ) $\times 10^6$ of the different proteins. Abbreviations: Alpha-2-glycoprotein 1, zinc-binding (AZGP1), Apolipoprotein (Apo), Bactericidal/permeability-increasing fold containing family A member 2 (BPIFA2), CD5 molecule like (CD5L), Extracellular matrix protein 1 (ECM1), Joining chain of multimeric IgA and IgM (JCHAIN), Serpin family G member 1 (SERPING1). Source: Paper III.
5. Discussion

This is the first long-term training study looking at metabolomics and proteomic changes in horses subjected to two different training programmes. It showed that introduction of high-intensity training interrupted the expected decline in IGF-1 concentrations which suggest that high-intensity training affects IGF-1 levels. Both the metabolomic and proteomic profile changed over time, but as a response to different training programs, mainly the metabolic profile was affected. The metabolomic analysis showed significant differences in metabolites involved in aerobic energy production, amino acid metabolism and potentially also changes in pH-buffering and vascular responses. The proteomic analysis identified significantly different proteins which were involved in pathways related to energy metabolism, circulation, bone formation and the immune system.

5.1 Effect of training on IGF-1

The hypothesis tested in Paper I was that IGF-1 concentrations in blood plasma of Standardbred horses are elevated by high-intensity training. The results showed that IGF-1 concentrations underwent a continuous decline, except in the month when high-intensity training was introduced, which is in accordance with previous findings that IGF-1 concentrations decline with age (Malinowski et al., 1996; Noble et al., 2007; Popot et al., 2001). The interruption in decline in IGF-1 concentrations when high-intensity training was introduced may suggest that high-intensity training stimulates IGF-1 release in horses. However, the results are not consistent with previous findings by Noble et al. (2007), who studied Thoroughbreds during a moderate-high training intensity programme for nine weeks and observed no change in IGF-1 concentrations. This could be due to the horses in that study
being much older (10±2 years) than those studied in Papers I-III, and hence being past their growth stage. However, it may also indicate that recurring high-intensity exercise bouts, as in this thesis, are needed to stimulate IGF-1 production. A study by Jackson et al. (2003) comparing two training groups over 20 weeks found a difference between the training groups, but interestingly it was the lightest training group (only walk) that had higher IGF-1 levels. This could be explained by the fact that nutrient intake and energy balance were not controlled for in that study, since these have been shown to affect plasma concentration of IGF-1 significantly (Salazar-Ortiz et al., 2014; Sticker et al., 1996). It is possible that some other factor caused the interruption in the decline in IGF-1 concentrations observed in Paper I, but seasonal variation seems unlikely because there was no disruption in the decline in IGF-1 concentrations in the following year (May 2012).

There were no differences in IGF-1 concentrations between the High and Low training groups, which indicates either that IGF-1 does not respond incrementally to increased training intensity or that the peak response in terms of IGF-1 was achieved even with the reduced training programme. Another explanation is that the difference in distance trained between the groups (30% shorter distances in high-intensity training) was not sufficiently large to result in differences in IGF-1 concentrations.

The proteomic analyses revealed that ALS, one of the carrier proteins that form complexes with IGF-1, showed a tendency (FDR-corrected $p=0.08$) to be significantly lower at 3.5 years of age compared with 1.5 years of age. This is in agreement with findings in studies on humans (Juul et al., 1998) that ALS levels reach a peak in puberty and then decrease with age, similarly to IGF-1. This is supported by that plasma ALS and IGF-1 concentrations were positively correlated ($r=0.78$, $p=0.002$) in horses at 3.5 years of age.

In conclusion introduction of high-intensity training induce IGF-1 release in horses but a 30% reduction of high-intensity training distance had no effect on IGF-1 levels.
5.2 Metabolomics

The metabolic profile of Standardbreds in the two different training groups (High, Low) differed significantly at 2 years of age and there were also changes in the metabolic profile over time at all ages studied (2, 2.5 and 3.5 years) compared with at 1.5 years of age (Paper II). Leucine concentration was significantly higher in the High group compared with the Low group at 2 years of age, and it was also higher over time in all ages compared with 1.5 years of age (see Figure 8). This is in agreement with pre-training results from a 12-week high-intensity training study by Klein et al. (2020), but contradicts findings in a study by Westermann et al. (2011) that horses trained for 18 weeks at moderate to high training intensity showed no differences in leucine concentrations. This could be because of differences in training intensities. Branched-chain amino acids are involved in aerobic energy metabolism in skeletal muscle (Lawrence, 1990), stimulate protein synthesis in skeletal muscle (Anthony et al., 2001) and may also stimulate glycogen synthesis (Morifuji et al., 2010), which are all important for performance. The results obtained in Paper II support this, because changes were observed both between the training groups and over time (with age).

Anserine and anserine synthesis concentrations were higher in the High group compared with the Low group, and also increased over the ages studied. Anserine is a methylated variant of carnosine and both are believed to play a major role in maintaining intracellular buffering and pH balance, while anserine may also have anti-oxidant, anti-glycation and anti-lipoxidation functions (Boldyrev et al., 2013; Mori M., 2015). All of these functions are important for exercise performance, but in particular there is an obvious need for pH buffering because during high-intensity training horses produce lactic acid in the skeletal muscle, which lowers the pH. The results in Paper II suggest that the duration of high-intensity training in each training session may be important for development of pH-buffering capacity.

Several of the triglyceride metabolites were present in lower plasma concentrations in the High group compared with the Low group (Paper II). However, on analysing the overall change in triglycerides over time, it emerged that most showed an increase with age, which is in agreement with findings by Klein et al. (2020). Fat is an important energy source for Standardbred horses in training and the activity of 3-hydroxyacyl-CoA-dehydrogenase, the last step in beta-oxidation, may increase with training
(Henckel, 1983). It is known to be associated with increased performance (Essen-Gustavsson & Lindholm, 1985).

