HIGH FAT INTAKE AND EQUINE LIPID METABOLISM

Vetopname en vetstofwisseling bij het paard

(met een samenvatting in het Nederlands)

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Abbreviations

ACAT, acyl-CoA: cholesterol acyltransferase;
ACC, acetyl-CoA carboxylase;
CE, cholesteryl esters;
CETP, cholesteryl ester transfer protein;
CS, citrate synthase;
CPT-I, carnitine palmitoyltransferase-I;
DGAT, diacylglycerol acyltranferase;
FAS, fatty acid synthase;
FFA, free fatty acids;
3-HAD, 3-hydroxy-acyl-CoA dehydrogenase;
HDL, high density lipoproteins;
HTGL, hepatic triacyl glycerol lipase;
HK, hexokinase;
LCAT, lecithin: cholesterol acyl transferase;
LDL, low density lipoproteins;
LPDS, lipoprotein deficient serum;
LPL, lipoprotein lipase;
PFK, phosphofructokinase;
TAG, triacylglycerol;
VLDL, very low density lipoproteins.

Contents

Chapter 1

INTRODUCTION

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Introduction

Domestication of the horse and its use in various sport disciplines has greatly changed its diet. Recently, the diet for horses, especially for (top level) performance horses, is very much in the spot light. Optimal performance is based on a combination of factors such as natural ability, health, training and diet. The primary goal of nutrition is to satisfy the energy and nutrient requirements of an animal. In addition, the composition of an otherwise adequate diet can also directly affect the performance level of the animal [9].

The aim of the present review is to discuss the rationale for feeding horses on diets with extra fat. To set the stage, the energy supply for exercise and fat metabolism will be described first. The basic information will be followed by an overview of the metabolic changes as induced by training and diet. It is concluded that training and feeding a high-fat diet may result in comparable metabolic adaptation.

Energy supply during exercise

Substrates

The major substrates for ATP production are carbohydrates and fats, whereas proteins normally contribute less [17,26]. The choice of substrate for energy production by muscle depends on a number of factors. Apart from the intensity and duration of the exercise, the training status of the horse plays an important role [7].
The contribution of carbohydrate or fat to the energy requirement can be established by using the respiratory quotient (RQ), provided that protein degradation does not occur. The RQ can be determined by measuring CO_2 and O_2 in the inspired and expired air and by subsequently dividing the amount of carbon dioxide produced by the amount of oxygen consumed [27]. The RQ is 0.7 and 1.0 for the oxidation of fat and carbohydrate, respectively. A RQ above 1.0 points at anaerobic metabolism. As the duration of exercise increases, the contribution to energy provision of fatty acids increases whereas that of carbohydrate decreases. As exercise becomes more intense, the oxygen consumption decreases due to a shift towards glycolytic catabolism of carbohydrate so that the RQ can increase to values above 1.0 [9,12,17].

Aerobic and anaerobic metabolism

Despite the fact that there are considerable differences in the types of athletic events that horses may perform, in metabolic terms exercise is divided into two types [30]: exercise that can be performed by aerobic metabolism only and exercise that also requires

anaerobic metabolism. The type of athletic performance dictates the extent of aerobic or anaerobic metabolism that is required. During aerobic metabolism, ATP is derived from the oxidation of both carbohydrate and fat. In anaerobic metabolism, energy is derived from the breakdown of glucose into lactate which does not require oxygen. In muscle, the creatine kinase reaction constitutes an additional metabolic pathway for the anaerobic generation of ATP. Although this source of energy is immediately available it is rapidly exhausted. Aerobic metabolism is a very efficient means of ATP generation. The exercise during aerobic metabolism is of moderate intensity, but it can be sustained for a long period. By recruiting anaerobic metabolism, heavy exercise can be realized, but it can only be sustained for a short period. Moreover, anaerobic metabolism is not an efficient means of ATP production. Aerobic metabolism yields 12-18 times more ATP per unit of glucose than does anaerobic metabolism [30].
The initial source of energy for an active muscle, following depletion of creatine phosphate, is muscle glycogen. However, there is only a limited amount of muscle glycogen. Thus, metabolism switches to blood-borne glucose and with an intense workload the supply of blood glucose increases to support the glucose demand by the muscle [27].

