



DOCTORAL THESIS NO. 2024:10
FACULTY OF VETERINARY MEDICINE AND ANIMAL SCIENCE

Metabolomic and proteomic changes in Standardbred horses in training

LISA JOHANSSON



Metabolomic and proteomic changes in Standardbred horses in training

Lisa Johansson

Faculty of Veterinary Medicine and Animal Science
Department of Anatomy, Physiology and Biochemistry
Uppsala



SWEDISH UNIVERSITY
OF AGRICULTURAL
SCIENCES

DOCTORAL THESIS

Uppsala 2024

Acta Universitatis Agriculturae Sueciae
2024:10

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

ISSN 1652-6880

ISBN (print version) 978-91-8046-284-6

ISBN (electronic version) 978-91-8046-285-3

<https://doi.org/10.54612/a.nndknetvqo>

© 2024 Lisa Johansson, <https://orcid.org/0009-0003-2901-3438>

Swedish University of Agricultural Sciences, Department of Anatomy, Physiology and Biochemistry, Uppsala, Sweden

The summary chapter of this thesis is licensed under CC BY NC ND 4.0, other licences or copyright may apply to illustrations and attached articles.

Print: SLU Grafisk Service, Uppsala 2023

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 blod-plasma-prover 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 träningsgrupper, Hög och Låg. Båda grupperna följde samma träningsprogram med skillnaden att träningsgruppen Låg hade 30% kortare hög-intensiv träningsdistans jämfört med träningsgrupp Hög. Båda grupperna tränades 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 åldrar (2, 2.5 och 3.5) jämfört med 1.5 års ålder. Metaboliterna som skilde signifikant för både träningsgrupper och över tid var associerade med aerob energiproduktion och aminosyrametabolismen, och kanske också med pH-buffring och kardiovaskulära förändringar. Proteomikanalysen visade inte på några signifikanta skillnader mellan träningsgrupperna men 17 proteiner skiljde sig signifikant 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

Contents

List of publications	7
Abbreviations	9
1. Introduction	13
1.1 Training effects in sport horses	13
1.2 Growth in sport horses	14
1.2.1 Insulin-like growth factor 1	15
1.3 Enzyme-linked immunosorbent assay	17
1.4 Mass spectrometry	17
1.4.1 Metabolomics	18
1.4.2 Proteomics	20
2. Aims of the thesis	21
3. Material and Methods	23
3.1 Horses and management	23
3.1.1 Training	24
3.2 Blood sample collection	25
3.3 IGF-1 analysis by ELISA	26
3.4 Metabolomics	26
3.5 Proteomics	27
3.6 Statistical analyses	27
4. Main results	29
4.1 IGF-1	29
4.2 Metabolomics	30
4.2.1 Differences between training groups	30
4.2.2 Changes over time	31
4.3 Proteomics	33
4.3.1 Changes over time	33

5.	Discussion	35
5.1	Effect of training on IGF-1	35
5.2	Metabolomics.....	37
5.3	Proteomics	39
6.	Future research	41
	References.....	43
	Popular science summary	51
	Populärvetenskaplig sammanfattning	53
	Acknowledgements	55

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

- I. Johansson, L., Ringmark, S., Skiöldebrand, E., Bergquist, J. and Jansson, A. (2022). Reduced high-intensity training distance in growing horses had no effect on IGF-1 concentrations, but training onset interrupted time-dependent IGF-1 decline. *Comparative Exercise Physiology* 18 (3), 201-209.
- 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)

Paper I is reproduced with the permission of the publisher.

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

Cer	Ceramide
CP	Crude protein
CV	Coefficient of variation
DG	Diglyceride
DiCA	Dodecanedioic acid
ECM1	Extracellular matrix protein 1
ELISA	Enzyme-linked immunosorbent assay
ESI	Electrospray ionisation
FA	Fatty acid
FDR	False discovery rate
FIA	Flow-injection analysis
GH	Growth hormone
HAD	3-hydroxyacyl-CoA dehydrogenas
HArg	Homoarginine
HexCer	Hexosylceramide
Ig	Immunoglobulin
IGF-1	Insulin-like growth factor 1
IGFBP	Insulin-like growth factor binding protein
IS	Internal standard
JCHAIN	Joining chain of multimeric IgA and IgM
Leu	Leucine
ME	Metabolisable energy
Met.SO	Methionine-sulfoxide
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry

NO	Nitric oxide
PC	Phosphatidylcholine
PUFA	Polyunsaturated fatty acids
SDMA	Symmetric dimethylarginine
SERPING1	Serpin family G member 1
TCA	Tricarboxylic acid cycle
TG	Triglyceride
UPLC	Ultra-high performance liquid chromatography
Val	Valine

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,

1985). 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.

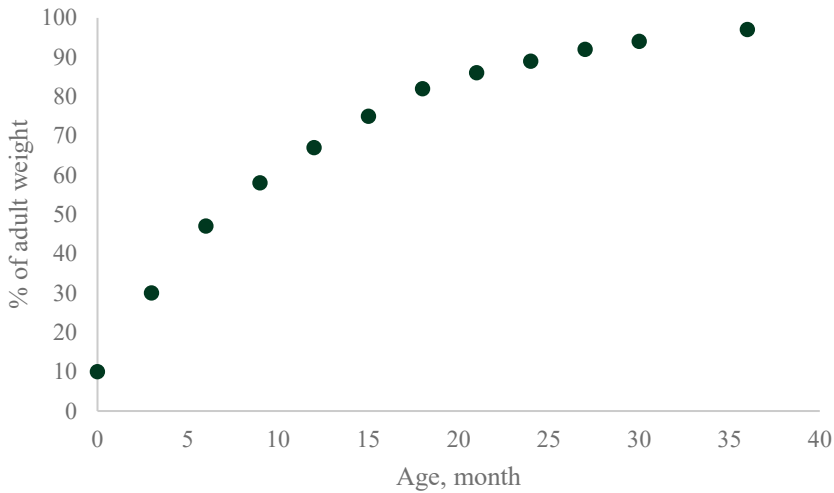


Figure 1. Changes over time in the growth rate of young horses. Source: (Jansson, 2013).

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

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).

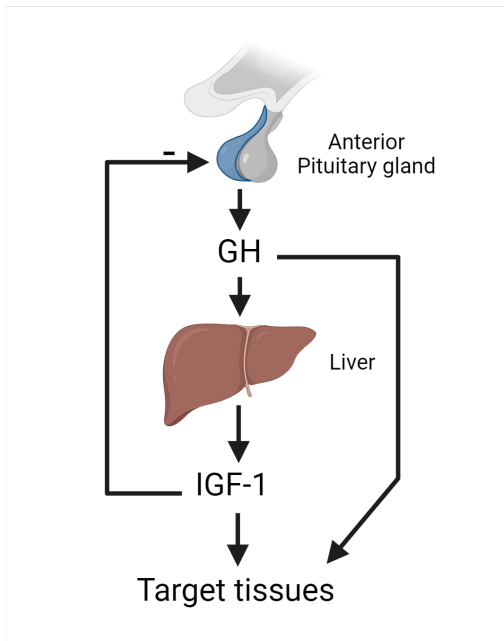


Figure 2. Growth hormone (GH) and insulin-like growth factor 1 (IGF-1) synthesis

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).

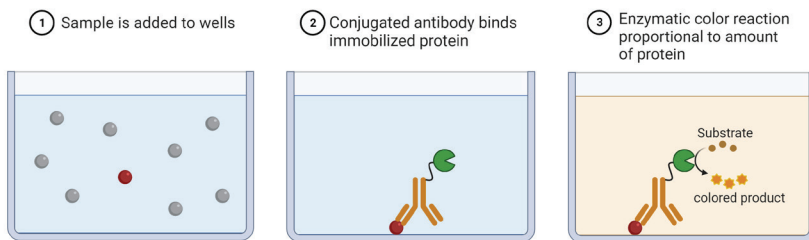


Figure 3. Steps involved in measuring protein concentration in a sample by enzyme-linked immunosorbent assay (ELISA).