Aconitic acid concentrations were higher in the High group compared with the Low group, and were higher at 2.5 and 3.5 years of age compared with 1.5 years. Aconitic acid is an intermediate in the citric cycle and it is well known that the activity of citrate synthase, which catalyses the first reaction in the citric cycle, increases with training (Essen-Gustavsson & Lindholm, 1985; Henckel, 1983; Hodgson, 1985; Roneus et al., 1992). The findings on aconitic acid levels support suggestions by Klein et al. (2020) that amino acid and lipid metabolism play pivotal roles in the response of equine skeletal muscle to training.

Homoarginine (HArg) concentrations and HArg synthesis were higher in the High group of horses compared with the Low group, and also increased over time at all ages. HArg is suggested to be one of the substrates for nitric oxide (NO) synthesis (Sibal et al., 2010; Tsikas & Wu, 2015). Nitric oxide has several biological functions, but one is as a potent vasodilator (Sibal et al., 2010). There is also evidence that NO production increases with physical activity and that NO both improves performance and promotes recovery (Oral, 2021). The horses in the High group in this thesis showed improved cardiovascular response from the age of 2.5 years until the end of the study, as reported previously (Ringmark et al., 2015).

In this thesis, differences in metabolic profile between the training groups were only seen at 2 years of age (Paper II). This could be because horses that were not deemed fit to train (according to the trainer) were allowed to skip training days. Horses in the High group skipped more training days than horses in the Low group, meaning that from the age of 2.5 years to the end of the study, the horses in the High group trained on average for the same cumulative distance as the horses in the Low group (Ringmark et al., 2016).

In conclusions the metabolomic analysis showed significant differences in metabolites involved in aerobic energy production, amino acid metabolism and potentially also changes in pH-buffering and vascular responses.
5.3 Proteomics

There were no significant differences in plasma proteomic response between the training groups (Paper III), which was surprising in light of the previously documented improvement in cardiovascular system in the High group compared with Low group from the age of 2.5 years (Ringmark et al., 2015).

In analyses of changes over time, plasma levels of 17 proteins were found to be significantly different at later measurement points compared with at 1.5 years of age (Paper III). At 2 years of age in comparison with 1.5 years, two proteins differed significantly. These were extracellular matrix protein 1 (ECM1), which was present in higher levels in horses at 2 years of age, and hemopexin, which was present in lower levels at 2 years of age. ECM1 is involved in angiogenesis (Han et al., 2001), skin differentiation, integrity and homeostasis (Sercu et al., 2009; Smits et al., 2000), and possibly also in endochondral bone formation (Deckers et al., 2001). Hemopexin is involved in binding and clearance of haem groups from the circulation (Smith & McCulloh, 2015).

At 2.5 years of age in comparison with 1.5 years, two proteins were present in significantly different levels. These were apolipoprotein (Apo) A-II and fetuin B, both of which showed higher levels at 2.5 years of age. Apo A-II is a key regulator of high-density lipoprotein structure and metabolism (Maïga et al., 2014). The function of fetuin B is still unclear, but it has a similar tissue distribution and is structurally similar to fetuin A and is therefore proposed to have similar functions, i.e. regulation of insulin, mineralisation of bones and involvement in systemic inflammation (Denecke et al., 2003).

At 3.5 years of age compared with 1.5 years, 13 proteins were present in significantly different levels. At 3.5 years of age, alpha-2-glycoprotein 1, zinc-binding (AZGP1), Apo A-II, Apo H, CD5 molecule like (CD5L), clusterin, fetuin B, joining chain of multimeric IgA and IgM (JCHAIN), kininogen 1 and prothrombin were present in higher levels, while bactericidal/permeability-increasing fold containing family A member 2 (BPIFA2), ceruloplasmin, lumican and serpin family G member 1 (SERPING1) were present in lower levels. AZGP1, Apo A-II and H are all involved in lipid metabolism (Maïga et al., 2014; Sodin-Semrl & Rozman, 2007; Wei et al., 2019) and it is logical for them to increase with a higher training load, because fat is an important energy source for Standardbred horses during training. The activity of the enzyme HAD, involved in the last
step of beta-oxidation, may increase with training (Henckel, 1983) and is associated with good performance (Essen-Gustavsson & Lindholm, 1985). Kininogen 1 and prothrombin are involved in clotting and wound healing (Degen & Sun, 1998; Lee et al., 2009), while CD5L and JCHAIN are involved in the immune system. The changes observed in these proteins may not have been caused by exercise and could be explained by the fact that all the horses had wounds (caused by physical interactions in the group housing system) or mud fever at least once during the study period. By the end of the study (>3 years), some horses also had health problems such as joint inflammation, wounds and fractures (trauma), which would be reflected in the proteomic profile.

In conclusions proteins involved in pathway related to energy metabolism, circulation, bone formation and the immune system changed significantly over time.
6. Future research

This thesis presents results from the first set of long-term training studies examining metabolomics and proteomic changes in horses subjected to different training programmes. A limitation of the work was that there was no control group subjected to no training at all. It would be interesting to repeat the studies but with a control group of the same age, in order to distinguish between metabolites and proteins associated with training and those associated with growth. The function of some proteins and metabolites is still unknown. Better knowledge of this could help explain the changes that occur in horses subjected to different training programmes and hopefully also help identify biomarkers that can be used to monitor training and assess whether horses are improving or not.

The horses that participated in this study were born in 2009, so most of them have already ended their racing career. It would be interesting to look at lifetime performance and number of starts by these horses and assess whether some metabolites or proteins are linked to better performance or lifetime earnings. Other data are also available for these horses, e.g. data on locomotion asymmetry from age 1.5 to 3.5 years. Studies comparing the asymmetry data with the metabolomics and proteomic data reported in this thesis should be performed to determine whether any biomarkers change when the horse become asymmetric (which could be a sign of pain-induced lameness). If such changes occur before the horse becomes lame, such biomarkers would be of great value in monitoring the training of horses by indicating when the training load becomes too much and needs to be adjusted.
References


Harness racing is a popular sport in Sweden and 12,000 Standardbred trotters compete each year in different races. In order to compete and possibly win races, Standardbred horses start training early (at around one year old). The aim of training is to increase the performance capacity and to adapt different tissues, such as cartilage, bone, muscle etc., to increasing training speeds and distances. However, increasing the training load too fast increases the risk of injuries. The most common cause of interruption in training in Standardbred and Thoroughbred horses is lameness.

This thesis looked at changes in metabolites (small molecules involved in cell metabolism) and proteins circulating in blood plasma in 16 young Standardbred horses in training from 1.5 to 3.5 years of age. The horses had the same training programme from September, when they were 1.5 years old, until March, when they were 2 years old. In March, high-intensity training (heart rate above 180 beats/minute) started and the horses were divided into two groups (High and Low). Both groups followed the same training programme, which was designed by three professional trainers, but the Low training group had 30% shorter high-intensity training distances than the High group. For example, during interval training sessions the High training group did six bouts and the Low training group did only four bouts, while the speed was the same for both groups. All horses were fed the same diet during the study. Blood samples were collected from the horses early in the morning at intervals over the two-year study period and analysed for metabolites, proteins and a hormone called IGF-1 (insulin-like growth hormone 1). IGF-1 stimulates the growth of many different tissues, such as bone, muscle, fat etc. The analysis revealed that the concentration of IGF-1 in horse blood plasma did not differ between the High and Low training groups. Plasma IGF-1 concentration is normally highest at puberty and
decreases with age. The horses in the study had already gone through puberty and the expected decline in IGF-1 concentration was observed, but with an interruption in the decline when the horses started high-intensity training. At that point, the IGF-1 concentrations returned temporarily to the levels seen when horses entered the study at 1.5 years of age. This suggests that high-intensity training may stimulate release of IGF-1 in horses.

Analysis of 850 different metabolites in blood plasma from the horses revealed differences between the training groups only when they were 2 years of age. This may be explained by horses deemed not fit to train by the trainer being allowed to skip a training session. Overall, horses in the High training group skipped more training sessions than horses in the Low training group, which meant that from the age of 2.5 years there was no difference in actual trained distance between the training groups. In contrast, analysis of changes in metabolites over time revealed many differences. The specific metabolites that changed over time differed between the training groups, but most are involved in energy production and amino acid metabolism, and possibly also the buffering capacity of cells (i.e. how well they can handle the lactic acid and protons produced during high-intensity training) and changes in the circulatory system.

Analysis of proteins in blood plasma from the horses did not show any differences between the training groups, but analysis of changes over time revealed significant changes in the concentrations of 17 proteins involved in energy production, bone formation, inflammation and wound healing. However, the inflammation and wound healing responses may not have been solely due to training, because the horses all had wounds (caused by physical interactions in the paddock) or mud fever at least once during the study. If these protein changes can be detected before the horse becomes lame, the training load could be adjusted in time to prevent injury, improving animal welfare.
Populärvetenskaplig sammanfattning

Travsport är populärt i Sverige och 12000 travare tävlar varje år i olika lopp. För att kunna vara med och tävla och förhoppningsvis vinna så startar träningen av travhästen tidigt (vid ett års ålder) och målet med träningen är att förbättra prestationssformågan och vänja olika vävnader, så som muskler, ben, ligament och leder, till ökad träningshastighet och distans. Men om träningsbelastningen ökas för mycket och för fort så ökar även risken för skador. Den vanligaste orsaken till avbrott i träningen för travare och galloppörer är hälta.

I min avhandling har jag tittat på förändringen av metaboliter (små molekyler som är inblandade i cellernas ämnesomsättning) och proteiner som cirkulerar i blodplasman hos 16 travare med olika träningsprogram från 1,5 till 3,5 års ålder. Hästarna hade samma träningsprogram från september när de var 1,5 år gamla till mars när de var 2 år gamla. I mars introducerades hög-intensiv träning (snabbjobb, hjärtfrekvens över 180 slag/min) och hästarna delades in i två olika träningsgrupper, Hög och Låg. Båda gruppena följde samma träningsprogram, vilket var designat av tre professionella tränare, men träningsgrupp Låg hade 30% minskad distans i snabbjobben. Till exempel om det var intervallträning gjorde träningsgrupp Hög 6 intervaller medan Låg gjorde 4 intervaller, men hastigheten var den samma för båda träningsgrupperna. Alla hästar fick samma foderstat under hela studien. Blodprover samlades in från hästarna vid åtta tillfällen och analyserades för metaboliter, proteiner och hormonet IGF-1 (insulin-like growth hormone 1).

IGF-1 är ett hormon som stimulerar tillväxt av flera olika vävnader så som muskler, ben, fett mm. När vi analyserade koncentrationen av IGF-1 och jämförde de två träningsgrupperna såg vi inga skillnader. I vanliga fall är IGF-1 som högst under puberteten och minskar sedan med ålder. I våra hästar
som redan hade gått igenom puberteten förväntade vi oss en minskning av IGF-1 med tiden men vi såg en uppgång av IGF-1 när snabbjobben introducerades. Detta tyder på att hög-intensiv träning stimulerar IGF-1 produktion i hästar. Vi vet att IGF-1 stimuleras av hög-intensiv träning i andra djurslag men resultat från studier på häst har varit motstridiga. Denna skillnad i resultat från studier på häst kan bero på att energi- och närings innehållet i deras foderstat inte var kontrollerad och vi vet att IGF-1 koncentrationen påverkas av foderstaten.