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 1. Summary of previous metabolomics studies in horses

	Year	Breed	Type of sample	Duration	No. of Animals
Bazzano <i>et al.</i>	2020	Standardbred	saliva, serum	1 exercise test	12
Jang <i>et al.</i>	2017	Thoroughbred	plasma, muscle, urine	1 exercise bout	3
Klein <i>et al.</i>	2020	Standardbred	muscle	12 week	8
Le Moyec <i>et al.</i>	2019	Arabian	plasma	1 Endurance race	16 (90km), 15 (120km), 9 (160km)
Le Moyec <i>et al.</i>	2014	Arabian	plasma	1 Endurance race	28
Luck <i>et al.</i>	2015	Arabian	plasma	1 Endurance. race	46 (young), 11 (mature)
Mach <i>et al.</i>	2017	Arabian	plasma	1 Endurance race	10
Ohmura <i>et al.</i>	2021	Thoroughbred	muscle	1 exercise bout	6
Ueda <i>et al.</i>	2019	Thoroughbred	plasma	1 race	60
Wang <i>et al.</i>	2022	Yili	plasma	1 race	8

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

	Year	Breed	Type of sample	Duration	No. of Animals
Bouwman <i>et al.</i>	2010	Standardbred	muscle	24 weeks	16
Ichibangase & Imai	2009	Thoroughbred	muscle	15 weeks	4
Scoppetta <i>et al.</i>	2012	Anglo-Arabian	plasma	1 Endurance race	8

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 ($\sim 9\text{m}^2$) for approximately 16 h per day from Monday to Thursday/Friday, while they spent the rest of the time together in a paddock ($\sim 20,000\text{m}^2$) 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*

Training type	High		Low	
	2 years old	3 years old	2 years old	3 years old
Heat	1-2 x 1600 m	2-3 x 1600 m	1-2 x 1100 m	2-3 x 1100 m
Interval	6 x 500-700 m	6 x 700 m	4 x 500-700 m	4 x 700 m
Uphill interval		6 x 600 m		4 x 600 m

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.

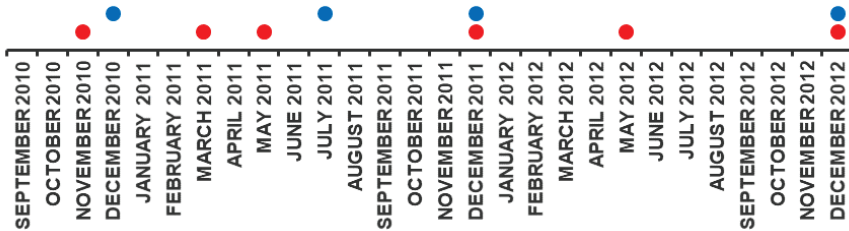


Figure 4. Time line showing blood samples used in Paper I (red dots) and blood samples used in Papers II and III (blue dots).

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 (Baldon, 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.

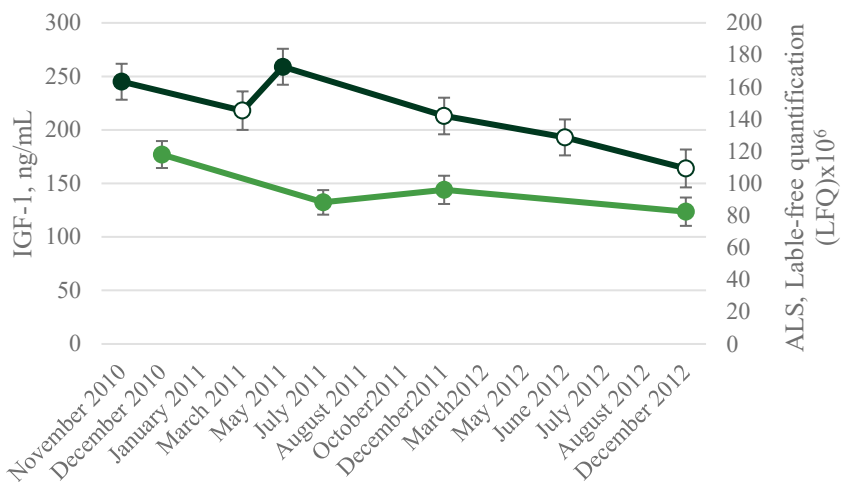


Figure 5. Changes over time in the concentrations (least-squares mean \pm standard error) of insulin-like growth factor 1 (IGF-1, dark green) and acid-labile subunit (ALS, green) in blood plasma samples from 16 Standardbred horses in training (high-intensity training was introduced in March 2011). Concentrations 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).

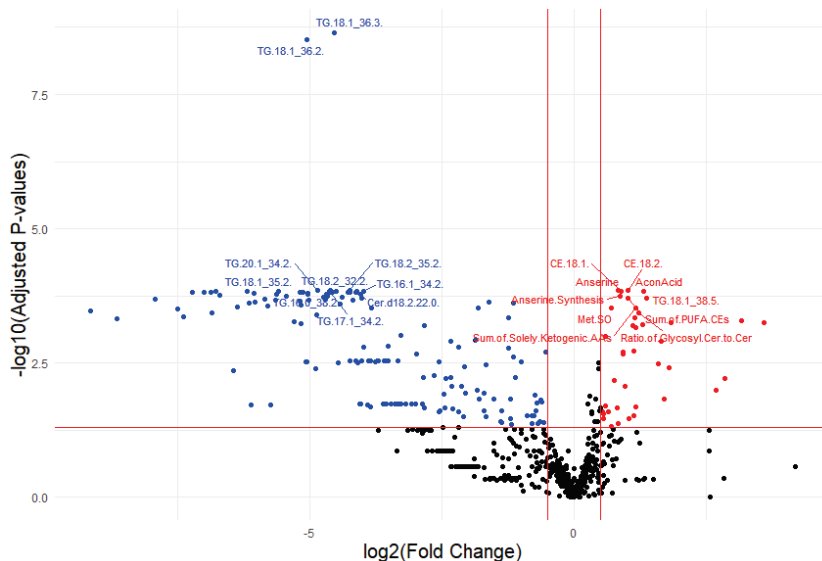


Figure 6. Volcano plot of metabolites present in higher (red), lower (blue) and unchanged (black) concentrations in plasma samples (taken at 2 years of age) from 16 Standardbred horses in high-intensity training (High group) than in samples from horses in lower-intensity training (Low group). Upper left 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.

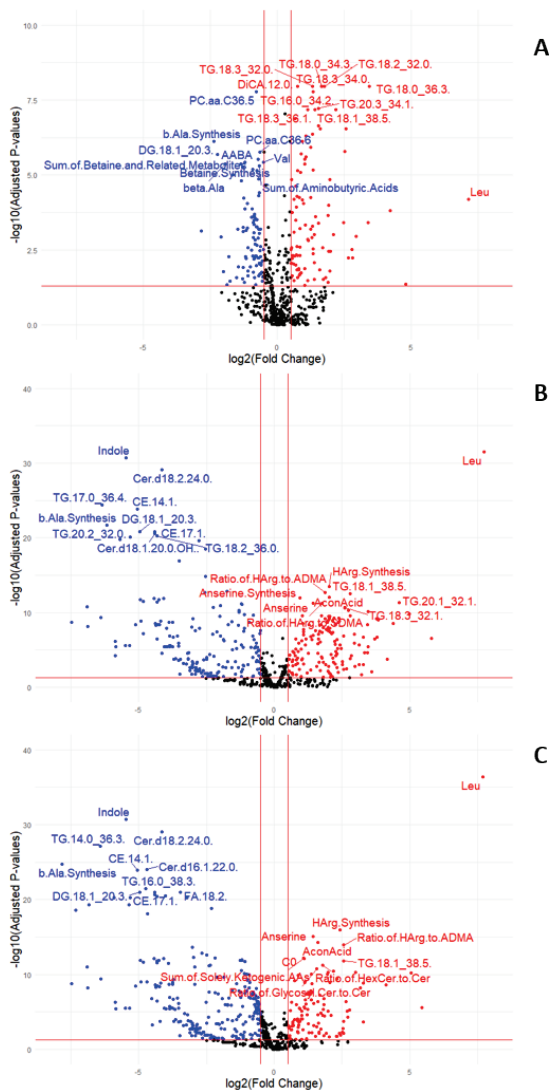


Figure 7. Volcano plot of plasma metabolites present in higher (red), lower (blue) and unchanged (black) values at (A) 2 years of age, (B) 2.5 years of age and (C) 3.5 years of age compared with 1.5 years of age, for 16 Standardbred horses in training. Upper left quadrant contains adjusted p -values <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).