Vi analyserade 850 olika metaboliter i hästarna och hittade bara skillnader mellan träningsgrupperna vid 2 års ålder. Detta skulle kunna bero på att hästar som inte var friska nog att träna, enligt tränaren, kunde stå över ett träningspass. Under studien visade det sig att hästar i träningsgrupp Hög stod över fler träningspass än hästarna i träningsgrupp Låg vilket ledde till att från 2,5 års ålder var det ingen skillnad mellan grupperna i den sammanlagda träningsdistansen som de verkligen tränade. Vi undersökte även om vi kunde se några skillnader i metaboliter över tid och hittade väldigt många som skiljde sig. De flesta av metaboliterna som skiljde såg åt mellan träningsgrupperna och över tid var inblandade i energiproduktion, aminosyrors ämnesomsättning och troligtvis också inblandade i cellens buffert kapacitet (dvs hur bra cellen kan hantera mjölksyra och protoner som bildas vid hög-intensiv träning) och förändringar i cirkulationssystemet.

Vi analyserade även proteiner men kunde inte se några skillnader mellan träningsgrupperna men för förändringar över tid var det 17 proteiner som skiljde sig. De var inblandade i energiproduktion, bentillväxt, inflammation och sårläkning. De proteiner som var inblandade i inflammation och sårläkning påverkades troligtvis inte bara av träningen eftersom alla hästar någon gång under studien hade sår (berodde på fysiska interaktioner i hagen) eller mugg vilket kan ha påverkat. Sammanfattningsvis visade studierna att både metaboliter och proteiner förändrades över tid, men skillnaden mellan träningsgrupper påverkade till största del bara metaboliterna.
The work presented in my thesis was performed at the Department of Anatomy, Physiology and Biochemistry, Faculty of Veterinary Medicine and Animal Science, Swedish University of Agricultural Sciences (SLU), Uppsala. I would like to thank the University, Faculty and the previous and current heads of department for giving me the opportunity to pursue my PhD-studies.

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1. Introduction

It is generally accepted that exercise can act as a stimulus for the hypothalamus to produce growth hormone-releasing hormone (GH-RH), which stimulates the release of growth hormone (GH) and, secondarily, the production of insulin-like growth factor 1 (IGF-1) by several tissues in humans and other species (De Graaf-Roelfsema et al., 2007; Kraemer and Ratamess, 2005). However, this is not well documented in horses. In humans, it is well known that high-intensity exercise increases plasma concentrations of growth hormone (Felsing et al., 1992; Jenkins, 2001; Pritzlaff et al., 1985). Introduction to high-intensity training could therefore also be expected to stimulate growth hormone and IGF-1 release in horses. While GH has a short half-life in plasma (20 min) (Faria et al., 1989), elimination of IGF-1 is slow (half-life 20 h) (Fortier et al., 2005), so analysis of IGF-1 could be more relevant when monitoring long-term growth stimuli responses. There are a few studies on the effect of training on plasma IGF-1 levels in horses, but the results are somewhat contradictory. For example, Jackson et al. (2003) observed a difference in the relative change in plasma IGF-1 concentrations between horses subjected to two different 20 weeks training programs, while Noble et al. (2007) observed no differences in horses subjected to different training programs. Interestingly,
in the study by Jackson et al. (2003), horses subjected to the lightest training program (only walk) had the highest (positive) IGF-1 changes. However, nutrient intake and energy balance were not controlled for in those studies, and it is generally known, including from studies in horses (Salazar-Ortiz et al., 2014; Sticker et al., 1995), that protein intake and energy balance can significantly affect IGF-1 plasma concentrations. To better understand the effect of high intensity training, studies with improved dietary control is therefore warranted and the aim of this study was to study long term IGF-1 concentrations in growing horses introduced to two different high intensity programs while fed the same controlled diet.

GH and IGF-1 target a number of cell types in various tissues, such as cartilage, bone, and skeletal muscle (Ballesteros et al., 2000; Verwilghen et al., 2009). Studies on GH-deficient animals and humans treated with IGF-1 have shown that the hormone stimulates longitudinal bone growth (Yakar and Isaksson, 2016). Fortier et al. (2005) analysed serum levels of IGF-1 and an IGF-1 carrier protein (IGFBP-3) in growing horses and concluded that concentrations peak at around 225 days of age, defining the onset of puberty, and then decline to steady-state levels at around 450 days, signalling the end of puberty. Those authors also observed correlations between structural changes (e.g. disappearance of cartilage canals) in articular-epiphyseal cartilage complex and IGF-1 and IGFBP-3 levels. In a study on horses representing a wider range of ages, IGF-1 concentrations showed a gradual decrease in mares and geldings from the age of one to 19 years (Noble et al., 2007).

An increase in GH and IGF-1 release due to increased exercise intensity would stimulate growth, and thereby possibly also alter conformation and muscle growth in growing horses. One possible effect of such changes is alterations in the locomotion pattern. Anecdotal observations by horse trainers support the suggestion that young Standardbred horses in training may show uneven growth (e.g. more rapid growth at the croup than at the withers) and periodically show flaccid and stumbling locomotion patterns, even at slow velocities.

The aims of this study were to: (1) determine the concentrations of plasma IGF-1 in response to high-intensity training in young Standardbred horses (kept under controlled dietary conditions); (2) compare plasma IGF-1 concentrations in horses subjected to two different levels of high-intensity training for 21 months; and (3) assess whether plasma IGF-1 concentrations are correlated to growth rates and locomotion asymmetry. The hypotheses tested were that: introduction to high-intensity training increases plasma concentrations of IGF-1; horses under a reduced high-intensity training program show an attenuated increase; and there are correlations between IGF-1 concentration, growth and locomotor patterns.

2. Material and methods

The study was performed at the Swedish National Centre for Trotting Education at Wängen, where the horses were cared for and trained by high school students under the supervision of professional trainers. The protocol was approved by Umeå Local Ethics Committee (A90-10, 2010-09-14).

Horses and management

Sixteen Standardbred colt yearlings from four Swedish breeders were used. The horses had mainly an American pedigree, but eight horses also had some (<27%) French ancestry. These eight horses were all tested by the SychoGait gene test (Capilet Genetics, Västerås, Sweden) and were all homozygous for the stop codon in the DMRT3 gene, which has been shown to negatively affect the ability for a balanced trot at high speed if heterozygous (Andersson et al., 2012). They entered the study in September 2010 as 1-year-olds and the study ended in December 2012 as 3-year-olds. The colts were all castrated in December 2010-January 2011. They were stabled individually (box stalls, ~9 m²) for approximately 14 h per day Monday to Thursday/Friday, and spent the rest of the time in a paddock with access to shelter. The horses had ad libitum access to water and haylage, both in the boxes and in the paddock. The haylage was analysed for energy and nutrient content, and the diet was supplemented with pelleted lucerne (Kraft AB, Malmö, Sweden), a commercial mineral supplement (Kraft AB, Malmö, Sweden) and NaCl to meet nutrient requirements (NRC, 2007). Mean daily energy and crude protein intake of the horses are summarised below (Table 1). The results of all haylage analyses and full data on feed, energy, and nutrient intake can be found in Ringmark et al. (2013, 2017). Hoof trimming and shoeing were performed every 5–6 weeks, while during wintertime (October/ November-March) permanently studs were fitted on the shoes (four 8-mm high studs per shoe).

Training

From September 2010 as 1-year-olds to March 2011 as 2-year olds, all horses were subjected to the same training program, which involved only occasional exercise at heart rate >180 beats/min. The goal was to train the horses to a level where they could trot with ease for 5-7 km at a velocity of 5.6 m/s (3 min/km). In March 2011 as 2-year olds, when regular twice-weekly high-intensity training was about to start, the horses were divided into two groups. These groups were balanced with respect to breeder and parameters known to affect performance, such as genetic potential (sire and mean pedigree index estimated with the Best Linear Unbiased Prediction (BLUP) method), percentage of French ancestry, inbreeding coefficient, age in days, abnormal radiographic findings, conformation, height at
withers, and proportion of type IIA/type IIB muscle fibres (Ringmark et al., 2015). Mean age of the horses in March 2011 was 657±31 days (range 595-713 days) and there was no difference between the training groups (P>0.05). High-intensity exercise was defined as training expected to cause a heart rate >180 beats per minute (heat training, interval training, and uphill interval training). One group was allocated to a control training program (group C) and the other to a reduced training program (group R) where the high-intensity exercise distances was reduced by 30%. Thus, for the remainder of the study, horses in group C performed heat training over 1,600 m, whereas horses in group R performed heat training over 1,100 m, and when horses in group C performed six intervals, horses in group R performed only four. The same velocity was aimed for with both groups. Full details about training distances and number of training sessions of these horses can be found in Ringmark et al. (2015).

Growth and feed intake recording

All growth measurement were performed by the same person. Body weight (BW) was recorded with a scale (weight indicator U-137, UNI Systems and Vågspecialisten, Skara, Sweden). Height at withers and height at croup was measured with a ruler with a precision of 0.5 cm. If the horses had spikes in the shoes 0.8 cm were subtracted. Body length was measured from the point of the shoulder to the point of the buttock using a folding ruler (positions identified by palpation of the tip of humerus (humeral tubercles) and tuber ischii). Circumference right below carpus (at cannon and splint bone tops) was measured with a tape measure on both left and right front limb and a difference of pelvis (minimum difference2 + maximum difference2) for head (front limbs, versus right maximal and minimum position of head was identical) was used to calculate the vector sums (VS): VS = √(maximum difference2 + minimum difference2) for head (front limbs, VSf). For hind limbs, pelvis differences was divided into pushoff (max difference of pelvis) and impact (minimum difference of pelvis) differences. Individual mean of VSf, pushoff and impact for Periods 1-4 were then calculated.

Table 1. Daily metabolisable energy (ME) intake and crude protein (CP) intake (per 100 kg body weight) and dietary CP/ME ratio in 16 Standardbred horses in training in Periods 1-4 (least squares mean ± standard error).1

<table>
<thead>
<tr>
<th></th>
<th>Period 1</th>
<th>Period 2</th>
<th>Period 3</th>
<th>Period 4</th>
</tr>
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<tbody>
<tr>
<td>ME, MJ/day</td>
<td>22±0.4</td>
<td>25±0.4***</td>
<td>26±0.4***</td>
<td>23±0.4*</td>
</tr>
<tr>
<td>CP, g/day</td>
<td>290±5</td>
<td>370±5***</td>
<td>350±5***</td>
<td>280±5*</td>
</tr>
<tr>
<td>CP/ME</td>
<td>13±0.1</td>
<td>15±0.1***</td>
<td>14±0.1***</td>
<td>12±0.1***</td>
</tr>
</tbody>
</table>

1 Significant differences compared with Period 1 are indicated by * (P<0.05) or *** (P<0.001).
Blood sample collection

On six occasions (November 2010, March 2011, May 2011, December 2011, May 2012, and December 2012), each within three days of a locomotion asymmetry evaluation, blood was collected early in the morning (05:00-06:00 h) before any activity had started in the stables. These blood samples were collected from the jugular vein in lithium heparin tubes (10 ml), using the vacutainer technique. The samples were directly centrifuged at room temperature (10 min, 2,700 rpm, 920×g) and the plasma was frozen (-20 °C) for later analysis of IGF-1. For correlation analysis, the IGF-1 concentration observed in the beginning of each period was used, and for periods with more observations (Periods 1 and 4) the mean IGF-1 concentration was calculated.