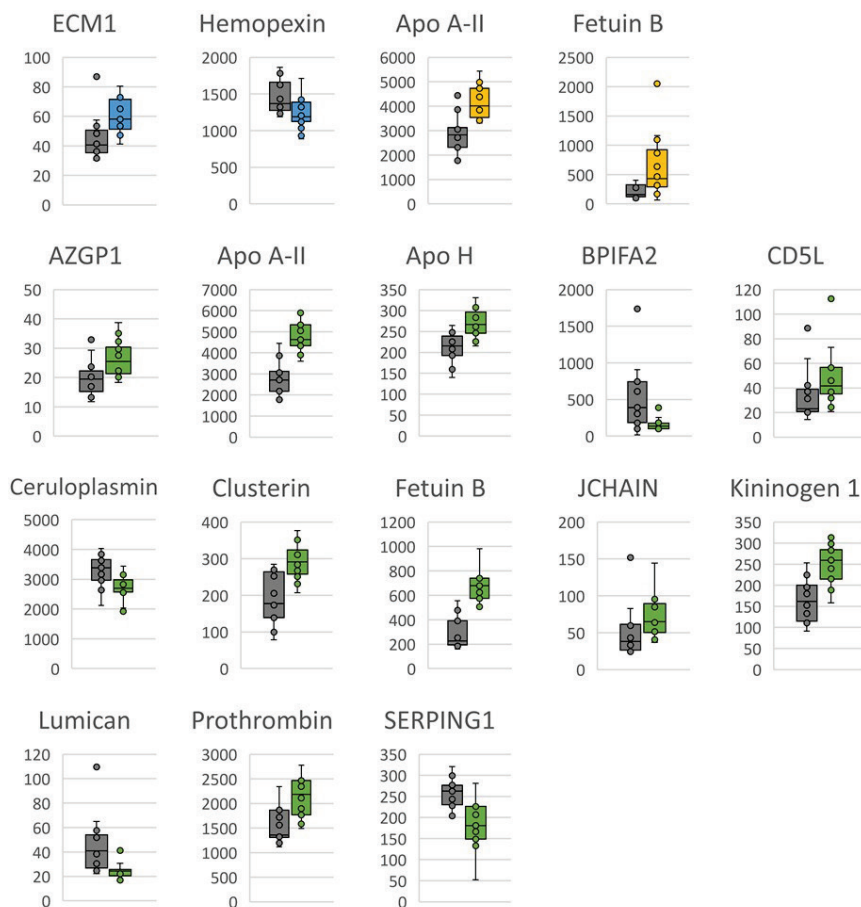


Figure 8. False discovery rate (FDR)-adjusted significantly different plasma proteins in 16 Standardbred horses in training at 2 years of age (blue), 2.5 years of age (yellow) and 3.5 years of age (green), compared with 1.5 years of age (grey). The y-axis shows label-free quantification (LFQ) $\times 10^6$ 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 conclusions 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

- Anderson, N. L., & Anderson, N. G. (2002). The human plasma proteome: history, character, and diagnostic prospects. *Mol Cell Proteomics*, *1*(11), 845-867. <https://doi.org/10.1074/mcp.r200007-mcp200>
- Anthony, J. C., Anthony, T. G., Kimball, S. R., & Jefferson, L. S. (2001). Signaling pathways involved in translational control of protein synthesis in skeletal muscle by leucine. *J Nutr*, *131*(3), 856S-860S. <https://doi.org/10.1093/jn/131.3.856S>
- Atsenova, N., Palova, N., Mehandjyiski, I., Neov, B., Radoslavov, G., & Hristov, P. (2022). The Sequence Analysis of Mitochondrial DNA Revealed Some Major Centers of Horse Domestications: The Archaeologist's Cut. *Journal of Equine Veterinary Science*, *109*. <https://doi.org/10.1016/j.jevs.2021.103830>
- Bailey, C. J., Reid, S. W., Hodgson, D. R., & Rose, R. J. (1999). Impact of injuries and disease on a cohort of two- and three-year-old thoroughbreds in training. *Veterinary Record*, *145*(17), 487-493. <https://doi.org/10.1136/vr.145.17.487>
- Ballesteros, M., Leung, K. C., Ross, R. J. M., Iismaa, T. P., & Ho, K. K. Y. (2000). Distribution and abundance of messenger ribonucleic acid for growth hormone receptor isoforms in human tissues. *Journal of Clinical Endocrinology & Metabolism*, *85*(8), 2865-2871. <https://doi.org/10.1210/jc.85.8.2865>
- Baskerville, C. L., Bamford, N. J., Harris, P. A., & Bailey, S. R. (2017). Comparison and validation of ELISA assays for plasma insulin-like growth factor-1 in the horse. *Open Veterinary Journal*, *7*(1), 75-80. <https://doi.org/10.4314/ovj.v7i1.12>
- Bazzano, M., Laghi, L., Zhu, C. L., Lotito, E., Sgariglia, S., Tesei, B., & Laus, F. (2020). Exercise Induced Changes in Salivary and Serum Metabolome in Trained Standardbred, Assessed by H-NMR. *Metabolites*, *10*(7). <https://doi.org/10.3390/metabo10070298>
- Benjamini, Y., & Hochberg, Y. (1995). Controlling the False Discovery Rate - a Practical and Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society Series B-Statistical Methodology*, *57*(1), 289-300. <https://doi.org/10.1111/j.2517-6161.1995.tb02031.x>
- Boldyrev, A. A., Aldini, G., & Derave, W. (2013). Physiology and pathophysiology of carnosine. *Physiol Rev*, *93*(4), 1803-1845. <https://doi.org/10.1152/physrev.00039.2012>

- Bouwman, F. G., van Ginneken, M. M. E., Noben, J. P., Royackers, E., de Graaf-Roelfsema, E., Wijnberg, I. D., van der Kolk, J. H., Mariman, E. C. M., & van Breda, E. (2010). Differential expression of equine muscle biopsy proteins during normal training and intensified training in young standardbred horses using proteomics technology. *Comparative Biochemistry and Physiology D-Genomics & Proteomics*, 5(1), 55-64. <https://doi.org/10.1016/j.cbd.2009.11.001>
- de Graaf-Roelfsema, E., Keizer, H. A., van Breda, E., Wijnberg, I. D., & van der Kolk, J. H. (2007). Hormonal responses to acute exercise, training and overtraining - A review with emphasis on the horse. *Veterinary Quarterly*, 29(3), 82-101. <https://doi.org/10.1080/01652176.2007.9695232>
- Deckers, M. M., Smits, P., Karperien, M., Ni, J., Tylzanowski, P., Feng, P., Parmelee, D., Zhang, J., Bouffard, E., Gentz, R., Lowik, C. W., & Merregaert, J. (2001). Recombinant human extracellular matrix protein 1 inhibits alkaline phosphatase activity and mineralization of mouse embryonic metatarsals in vitro. *Bone*, 28(1), 14-20. [https://doi.org/10.1016/s8756-3282\(00\)00428-2](https://doi.org/10.1016/s8756-3282(00)00428-2)
- Degen, S. J. F., & Sun, W. Y. (1998). The biology of prothrombin. *Critical Reviews in Eukaryotic Gene Expression*, 8(2), 203-224. <https://doi.org/10.1615/CritRevEukarGeneExpr.v8.i2.60>
- Denecke, B., Graber, S., Schafer, C., Heiss, A., Woltje, M., & Jahnen-Dechent, W. (2003). Tissue distribution and activity testing suggest a similar but not identical function of fetuin-B and fetuin-A. *Biochem J*, 376(Pt 1), 135-145. <https://doi.org/10.1042/BJ20030676>
- Domon, B., & Aebersold, R. (2006). Review - Mass spectrometry and protein analysis. *Science*, 312(5771), 212-217. <https://doi.org/10.1126/science.1124619>
- Dyson, P. K., Jackson, B. F., Pfeiffer, D. U., & Price, J. S. (2008). Days lost from training by two- and three-year-old Thoroughbred horses: a survey of seven UK training yards. *Equine Vet J*, 40(7), 650-657. <https://doi.org/10.2746/042516408x363242>
- Essen-Gustavsson, B., & Lindholm, A. (1985). Muscle fibre characteristics of active and inactive standardbred horses. *Equine Vet J*, 17(6), 434-438. <https://doi.org/10.1111/j.2042-3306.1985.tb02549.x>
- Essen-Gustavsson, B., McMiken, D., Karlstrom, K., Lindholm, A., Persson, S., & Thornton, J. (1989). Muscular Adaptation of Horses during Intensive Training and Detraining. *Equine Veterinary Journal*, 21(1), 27-33. <https://doi.org/10.1111/j.2042-3306.1989.tb02085.x>
- Evans, D. L. (1985). Cardiovascular Adaptations to Exercise and Training. *Veterinary Clinics of North America-Equine Practice*, 1(3), 513-531. [https://doi.org/10.1016/S0749-0739\(17\)30748-4](https://doi.org/10.1016/S0749-0739(17)30748-4)

- Faria, A. C. S., Veldhuis, J. D., Thorner, M. O., & Vance, M. L. (1989). Half-Time of Endogenous Growth-Hormone (Gh) Disappearance in Normal Man after Stimulation of Gh Secretion by Gh-Releasing Hormone and Suppression with Somatostatin. *Journal of Clinical Endocrinology & Metabolism*, 68(3), 535-541. <https://doi.org/10.1210/jcem-68-3-535>
- Fortier, L. A., Kornatowski, M. A., Mohammed, H. O., Jordan, M. T., O'Cain, L. C., & Stevens, W. B. (2005). Age-related changes in serum insulin-like growth factor I, insulin-like growth factor-I binding protein-3 and articular cartilage structure in Thoroughbred horses. *Equine Veterinary Journal*, 37(1), 37-42. <https://doi.org/10.2746/0425164054406838>
- Goldansaz, S. A., Guo, A. C., Sajed, T., Steele, M. A., Plastow, G. S., & Wishart, D. S. (2017). Livestock metabolomics and the livestock metabolome: A systematic review. *PLoS One*, 12(5), e0177675. <https://doi.org/10.1371/journal.pone.0177675>
- Han, Z. Q., Ni, J., Smits, P., Underhill, C. B., Xie, B., Chen, Y. X., Liu, N. F., Tylzanowski, P., Parmelee, D., Feng, P., Ding, I., Gao, F., Gentz, R., Huylebroeck, D., Merregaert, J., & Zhang, L. R. (2001). Extracellular matrix protein 1 (ECM1) has angiogenic properties and is expressed by breast tumor cells. *Faseb Journal*, 15(6), 988-994. <https://doi.org/10.1096/fj.99-0934com>
- Henckel, P. (1983). Training and growth induced changes in the middle gluteal muscle of young Standardbred trotters. *Equine Vet J*, 15(2), 134-140. <https://doi.org/10.1111/j.2042-3306.1983.tb01736.x>
- Hodgson, D. R. (1985). Energy considerations during exercise. *Vet Clin North Am Equine Pract*, 1(3), 447-460. [https://doi.org/10.1016/s0749-0739\(17\)30744-7](https://doi.org/10.1016/s0749-0739(17)30744-7)
- Ichibangase, T., & Imai, K. (2009). Application of Fluorogenic Derivatization-Liquid Chromatography-Tandem Mass Spectrometric Proteome Method to Skeletal Muscle Proteins in Fast Thoroughbred Horses. *Journal of Proteome Research*, 8(4), 2129-2134. <https://doi.org/10.1021/pr801004s>
- Jackson, B. F., Goodship, A. E., Eastell, R., & Price, J. S. (2003). Evaluation of serum concentrations of biochemical markers of bone metabolism and insulin-like growth factor I associated with treadmill exercise in young horses. *American Journal of Veterinary Research*, 64(12), 1549-1556. <https://doi.org/10.2460/ajvr.2003.64.1549>
- Jang, H. J., Kim, D. M., Kim, K. B., Park, J. W., Choi, J. Y., Oh, J. H., Song, K. D., Kim, S., & Cho, B. W. (2017). Analysis of metabolomic patterns in thoroughbreds before and after exercise. *Asian-Australas J Anim Sci*, 30(11), 1633-1642. <https://doi.org/10.5713/ajas.17.0167>
- Jansson, A. (2013). *Utfodringsrekommendationer för häst*. Institutionen för husdjurens utfodring och vård, Sveriges lantbruksuniversitet.

- Jiang, L. H., Wang, M., Lin, S., Jian, R. Q., Li, X., Chan, J., Dong, G. L., Fang, H. Y., Robinson, A. E., Snyder, M. P., & Consortium, G. (2020). A Quantitative Proteome Map of the Human Body. *Cell*, *183*(1), 269-+. <https://doi.org/10.1016/j.cell.2020.08.036>
- Juul, A., Moller, S., Mosfeldt-Laursen, E., Rasmussen, M. H., Scheike, T., Pedersen, S. A., Kastrup, K. W., Yu, H., Mistry, J., Rasmussen, S., Müller, J., Henriksen, J., & Skakkebaek, N. E. (1998). The acid-labile subunit of human ternary insulin-like growth factor binding protein complex in serum: Hepatosplanchnic release, diurnal variation, circulating concentrations in healthy subjects, and diagnostic use in patients with growth hormone deficiency. *Journal of Clinical Endocrinology & Metabolism*, *83*(12), 4408-4415. <https://doi.org/10.1210/jc.83.12.4408>
- Khoramipour, K., Sandbakk, O., Keshteli, A. H., Gaeini, A. A., Wishart, D. S., & Chamari, K. (2022). Metabolomics in Exercise and Sports: A Systematic Review. *Sports Med*, *52*(3), 547-583. <https://doi.org/10.1007/s40279-021-01582-y>
- Klein, D. J., Anthony, T. G., & McKeever, K. H. (2021). Metabolomics in equine sport and exercise. *J Anim Physiol Anim Nutr (Berl)*, *105*(1), 140-148. <https://doi.org/10.1111/jpn.13384>
- Klein, D. J., McKeever, K. H., Mirek, E. T., & Anthony, T. G. (2020). Metabolomic Response of Equine Skeletal Muscle to Acute Fatiguing Exercise and Training. *Front Physiol*, *11*, 110. <https://doi.org/10.3389/fphys.2020.00110>
- Kraemer, W. J., & Ratamess, N. A. (2005). Hormonal responses and adaptations to resistance exercise and training. *Sports Medicine*, *35*(4), 339-361. <https://doi.org/10.2165/00007256-200535040-00004>
- Lawrence, L. M. (1990). Nutrition and fuel utilization in the athletic horse. *Vet Clin North Am Equine Pract*, *6*(2), 393-418. [https://doi.org/10.1016/s0749-0739\(17\)30548-5](https://doi.org/10.1016/s0749-0739(17)30548-5)
- Le Moyec, L., Robert, C., Triba, M. N., Billat, V. L., Mata, X., Schibler, L., & Barrey, E. (2014). Protein Catabolism and High Lipid Metabolism Associated with Long-Distance Exercise Are Revealed by Plasma NMR Metabolomics in Endurance Horses. *PLoS One*, *9*(3). <https://doi.org/10.1371/journal.pone.0090730>
- Le Moyec, L., Robert, C., Triba, M. N., Bouchemal, N., Mach, N., Rivière, J., Zalachas-Rebours, E., & Barrey, E. (2019). A First Step Toward Unraveling the Energy Metabolism in Endurance Horses: Comparison of Plasma Nuclear Magnetic Resonance Metabolomic Profiles Before and After Different Endurance Race Distances. *Frontiers in Molecular Biosciences*, *6*. <https://doi.org/10.3389/fmolb.2019.00045>
- Lee, C., Bongcam-Rudloff, E., Sollner, C., Jahnke-Dechent, W., & Claesson-Welsh, L. (2009). Type 3 cystatins; fetuins, kininogen and histidine-rich

- glycoprotein. *Frontiers in Bioscience-Landmark*, 14, 2911-2922. <https://doi.org/10.2741/3422>
- Luck, M. M., Le Moyec, L., Barrey, E., Triba, M. N., Bouchemal, N., Savarin, P., & Robert, C. (2015). Energetics of endurance exercise in young horses determined by nuclear magnetic resonance metabolomics. *Frontiers in Physiology*, 6. <https://doi.org/10.3389/fphys.2015.00198>
- Mach, N., Ramayo-Caldas, Y., Clark, A., Moroldo, M., Robert, C., Barrey, E., López, J. M., & Le Moyec, L. (2017). Understanding the response to endurance exercise using a systems biology approach: combining blood metabolomics, transcriptomics and miRNomics in horses. *Bmc Genomics*, 18. <https://doi.org/10.1186/s12864-017-3571-3>
- Maïga, S. F., Kalopissis, A. D., & Chabert, M. (2014). Apolipoprotein A-II is a key regulatory factor of HDL metabolism as appears from studies with transgenic animals and clinical outcomes. *Biochimie*, 96, 56-66. <https://doi.org/10.1016/j.biochi.2013.08.027>
- Malinowski, K., Christensen, R. A., Hafs, H. D., & Scanes, C. G. (1996). Age and breed differences in thyroid hormones, insulin-like growth factor (IGF)-I and IGF binding proteins in female horses. *Journal of Animal Science*, 74(8), 1936-1942. <https://doi.org/10.2527/1996.7481936x>
- McGowan, C. M., Golland, L. C., Evans, D. L., Hodgson, D. R., & Rose, R. J. (2002). Effects of prolonged training, overtraining and detraining on skeletal muscle metabolites and enzymes. *Equine Veterinary Journal*, 34, 257-263. <https://doi.org/10.1111/j.2042-3306.2002.tb05429.x>
- Miller, I., Friedlein, A., Tsangaris, G., Maris, A., Fountoulakis, M., & Gemeiner, M. (2004). The serum proteome of Equus caballus. *Proteomics*, 4(10), 3227-3234. <https://doi.org/10.1002/pmic.200400846>
- Mori M., M. D., Konoha-Mizuno K., Sadakane Y., Kawahara M. (2015). Carnosine concentration in the muscle of thoroughbred horses and its implications in exercise performance. *Trace Nutrients Research*, 32, 49-53. https://doi.org/10.51029/jtnrs.32.0_49
- Morifuji, M., Kanda, A., Koga, J., Kawanaka, K., & Higuchi, M. (2010). Post-exercise carbohydrate plus whey protein hydrolysates supplementation increases skeletal muscle glycogen level in rats. *Amino Acids*, 38(4), 1109-1115. <https://doi.org/10.1007/s00726-009-0321-0>
- National Research Council. (2007). *Nutrient Requirements of Horses: Sixth Revised Edition*. The National Academies Press. <https://doi.org/10.17226/11653>
- Noble, G. K., Houghton, E., Roberts, C. J., Faustino-Kemp, J., de Kock, S. S., Swanepoel, B. C., & Sillence, M. N. (2007). Effect of exercise, training, circadian rhythm, age, and sex on insulin-like growth factor-1 in the horse. *Journal of Animal Science*, 85(1), 163-171. <https://doi.org/10.2527/jas.2006-210>