Insulin-like growth factor-1 ELISA

IGF-1 levels were determined using an ELISA kit (Immunodiagnostic Systems, Boldon, UK) manufactured for human plasma, but validated for horse plasma (Baskerville et al., 2017). The homology of IGF-1 protein sequence for humans and horses is 100% (Otte et al., 1996). The analysis was performed according to the manufacturer’s instructions. All samples from the same horse were run on the same plate and horses from both training groups were included on all plates. The samples were run in duplicate and the intra-assay coefficient of variation (CV) for the ELISA plates was 4, 5, 5 and 7%, respectively, and the inter-assay CV was 8%. The detection range was 10-1,200 ng/ml.

Data and statistical analyses

All analyses except correlation analysis were carried out in R (v4.0.3, R Core Team, 2020) using the packages nlme (v3.1-149) and emmeans (v1.5.3). Normal distribution of data was verified by residual plots and if the data deviated from normality, they were log-transformed except for IGF-1 concentrations, and therefore data from the two groups were pooled (Figure 1). There was a significant decrease in IGF-1 level over time (P<0.0001) except for a notable exception in May 2011 (Period 2), when the IGF-1 concentration was not different from the start level in Period 1 (Figure 1). That temporary disruption in decline in plasma IGF-1 concentration coincided with the introduction of systematic high-intensity exercise (Figure 1).

Growth rate and changes in body measurement

There were no significant differences in growth parameters and body measurements between the training groups, and therefore the data were pooled (Table 2). Compared with Period 1, growth rate in height at withers decreased and was lower in all remaining periods (Table 2). The growth rate in height at croup and change in BW showed a similar pattern except in Period 3, when the changes were not significantly different from those in Period 1. Growth in body length and front limb circumference showed a different response, in that the changes were equally high in Periods 1 and 2 (no significant difference), while lower rates were first observed in Period 3. The growth rate of m. longissimus increased in Periods 2 and 3 compared with Period 1. The change in fat thickness at croup was greater in all periods compared with Period 1, but no differences were observed in changes in BCS between Period 1 and 2, while after that the change was lower than in Period 1 (Table 2).

Locomotion asymmetry

As reported by Ringmark et al. (2016), there were no significant differences in locomotion asymmetry pattern between the two training groups, and therefore the data were pooled. Compared with Period 1, hind limb asymmetry for both pushoff and impact was elevated in Period 2 (P=0.02 and P=0.0008, respectively), but not in Period 3 (Table 3). Hind limb asymmetry increased again in Period 4 for pushoff and impact (P=0.0008 and P=0.004, respectively), but there were no differences between Periods 2, 3, and 4 (P>0.05). Front limb asymmetry was significantly elevated in all periods compared with Period 1 (P<0.0001, P=0.0003, and P<0.0001 for Period 2, 3, and 4, respectively), but there were no differences between Periods 2, 3, and 4 (Table 3).

Correlations

There was a positive correlation between IGF-1 concentrations and BCS and a tendency for a negative correlation between IGF-1 and body length, but IGF-1
Figure 1. Insulin-like growth factor 1 (IGF-1) in 16 Standardbred horses in training from November 2010 as one-year olds to December 2012 at three years of age (least-squares mean ± standard error). Unfilled markers are significantly different ($P<0.05$) from the first observation in November 2010. Dark grey background: training intensity below <180 bpm, White background: regular high intensity exercise at >180 bpm.

Table 2. Changes in body weight, height at withers and croup, body length, front limb circumference, depth of m. longissimus dorsi, subcutaneous fat thickness at croup, and body condition score (scale 1-9) in 16 growing Standardbred horses in training during Periods 1-4 (least squares mean ± standard error).

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<tbody>
<tr>
<td></td>
<td>1-2 yrs</td>
<td>2 yrs</td>
<td>2 yrs</td>
<td>3 yrs</td>
</tr>
<tr>
<td>Height at withers (cm/month)</td>
<td>1.0±0.04</td>
<td>0.2±0.04***</td>
<td>0.6±0.04***</td>
<td>0.1±0.05***</td>
</tr>
<tr>
<td>Height at croup (cm/month)</td>
<td>0.7±0.06</td>
<td>-0.1±0.06***</td>
<td>0.6±0.06</td>
<td>0.1±0.07***</td>
</tr>
<tr>
<td>Body length (cm/month)</td>
<td>1.1±0.02</td>
<td>0.7±0.2</td>
<td>-0.1±0.2***</td>
<td>0.3±0.2’</td>
</tr>
<tr>
<td>Front limb circ. (cm/month)</td>
<td>0.1±0.02</td>
<td>0.1±0.02</td>
<td>-0.1±0.02’</td>
<td>-0.1±0.02</td>
</tr>
<tr>
<td>m. longissimus (mm/month)</td>
<td>-1.4±0.5</td>
<td>0.9±0.5**</td>
<td>0.4±0.5’</td>
<td>-0.1±0.5</td>
</tr>
<tr>
<td>Fat at croup (mm/month)</td>
<td>-0.2±0.04</td>
<td>0.2±0.04***</td>
<td>-0.1±0.04’</td>
<td>0.1±0.04***</td>
</tr>
<tr>
<td>Body condition score2</td>
<td>0.1±0.03</td>
<td>0.1±0.03</td>
<td>-0.1±0.03’</td>
<td>-0.1±0.03’</td>
</tr>
<tr>
<td>Weight (kg/month)</td>
<td>5.8±0.06</td>
<td>2.4±0.6***</td>
<td>6.1±0.6</td>
<td>0.5±0.6’</td>
</tr>
</tbody>
</table>

1 Significant differences from Period 1 are indicated by * ($P<0.05$), ** ($P<0.01$) or *** ($P<0.001$).
2 Scale from Henneke et al. (1983).