- Ohmura, H., Mukai, K., Takahashi, Y., & Takahashi, T. (2021). Metabolomic analysis of skeletal muscle before and after strenuous exercise to fatigue. *Scientific Reports*, *11*(1). <https://doi.org/10.1038/s41598-021-90834-y>
- Oral, O. (2021). Nitric oxide and its role in exercise physiology. *The Journal of sports medicine and physical fitness*, *61*(9), 1208-1211. <https://doi.org/10.23736/s0022-4707.21.11640-8>
- Otte, K., Rozell, B., Gessbo, A., & Engstrom, W. (1996). Cloning and sequencing of an equine insulin-like growth factor I cDNA and its expression in fetal and adult tissues. *General and Comparative Endocrinology*, *102*(1), 11-15. <https://doi.org/10.1006/gcen.1996.0040>
- Popot, M. A., Bobin, S., Bonnaire, Y., Delahaut, P. H., & Closset, J. (2001). IGF-I plasma concentrations in non-treated horses and horses administered with methionyl equine somatotropin. *Research in Veterinary Science*, *71*(3), 167-173. <https://doi.org/10.1053/rvsc.2001.0505>
- Ringmark, S. (2014). *A Forage-Only Diet and Reduced High Intensity Training Distance in Standardbred Horses: Growth, Health and Performance*. [Diss, Swedish University of Agricultural Sciences]. Uppsala. <https://res.slu.se/id/publ/61636>
- Ringmark, S., Jansson, A., Lindholm, A., Hedenstrom, U., & Roepstorff, L. (2016). A 2.5 year study on health and locomotion symmetry in young Standardbred horses subjected to two levels of high intensity training distance. *Vet J*, *207*, 99-104. <https://doi.org/10.1016/j.tvjl.2015.10.052>
- Ringmark, S., Lindholm, A., Hedenstrom, U., Lindinger, M., Dahlborn, K., Kvarn, C., & Jansson, A. (2015). Reduced high intensity training distance had no effect on VLa4 but attenuated heart rate response in 2-3-year-old Standardbred horses. *Acta Vet Scand*, *57*(1), 17. <https://doi.org/10.1186/s13028-015-0107-1>
- Roneus, M., Essen-Gustavsson, B., Lindholm, A., & Persson, S. G. (1992). Skeletal muscle characteristics in young trained and untrained standardbred trotters. *Equine Vet J*, *24*(4), 292-294. <https://doi.org/10.1111/j.2042-3306.1992.tb02838.x>
- Salazar-Ortiz, J., Monget, P., & Guillaume, D. (2014). The influence of nutrition on the insulin-like growth factor system and the concentrations of growth hormone, glucose, insulin, gonadotropins and progesterone in ovarian follicular fluid and plasma from adult female horses (*Equus Caballus*). *Reproductive Biology and Endocrinology*, *12*. <https://doi.org/10.1186/1477-7827-12-72>
- Scoppetta, F., Tartaglia, M., Renzone, G., Avellini, L., Gaiti, A., Scaloni, A., & Chiaradia, E. (2012). Plasma protein changes in horse after prolonged physical exercise: a proteomic study. *J Proteomics*, *75*(14), 4494-4504. <https://doi.org/10.1016/j.jprot.2012.04.014>

- Sercu, S., Lambeir, A. M., Steenackers, E., El Ghalbzouri, A., Geentjens, K., Sasaki, T., Oyama, N., & Merregaert, J. (2009). ECM1 interacts with fibulin-3 and the beta 3 chain of laminin 332 through its serum albumin subdomain-like 2 domain. *Matrix Biology*, 28(3), 160-169. <https://doi.org/10.1016/j.matbio.2009.02.003>
- Sibal, L., C Agarwal, S., D Home, P., & H Boger, R. (2010). The Role of Asymmetric Dimethylarginine (ADMA) in Endothelial Dysfunction and Cardiovascular Disease. *Current Cardiology Reviews*, 6(2), 82-90. <https://doi.org/10.2174/157340310791162659>
- Smith, A., & McCulloh, R. J. (2015). Hemopexin and haptoglobin: allies against heme toxicity from hemoglobin not contenders. *Front Physiol*, 6, 187. <https://doi.org/10.3389/fphys.2015.00187>
- Smits, P., Poumay, Y., Karperien, M., Tylzanowski, P., Wauters, J., Huylebroeck, D., Ponc, M., & Merregaert, J. (2000). Differentiation-dependent alternative splicing and expression of the extracellular matrix protein 1 gene in human keratinocytes. *J Invest Dermatol*, 114(4), 718-724. <https://doi.org/10.1046/j.1523-1747.2000.00916.x>
- Sodin-Semrl, S., & Rozman, B. (2007). β -Glycoprotein I and its clinical significance: From gene sequence to protein levels. *Autoimmunity Reviews*, 6(8), 547-552. <https://doi.org/10.1016/j.autrev.2007.02.002>
- Sticker, L. S., Thompson, D. L., Bunting, L. D., & Fernandez, J. M. (1996). Dietary protein and energy restriction in mares: Rapid changes in plasma metabolite and hormone concentrations during dietary alteration. *Journal of Animal Science*, 74(6), 1326-1335. <https://doi.org/10.2527/1996.7461326x>
- Swedish Trotting Association. (2023). *Database of Swedish Trotting Association* www.travsport.se
- Tsikis, D., & Wu, G. (2015). Homoarginine, arginine, and relatives: analysis, metabolism, transport, physiology, and pathology. *Amino Acids*, 47(9), 1697-1702. <https://doi.org/10.1007/s00726-015-2055-5>
- Ueda, T., Tozaki, T., Nozawa, S., Kinoshita, K., & Gawahara, H. (2019). Identification of metabolomic changes in horse plasma after racing by liquid chromatography-high resolution mass spectrometry as a strategy for doping testing. *J Equine Sci*, 30(3), 55-61. <https://doi.org/10.1294/jes.30.55>
- Verwilghen, D. R., Vanderheyden, L., Franck, T., Busoni, V., Enzerink, E., Gangl, M., Lejeune, J. P., van Galen, G., Grulke, S., & Serteyn, D. (2009). Variations of plasmatic concentrations of Insulin-like Growth Factor-I in post-pubescent horses affected with developmental osteochondral lesions. *Veterinary Research Communications*, 33(7), 701-709. <https://doi.org/10.1007/s11259-009-9219-2>
- Vigre, H., Chriél, M., Hesselholt, M., Falk-Ronne, J., & Ersboll, A. K. (2002). Risk factors for the hazard of lameness in Danish Standardbred trotters.

- Preventive Veterinary Medicine*, 56(2), 105-117.
[https://doi.org/10.1016/S0167-5877\(02\)00158-7](https://doi.org/10.1016/S0167-5877(02)00158-7)
- Wang, T. L., Zeng, Y. Q., Ma, C. X., Meng, J., Wang, J. W., Ren, W. L., Wang, C. K., Yuan, X. X., Yang, X. X., & Yao, X. K. (2023). Plasma Non-targeted Metabolomics Analysis of Yili Horses Raced on Tracks With Different Surface Hardness. *Journal of Equine Veterinary Science*, 121.
<https://doi.org/10.1016/j.jevs.2022.104197>
- Wei, X., Liu, X., Tan, C. H., Mo, L. J., Wang, H., Peng, X., Deng, F., & Chen, L. F. (2019). Expression and Function of Zinc-2-Glycoprotein. *Neuroscience Bulletin*, 35(3), 540-550. <https://doi.org/10.1007/s12264-018-00332-x>
- Westermann, C. M., Dorland, L., Wijnberg, I. D., de Sain-van der Velden, M. G. M., van Breda, E., Barneveld, A., de Graaf-Roelfsema, E., Keizer, H. A., & van der Kolk, J. H. (2011). Amino acid profile during exercise and training in Standardbreds. *Res Vet Sci*, 91(1), 144-149.
<https://doi.org/10.1016/j.rvsc.2010.08.010>
- Yakar, S., & Isaksson, O. (2016). Regulation of skeletal growth and mineral acquisition by the GH/IGF-1 axis: Lessons from mouse models. *Growth Hormone & Igf Research*, 28, 26-42.
<https://doi.org/10.1016/j.ghir.2015.09.004>
- Yakar, S., Werner, H., & Rosen, C. J. (2018). Insulin-like growth factors: actions on the skeleton. *Journal of Molecular Endocrinology*, 61(1), T115-T137.
<https://doi.org/10.1530/Jme-17-0298>
- Zhou, B., Xiao, J. F., Tuli, L., & Resson, H. W. (2012). LC-MS-based metabolomics. *Molecular Biosystems*, 8(2), 470-481.
<https://doi.org/10.1039/c1mb05350g>

Popular science summary

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 prestationsförmågan 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 galoppörer 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 grupperna 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 näringsinnehållet 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, aminosyror ämnesomsättning och troligtvis också inblandade i cellens buffertkapacitet (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 sårhäkning. De proteiner som var inblandade i inflammation och sårhäkning 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.

Acknowledgements

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.

I would also like to thank all the sponsors which made this study possible, Wången AB, Trioplast AB, Dow Chemicals, Swedish Trotting Association, Swedish Horse Council Foundation, Swedish Horse Racing Totalisator Board and Swedish University of Agricultural Sciences, without you it would not have been possible. I have met and worked with a lot of people during my PhD-studies and I would like to thank each and every one of you but I would like to thank some of you a bit extra.

Anna Jansson tack för att du gav mig möjligheten att göra det här projektet och för all support, peppning, goda råd och för all kunskap du har gett mig. Det har alltid kännas som att du har haft tid för både små stora frågor även när du har haft mycket annat på gång. Hade inte kunnat göra detta utan dig!

Sara Ringmark tack för att du alltid är positiv och kommer med förslag och råd även om saker som inte handlar om jobbet. Du har varit en ovärderlig hjälp i mitt skrivande speciellt när det har gått lite trögt.

Jonas Bergquist och Eva Skiöldebrand, tack för ert engagemang och stöd och för alla bra idéer och förslag, jag har lärt mig massor.

Emma Nilsson, Denise Söderroos, Nina Känsälä, Lotten Wahlund, Birgitta Staaf Larsson and Lea Managos, tack för intressanta och lärorika diskussioner på våra journal clubs, har lärt mig väldigt mycket om ämnen som jag inte kunde sen innan.

Skulle också vilja tacka hela AFB och alla kollegor för att ni fick mig att känna mig välkommen när jag var ny doktorand och för alla roliga och intressanta samtalsämnen på luncherna och vid röda soffan som vi har haft under åren.

Jag skulle också vilja tacka Kumari Ubhayasekera och Anna Widgren från Mass Spectrometry Based Metabolomics Facility på Uppsala Universitet för all er hjälp med analyserna av alla prover.

Ett stort tack till Claudia von Brömssen som har svarat på alla mina statistiska frågor och hjälpt mig att förstå R och även hjälpt mig att bygga olika funktioner.

Jag vill även tacka mina nära och kära för all er hjälp. Mamma, Pappa och syrran, ni har lyssnat, peppat och haft förståelse och jag vill tacka er för all support och uppmuntran. Benne, tack för all tålamod och uppmuntran.

Reduced high-intensity training distance in growing horses had no effect on IGF-1 concentrations, but training onset interrupted time-dependent IGF-1 decline

L. Johansson^{1*}, S. Ringmark¹, E. Skiöldebrand², J. Bergquist³ and A. Jansson¹

¹Swedish University of Agricultural Sciences, Department of Anatomy, Physiology and Biochemistry, Ulls väg 26, Uppsala 75007, Sweden; ²Swedish University of Agricultural Sciences, Department of Biomedical Sciences and Veterinary Public Health, Ulls väg 26, Uppsala 75007, Sweden; ³Uppsala University, Department of Chemistry-BMC, Analytical Chemistry and Neurochemistry, Husargatan 3, Uppsala 75237, Sweden; lisa.johansson@slu.se

Received: 3 September 2021 / Accepted: 27 January 2022

© 2022 Wageningen Academic Publishers



RESEARCH ARTICLE

Abstract

This study investigated plasma insulin like growth factor (IGF)-1 concentrations in 16 young Standardbred horses introduced to systematic high-intensity training at two different levels of intensity. Growth and locomotion asymmetry and correlations between these and plasma IGF-1 concentrations were also examined. From September as 1-year olds to March as 2-year olds (Period 1), all horses were subjected to the same submaximal training program. In March (start of Period 2), the horses were divided into two groups (n=8) and one group was introduced to regular high-intensity training. The other group was introduced to a program where the high-intensity exercise distances were reduced by 30%. These two training programs were maintained for the remaining 21 months of the study (Periods 2, 3, and 4). There was no effect of training group on plasma IGF-1 concentrations. A continuous decline in IGF-1 levels was observed throughout the study ($P < 0.0001$), with one notable interruption in Period 2 when the IGF-1 concentration remained at the level seen at the start of Period 1. Growth rate of body length was equally high in Periods 1 and 2 ($P > 0.05$). Front and hind limb asymmetry was elevated in Period 2 compared with Period 1. There were positive correlations between IGF-1 concentrations and changes in body condition score, and a negative correlation between IGF-1 concentration and weight. These results indicate that introduction to high-intensity training induces IGF-1 release in horses, but that a 30% difference in the distances used in 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 influence growth pattern and locomotion asymmetry, but further studies are needed to assess causality.

Keywords: locomotion symmetry, growth, body length, exercise

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 very 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 Wången, 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 ancestors. These eight horses were all tested by the SychroGait 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 (Krafft AB, Malmö, Sweden), a commercial mineral supplement (Krafft 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 measurements were performed by the same person. Body weight (BW) was recorded with a scale (weight indicator U-137, UNI Systems and Vågsspecialisten, 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 mean was calculated. Thickness of *m. longissimus dorsi* and subcutaneous fat at croup was measured with a DP-6600 Vet ultrasound system (Mindray Medical International, Shenzhen, China P.R.) using 7.5 MHz, 38 mm linear probe. The measurement of *m. longissimus dorsi* were taken above the 18th rib by the first lumbar vertebra. The thickness of fat at croup was measured 5 cm from the middle line at the croup. Both thickness of *m. longissimus dorsi* and subcutaneous fat at croup were measured three times and a mean was calculated. To evaluate the body condition score (BCS, adopted from Henneke *et al.*, 1983) the body were

divided into four sections; neck and shoulders, back, ribs and tailhead, and given a score with 0.5 precision (Ringmark *et al.*, 2013). BCS was then calculated as the mean of the different sections. In the present study, the change in these observations (i.e. growth rate) was calculated for four periods: Period 1: October 2010 to March 2011 (before high-intensity training); Period 2: March 2011 to August 2011 (first 6 months with high-intensity training); Period 3: August 2011 to December 2011; and Period 4: December 2011 to December 2012 (longer than previous periods since low growth rate was expected). Mean growth rate was calculated as the difference between the last observed measurement in the period and the first measurement, divided by the number of months in the period.

Individual feed intake was measured for three consecutive days on 12 occasions and daily metabolisable energy (ME) and crude protein (CP) intake were calculated based on feed analyses (Ringmark *et al.*, 2017). The mean values obtained for the four periods are shown in Table 1. The dietary CP/ME ratio was planned to decrease, as horses reach maturity at the age of 3 years (NRC, 2007). There were no differences in energy and CP intake between training groups C and R (Ringmark *et al.*, 2017).

Locomotion asymmetry

Locomotion asymmetry in front and hind limbs was measured on 17 occasions, approximately in alternate months (there were four months between measurements in one case), starting in September as 1-year-olds and ending in December as 3-year-olds. Locomotion asymmetry was evaluated during trot at hand for ~100 m on a hard surface (packed gravel), using a sensor-based system (Lameness Locator; Equinosis LLC, Columbia, MO, USA) similar to that described by Keegan *et al.* (2011). The difference in left versus right maximal and minimum position of head was used to calculate the vector sums (VS): $VS = \sqrt{(\text{maximum difference}^2 + \text{minimum difference}^2)}$ 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 \pm standard error).¹

	Period 1	Period 2	Period 3	Period 4
ME, MJ/day	22 \pm 0.4	25 \pm 0.4***	26 \pm 0.4***	23 \pm 0.4*
CP, g/day	290 \pm 5	370 \pm 5***	350 \pm 5***	280 \pm 5*
CP/ME	13 \pm 0.1	15 \pm 0.1***	14 \pm 0.1***	12 \pm 0.1***

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 hind limb pushoff and impact differences in locomotion asymmetries that were not square-transformed. Differences were considered statistically significant at $P < 0.05$. Results are presented as least square means \pm standard error unless otherwise stated. The IGF-1 values were log-transformed and analysed using a mixed model that included effect of date, training group, and horse, which was considered random and repeated. Changes in body measurements, VSF, hind limb pushoff and impact asymmetries were analysed with a mixed model where period and training group were included and where horse was considered random and repeated. Correlation analysis was performed on individual mean values per period using PROC CORR (Pearson's correlation coefficient) to calculate correlations (Statistical Analysis System package, version 9.4, SAS Inst. Inc., Cary, NC, USA).