Table 3. Mean hind and front limb asymmetry in 16 growing Standardbred horses in training during Periods 1-4 (least squares mean ± standard error).

<table>
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<tbody>
<tr>
<td></td>
<td>1-2 yrs (n=89)2</td>
<td>2 yrs (n=48)</td>
<td>2 yrs (n=32)</td>
<td>3 yrs (n=94)</td>
</tr>
<tr>
<td>Hind limb pushoff asymmetry (mm)</td>
<td>3.4±0.50</td>
<td>4.6±0.60</td>
<td>4.7±0.70</td>
<td>4.8±0.50</td>
</tr>
<tr>
<td>Hind limb impact asymmetry (mm)</td>
<td>3.2±0.50</td>
<td>5.4±0.60</td>
<td>3.9±0.60</td>
<td>4.6±0.50</td>
</tr>
<tr>
<td>Front limb asymmetry (vector sum, mm)</td>
<td>10.0±1.00</td>
<td>19.3±1.30</td>
<td>15.4±1.60</td>
<td>17.0±1.00</td>
</tr>
</tbody>
</table>

1 Values in a row with different superscript letters are significantly different ($P<0.05$).
2 n = number of observations during the period.
Correlation analysis performed on data from Periods 1-4. There was a negative correlation between IGF-1 concentration and weight (Table 4). There was also a negative correlation between IGF-1 concentration and hind limb pushoff asymmetry and a tendency for a negative correlation for hind limb impact asymmetry, but no correlation with front limb asymmetry (Table 4). IGF-1 concentrations showed no correlation to ME or CP intake, but a positive correlation to CP/ME intake ratio (Table 4).

4. Discussion

The main aims of this study were to describe and compare plasma concentrations of IGF-1 in growing Standardbred horses when introduced to two high-intensity training programs for 21 months. The starting hypothesis was that high-intensity training would elevate IGF-1 concentrations. The results showed that there was a significant interruption of the decline in IGF-1 levels at the time when high-intensity exercise training was introduced. This could indicate that high-intensity training stimulates IGF-1 release in horses. To our knowledge, this is the first study to describe this in growing horses subjected to high-intensity training. The results are somewhat in contradiction to observations made by Noble et al. (2007), who observed no acute changes in plasma IGF-1 concentrations after race-like exercise in adult Thoroughbred horses (10±2 years). The reason for this discrepancy is unclear, but it could be due to the response differing depending on age of the horse (growing or not) or to recurrent high-intensity exercise bouts being required to stimulate IGF-1 production (e.g. twice weekly as in the present study). Although the interruption of the decline in IGF-1 concentration coincided with the introduction of high intensity training it cannot be excluded that it was due to other reasons. A seasonal effect seems however, unlikely since there was no second interruption at the same time next year (May 2012).

Although IGF-1 release may have been stimulated when high-intensity exercise was introduced, there was no difference between the two training intensity levels (R and C). This indicates that IGF-1 release does not display an incremental response to increasing exercise intensity, or perhaps that the peak response was achieved already at the intensity of the group R horses. Another explanation could be that the difference in high-intensity training evaluated (30% shorter distance) was not enough to stimulate clear differences in IGF-1 release.

As mentioned, IGF-1 levels showed a continuous decline throughout the study apart from the temporary elevation when high-intensity exercise was introduced. This observation is in accordance with previous studies which have linked IGF-1 levels to age (Malinowski et al., 1996; Noble et al., 2007; Popot et al., 2001). It is also in accordance with Fortier et al. (2005), who observed a peak at the age of 225 days, which is before our horses entered the study.

An additional aim of this study was to investigate whether changes in plasma IGF-1 concentrations are linked or correlated to growth pattern and possibly changes in locomotion patterns. Our horses entered the study at the age of 15.5±1 months, by which time horses generally have reached 75% of their adult body weight and >90% of their adult height at withers (Martin-Rosset, 2004; NRC, 2007). At this age, daily growth rates can be expected to be less than 400 g day and show a continuous decline as horses get older (NRC, 2007). A decline in growth rate compared with Period 1 (i.e. before high-intensity training was introduced) was observed for height at withers and BW, and for height at croup for all periods except Period 3. However, growth rate of body length and front limb circumference showed a different response, with the rates being equally high in Periods 1 and 2 (no significant difference). Altogether, this provides some support for the suggestion that IGF-1 release triggered growth in some bones. IGF-1 is the major regulator of growth and controls elongation of long bones (such as humerus, radius/ulna, femur, and tibia/fibula (but likely also the vertebral column (Adem et al., 1994)) by promoting chondrocyte proliferation and hypertrophy (Racine and Serrat, 2020). Growth was possible in these