3. Results

Insulin-like growth factor-1 concentration

There was no significant effect of training group on plasma 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

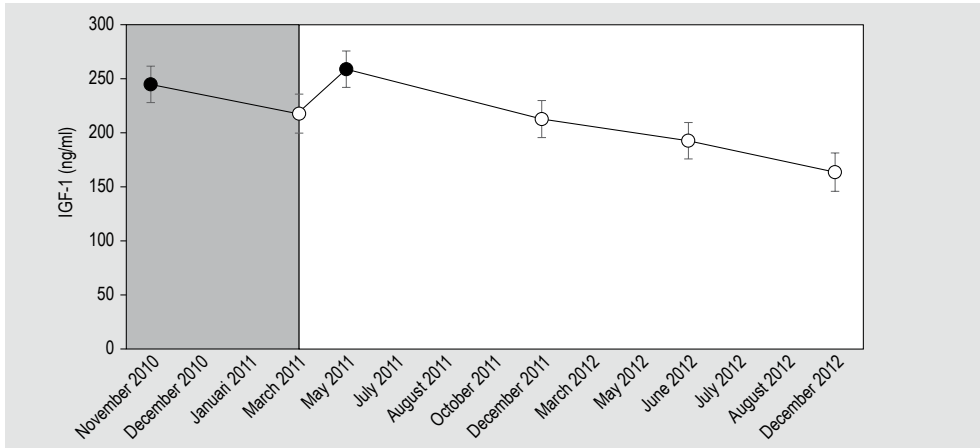


Figure 1. Insulin-like growth factor 1 (IGF-1) in 16 Standardbred horses in training from November 2010 as one-year olds to December 2012 at three years of age (least-squares mean \pm standard error). Unfilled markers are significantly different ($P < 0.05$) from the first observation in November 2010. 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 \pm standard error).¹

	Period 1 (Oct-Mar) 1-2 yrs	Period 2 (Mar-Aug) 2 yrs	Period 3 (Aug-Dec) 2 yrs	Period 4 (Dec-Dec) 3 yrs
Height at withers (cm/month)	1.0 \pm 0.04	0.2 \pm 0.04***	0.6 \pm 0.04***	0.1 \pm 0.05***
Height at croup (cm/month)	0.7 \pm 0.06	-0.1 \pm 0.06***	0.6 \pm 0.06	0.1 \pm 0.07***
Body length (cm/month)	1.1 \pm 0.2	0.7 \pm 0.2	-0.1 \pm 0.2***	0.3 \pm 0.2 [†]
Front limb circ. (cm/month)	0.1 \pm 0.02	0.1 \pm 0.02	-0.1 \pm 0.02 [†]	-0.1 \pm 0.02
<i>m. longissimus</i> (mm/month)	-1.4 \pm 0.5	0.9 \pm 0.5**	0.4 \pm 0.5 [†]	-0.1 \pm 0.5
Fat at croup (mm/month)	-0.2 \pm 0.04	0.2 \pm 0.04***	-0.1 \pm 0.04 [†]	0.1 \pm 0.04***
Body condition score ²	0.1 \pm 0.03	0.1 \pm 0.03	-0.1 \pm 0.03 [†]	-0.1 \pm 0.03**
Weight (kg/month)	5.8 \pm 0.6	2.4 \pm 0.6***	6.1 \pm 0.6	0.5 \pm 0.6***

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 \pm standard error).¹

	Period 1 (Oct-Mar) 1-2 yrs (n=89) ²	Period 2 (Mar-Aug) 2 yrs (n=48)	Period 3 (Aug-Dec) 2 yrs (n=32)	Period 4 (Dec-Dec) 3 yrs (n=94)
Hind limb pushoff asymmetry (mm)	3.4 \pm 0.5 ^a	4.6 \pm 0.6 ^b	4.7 \pm 0.7 ^a	4.8 \pm 0.5 ^b
Hind limb impact asymmetry (mm)	3.2 \pm 0.5 ^a	5.4 \pm 0.6 ^b	3.9 \pm 0.6 ^a	4.6 \pm 0.5 ^b
Front limb asymmetry (vector sum, mm)	10.0 \pm 1.0 ^a	19.3 \pm 1.3 ^b	15.4 \pm 1.6 ^b	17.0 \pm 1.0 ^b

¹ Values in a row with different superscript letters are significantly different ($P < 0.05$).

² n = number of observations during the period.

concentrations were not correlated to height at withers or croup, depth of *m. longissimus dorsi*, front limbs circumference or fat thickness at croup (Table 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. However, 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 <i>m. longissimus</i>	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 circ.	-0.013	0.92
Fat at croup	-0.14	0.26
Body condition score	0.34	0.006
Front limb asymmetry	0.010	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

bones 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.

Acknowledgements

This work was supported by the Swedish University of Agricultural Sciences, the National Trotting School Wängen, Trioplast AB, Dow Chemicals, Swedish Trotting Association, Swedish Horse Racing Totalisator Board, and the Swedish Horse Council Foundation. All authors would also like to thank all staff and students at Wängen for management of the horses, Astrid Gumucio for support with laboratory analyses, Claudia von Brömmssen for statistical assistance, and Mary McAfee for language help.

Conflict of interest

All authors declare that they have no conflict of interest.