| Table 4. Correlations between insulin-like growth factor 1 (IGF-1) and front and hind asymmetries, body weight (BW), height at withers and croup, body length, front limb circumference, depth of m. longissimus dorsi, subcutaneous fat thickness at croup, body condition score (scale 1-9), daily metabolisable energy (ME) and crude protein (CP) intake/100 kg BW and dietary CP/ME ratio in 16 growing Standardbred horses in training. Correlation analysis performed on data from Periods 1-4. |
|---|---|---|
| Correlation | P-value |
| Body length | -0.23 | 0.074 |
| Depth m. longissimus | 0.040 | 0.75 |
| Weight | -0.28 | 0.029 |
| Height at withers | -0.18 | 0.16 |
| Height at croup | -0.12 | 0.37 |
| Front limb cire. | -0.013 | 0.92 |
| Fat at croup | -0.14 | 0.26 |
| Body condition score | 0.34 | 0.006 |
| Front limb asymmetry | 0.100 | 0.94 |
| Hind limb pushoff asymmetry | -0.25 | 0.045 |
| Hind limb impact asymmetry | -0.23 | 0.067 |
| CP intake | 0.14 | 0.26 |
| ME intake | -0.074 | 0.56 |
| CP/ME | 0.34 | 0.007 |

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bodies since fusion is not completed in horses until around 3 years of age (Strand et al., 2007). The uneven growth can be seen by comparing the relationship between length and height during Periods 1 and 2. In Period 1, body length was 100% of height at withers whereas in Period 2 it was 102%, i.e. the horses were 3 cm longer than their height (Ringmark et al., 2013, 2017). This relationship (longer than tall) was present also at the age of 3 years in these horses (Ringmark et al., 2017). Accordingly, our data show that the body proportions of the horses changed following the point when high-intensity exercise was introduced and that these changes may have been IGF-1-stimulated. However, further studies are needed to confirm the importance of high-intensity exercise for alterations in IGF-1 release and growth patterns in growing horses.

Interestingly, the growth rate of m. longissimus increased in Periods 2 and 3 compared with Period 1. However, it is unlikely that IGF-1 was the long-term trigger for this growth, since the elevated growth continued many months after the peak in IGF-1 was observed. There was also a small, but significant, change (positive compared with Period 1) in body fat thickness at the croup throughout the study, but this pattern was not reflected in the body condition scoring. We have no explanation for this contradiction, but the changes were very small and may not have had any biological impact. We concluded that growth rates did not differ between the training groups (C, R), which was as expected since there was no difference in IGF-1 levels.

Changes in front limb circumference were small and clearly within the range of error of the method (tape measure with 1 mm precision). Growth was expected to be small, but front limb circumference is also highly affected by several other factors, e.g. the size of the cannon bone and tendons, the thickness of the horse’s coat, and possible swelling.

The correlations observed between IGF-1 and BW and BCS most likely reflect the long-term parallel processes of decreasing IGF-1 levels, increased BW and decrease in BCS as horses approach race fitness by the end of the study. If horses go through a period with uneven growth, this could be expected to affect their locomotion pattern. In this study, locomotion pattern was altered when high-intensity training was introduced, with both hind and front limb asymmetry increasing in Period 2. For hind limb asymmetry, the elevation was temporary and during the subsequent six-month period (Period 3) no elevation was observed. In contrast, mean front limb asymmetry remained elevated for Period 3 and 4. However, front limb asymmetry showed large variation between recording occasions, as previously reported by Ringmark et al. (2016), with the highest peak observed in April of Period 2. Front limb asymmetry then declined in Period 3 and on one occasion was not different from that observed in Period 1.

In Period 4 there was another peak, but by the end of that period the level did not differ from that observed in Period 1. However, when the data were pooled into longer periods (each including several recording occasions), this pattern was only observed as numerical differences. It is possible that the temporary increase in IGF-1 and the growth pattern observed during Period 2 contributed to the changes in locomotion pattern during that period, but other factors most likely had a greater influence, e.g. true lameness or muscle soreness. The horses also showed an elevated response to flexion tests during Period 2 (Ringmark et al., 2016). One possible explanation that has been offered previously is that unaccustomed exercise elevates aspartate aminotransferase (AST) levels, indicating muscle damage (Mack et al., 2014) and possibly soreness. To better understand the effect of growth on changes in locomotion pattern further studies are needed, preferably with more detailed objective locomotion asymmetry measures.

In the present study, the horses were fed a standardised diet ad libitum and it was interesting to observe that daily energy intake increased when high-intensity exercise was introduced, while body fat and body condition remained stable. Since IGF-1 levels can be influenced by energy and protein intake, it is important to control and monitor feed intake. If the increased requirement for energy and protein with increasing exercise is not met, the body will end up in a catabolic condition, which will have significant lowering effects on IGF-1 levels (Chelikani et al., 2004; Kiani, 2013; Salazar-Ortiz et al., 2014; Sticker et al., 1995). In the present study, the variations in CP intake were due to variations in the CP/ME ratio of the different haylage batches used throughout the study.

In conclusion, the results of this study indicate that introduction to high-intensity training induces IGF-1 release in horses, but that a 30% difference in the volume of high-intensity training does not affect IGF-1 levels. The temporary interruption in decline in IGF-1 release with the onset of high-intensity training may have influenced growth pattern in the horses, but further studies are needed to assess causality.

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Conflict of interest

All authors declare that they have no conflict of interest.

References


High intensity training distance and plasma IGF-1 concentrations


The aim of this thesis was to identify metabolomic and proteomic changes in 1.5-3.5 year-old Standardbred horses subjected to two different high-intensity training programmes. Metabolomic differences between the training groups were only observed at 2 years of age, and were associated with energy production, amino acid metabolism, pH-buffering and vascular responses. Both the metabolomic and proteomic profile in Standardbred horses changed over time.

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SLU generates knowledge for the sustainable use of biological natural resources. Research, education, extension, as well as environmental monitoring and assessment are used to achieve this goal.