References

- Adem, A., Ekblom, J., Gillberg, P.G., Jossan, S.S., Höög, A., Winblad, B., Aquilinius, S.M., Wang, L.H. and Sara, V., 1994. Insulin-like growth factor-1 receptors in human spinal cord: changes in amyotrophic lateral sclerosis. *Journal of Neural Transmission* 97: 73-84. <https://doi.org/10.1007/BF01277964>
- Andersson, L.S., Larhammar, M., Memic, F., Wootz, H., Schwochow, D., Rubin, C.J., Patra, K., Arnason, T., Wellbring, L., Hjälm, G., Imsland, F., Petersen, J.L., McCue, M.E., Mickelson, J.R., Cothran, G., Ahituv, N., Roepstorff, L., Mikko, S., Vallstedt, A., Lindgren, G., Andersson, L. and Kullander, K., 2012. Mutations in DMRT3 affect locomotion in horses and spinal circuit function in mice. *Nature* 488: 642-646. <https://doi.org/10.1038/nature11399>
- Ballesteros, M., Leung, K.C., Ross, R.J., Iismaa, T.P. and Ho, K.K., 2000. Distribution and abundance of messenger ribonucleic acid for growth hormone receptor isoforms in human tissues. *Journal of Clinical Endocrinology Metabolism* 85: 2865-2871. <https://doi.org/10.1210/jcem.85.8.6711>
- Baskerville, C.L., Bamford, N.J., Harris, P.A. and Bailey, S.R., 2017. Comparison and validation of ELISA assays for plasma insulin-like growth factor-1 in the horse. *Open Veterinary Journal* 7: 75-80. <https://doi.org/10.4314/ovj.v7i1.12>
- Chelikani, P.K., Ambrose, J.D., Keisler, D.H. and Kennelly, J.J., 2004. Effect of short-term fasting on plasma concentrations of leptin and other hormones and metabolites in dairy cattle. *Domestic Animal Endocrinology* 26: 33-48. <https://doi.org/10.1016/j.domaniend.2003.08.003>
- De Graaf-Roelofsma, E., Keizer, H.A., Van Breda, E., Wijnberg, I.D. and Van der Kolk, J.H., 2007. Hormonal responses to acute exercise, training and overtraining. A review with emphasis on the horse. *Veterinary Quarterly* 29: 82-101. <https://doi.org/10.1080/01652176.2007.9695232>
- Faria, A.C., Veldhuis, J.D., Thorner, M.O. and Vance, M.L., 1989. Half-time of endogenous growth hormone (GH) disappearance in normal man after stimulation of GH secretion by GH-releasing hormone and suppression with somatostatin. *Journal of Clinical Endocrinology Metabolism* 68: 535-541. <https://doi.org/10.1210/jcem-68-3-535>
- Felsing, N.E., Brasel, J.A. and Cooper, D.M., 1992. Effect of low and high intensity exercise on circulating growth hormone in men. *Journal of Clinical Endocrinology Metabolism* 75: 157-162. <https://doi.org/10.1210/jcem.75.1.1619005>
- Fortier, L.A., Kornatowski, M.A., Mohammed, H.O., Jordan, M.T., O' Cain, L.C. and Stevens, W.B., 2005. Age-related changes in serum insulin-like growth factor I, insulin-like growth factor-I binding protein-3 and articular cartilage structure in Thoroughbred horses. *Equine Veterinary Journal* 37: 37-42. <https://doi.org/10.2746/0425164054406838>
- Henneke, D.R., Potter, G.D., Kreider, J.L. and Yeates, B.F., 1983. Relationship between condition score, physical measurements and body-fat percentage in mares. *Equine Veterinary Journal* 15: 371-372. <https://doi.org/10.1111/j.2042-3306.1983.tb01826.x>
- Jackson, B.F., Goodship, A.E., Eastell, R. and Price, J.S., 2003. Evaluation of serum concentrations of biochemical markers of bone metabolism and insulin-like growth factor I associated with treadmill exercise in young horses. *American Journal of Veterinary Research* 64: 1549-1556. <https://doi.org/10.2460/ajvr.2003.64.1549>
- Jenkins, P.J., 2001. Growth hormone and exercise: physiology, use and abuse. *Growth Hormone and IGF Research* 11, Suppl. 1: S71-77. [https://doi.org/10.1016/s1096-6374\(01\)80012-5](https://doi.org/10.1016/s1096-6374(01)80012-5)
- Keegan, K.G., Kramer, J., Yonezawa, Y., Maki, H., Pai, F., Dent, E.V., Kellerman, T.E., Wilson, D.A. and Reed, S.K., 2011. Assessment of repeatability of a wireless inertial sensor-based lameness evaluation system for horses. *American Journal of Veterinary Research* 72: 1156-1163. <https://doi.org/10.2460/ajvr.72.9.1156>
- Kiani, A., 2013. Temporal changes in plasma concentration of leptin, IGF-1, insulin and metabolites under extended fasting and re-feeding conditions in growing lambs. *Journal of Clinical Endocrinology Metabolism* 11: 34-40. <https://doi.org/10.5812/ijem.6529>
- Kraemer, W.J. and Ratamess, N.A., 2005. Hormonal responses and adaptations to resistance exercise and training. *Sports Medicine* 35: 339-361. <https://doi.org/10.2165/00007256-200535040-00004>
- Mack, S.J., Kirkby, K., Malalana, F. and McGowan, C.M., 2014. Elevations in serum muscle enzyme activities in racehorses due to unaccustomed exercise and training. *Veterinary Record* 174: 145. <https://doi.org/10.1136/vr.101669>
- Malinowski, K., Christensen, R.A., Hafs, H.D. and Scanes, C.G., 1996. Age and breed differences in thyroid hormones, insulin like growth factor (IGF-1) and IGF binding proteins in female horses. *Journal of Animal Science* 74: 1936-1942. <https://doi.org/10.2527/1996.7481936x>
- Martin-Rosset, W., 2004. Growth and development in the equine. In: 2nd European Workshop on Equine Nutrition. Dijon, France.
- Noble, G.K., Houghton, E., Roberts, C.J., Faustino-Kemp, J., De Kock, S.S., Swanepoel, B.C. and Silrence, M.N., 2007. Effect of exercise, training, circadian rhythm, age, and sex on insulin-like growth factor-1 in the horse. *Journal of Animal Science* 85: 163-171. <https://doi.org/10.2527/jas.2006-210>
- National Research Council (NRC), 2007. Nutrient requirements of horses. National Academies Press, Washington, DC, USA.
- Otte, K., Rozell, B., Gessbo, Å. and Engström, W., 1996. Cloning and sequencing of an equine insulin-like growth factor I cDNA and its expression in fetal and adult tissues. *General and Comparative Endocrinology* 102: 11-15. <https://doi.org/10.1006/gcen.1996.0040>
- Popot, M.A., Bobin, S., Bonnaire, Y., Delahaut, P.H. and Closset, J., 2001. IGF-I plasma concentrations in non-treated horses and horses administered with methionyl equine somatotropin. *Research in Veterinary Science* 71: 167-173. <https://doi.org/10.1053/rvsc.2001.0505>
- Pritzlaff, C.J., Wideman, L., Weltman, J.Y., Abbott, R.D., Gutgesell, M.E., Hartman, M.L., Veldhuis, J.D. and Weltman, A., 1985. Impact of acute exercise intensity on pulsatile growth hormone release in men. *Journal of Applied Physiology* 87: 498-504. <https://doi.org/10.1152/jappl.1999.87.2.498>
- Racine, H.L. and Serrat, M.A., 2020. The actions of IGF-1 in the growth plate and its role in postnatal bone elongation. *Current Osteoporosis Reports* 18: 210-227. <https://doi.org/10.1007/s11914-020-00570-x>

- Ringmark, S., Jansson, A., Lindholm, A., Hedenström, U. and Roepstorff, L., 2016. A 2.5 year study on health and locomotion symmetry in young Standardbred horses subjected to two levels of high intensity training distance. *Veterinary Journal* 207: 99-104. <https://doi.org/10.1016/j.tvjl.2015.10.052>
- Ringmark, S., Lindholm, A., Hedenström, U., Lindinger, M., Dahlborn, K., Kvarn, C. and Jansson, A., 2015. Reduced high intensity training distance had no effect on VL4 but attenuated heart rate response in 2-3-year-old Standardbred horses. *Acta Veterinaria Scandinavica* 57: 17. <https://doi.org/10.1186/s13028-015-0107-1>
- Ringmark, S., Revold, T. and Jansson, A., 2017. Effects of training distance on feed intake, growth, body condition and muscle glycogen content in young Standardbred horses fed a forage-only diet. *Animal* 11: 1718-1726. <https://doi.org/10.1017/S1751731117000593>
- Ringmark, S., Roepstorff, L., Essèn-Gustavsson, B., Revold, T., Lindholm, A., Hedenström, U., Rundgren, M., Ögren, G. and Jansson, A., 2013. Growth, training response and health in Standardbred yearlings fed a forage-only diet. *Animal* 7: 746-753. <https://doi.org/10.1017/S1751731112002261>
- Salazar-Ortiz, J., Monget, P. and Guillaume, D., 2014. The influence of nutrition on the insulin-like growth factor system and the concentrations of growth hormone, glucose, insulin, gonadotropins and progesterone in ovarian follicular fluid and plasma from adult female horses (*Equus caballus*). *Reproductive Biology and Endocrinology* 12: 72. <https://doi.org/10.1186/1477-7827-12-72>
- Sticker, L.S., Thompson, D.L., Fernandez, J.M., Bunting, L.D. and DePew, C.L., 1995. Dietary protein and(or) energy restriction in mares: plasma growth hormone, IGF-I, prolactin, cortisol, and thyroid hormone responses to feeding, glucose, and epinephrine. *Journal of Animal Science* 73: 1424-1432. <https://doi.org/10.2527/1995.7351424x>
- Strand, E., Braathen, L.C., Hellsten, M.C., Huse-Olsen, L. and Bjornsdottir, S., 2007. Radiographic closure time of appendicular growth plates in the Icelandic horse. *Acta Veterinaria Scandinavica* 49: 19. <https://doi.org/10.1186/1751-0147-49-19>
- Verwilghen, D.R., Vanderheyden, L., Franck, T., Busoni, V., Enzerink, E., Gangl, M., Lejeune, J.-P., Van Galen, G., Grulke, S. and Serteyn, D., 2009. Variations of plasmatic concentrations of insulin-like growth factor-I in post-pubescent horses affected with developmental osteochondral lesions. *Veterinary Research Communications* 33: 701-709. <https://doi.org/10.1007/s11259-009-9219-2>
- Yakar, S. and Isaksson, O., 2016. Regulation of skeletal growth and mineral acquisition by the GH/IGF-1 axis: lessons from mouse models. *Growth Hormone and IGF Research* 28: 26-42. <https://doi.org/10.1016/j.ghir.2015.09.004>

ACTA UNIVERSITATIS AGRICULTURAE SUECIAE

DOCTORAL THESIS NO. 2024:10

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 received here postgraduate education at the Department of Anatomy, Physiology and Biochemistry, Swedish University of Agricultural Sciences (SLU) in Uppsala. She received her Master of Science degree in biology at Uppsala University.

Acta Universitatis Agriculturae Sueciae presents doctoral theses from the Swedish University of Agricultural Sciences (SLU).

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.

ISSN 1652-6880

ISBN (print version) 978-91-8046-284-6

ISBN (electronic version) 978-91-8046-285-3