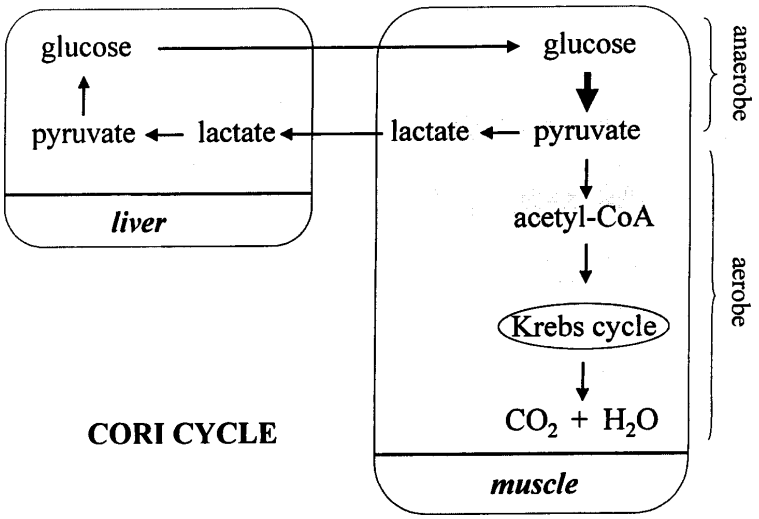

Figure 1 Aerobic and anaerobic metabolism of glucose and the production of pyruvate and lactate in muscle and liver [4]

Glucose can be metabolized both aerobically and anaerobically (Fig. 1). Aerobic metabolism is more profitable as to energy production, but it proceeds at a much slower rate than anaerobic metabolism. The glucose needed for muscle activity is derived from the liver and blood glucose is maintained through glycogenolysis. It is only after several hours of submaximal exercise that liver glycogen is depleted and that the blood glucose level will decrease [29].

Fat constitutes the most important source of energy in the body. The total amount of energy present in the form of body fat is 30-60 times that of stored carbohydrate [26,28]. Fat can only be degraded aerobically. The fats available to the muscle for combustion are triacylglycerol (TAG) stores in the muscle, blood-borne non-esterified fatty acids (NEFA) and TAG present in circulating chylomicrons and very-low-density lipoproteins (VLDL). The NEFA 's are released from adipose tissue bound to albumin for transport through the blood. During prolonged exercise the concentration of NEFA 's in the blood increases considerably, thus enhancing their availability to muscles for combustion. Circulating TAG is synthesized in the liver and secreted into the blood as VLDL or absorbed by the intestine from ingested feed and secreted into the lymphatic system as component of chylomicrons. The uptake of circulating TAG by muscle and adipose tissue is regulated by lipoprotein lipase (LPL). The activity exhibited by the animal (rest or exercise) and its nutritional status (fasting or feeding) determines whether endothelial LPL is most active in adipose or in muscle tissue [26].

Protein normally contributes little to the energy demand during exercise and from an energy point of view, its breakdown is not profitable because energy is required to synthesize its waste product, urea [7,18]. Therefore, proteins will not be discussed here any further.

Energy utilization during exercise

Theoretically, utilization of energy in a contracting muscle during exercise can be described as follows [3,27,30].

- The amount of ATP and creatine phosphate in muscle cells is very limited; the total amount would be utilized within one minute,
- At the onset of exercise, a combination of fat and carbohydrate sources is oxidized to satisfy the increased demand for ATP,
- Anaerobic metabolism serves as a buffer because aerobic metabolism, independently of the amount of available oxygen, lags behind the demand for ATP, (Fig. 2).

The contribution of anaerobic metabolism to energy demand will not only depend

on the intensity at which the exercise starts, but also on the speed at which aerobic metabolism can be adjusted. The latter can be influenced by training and diet composition [3,30].

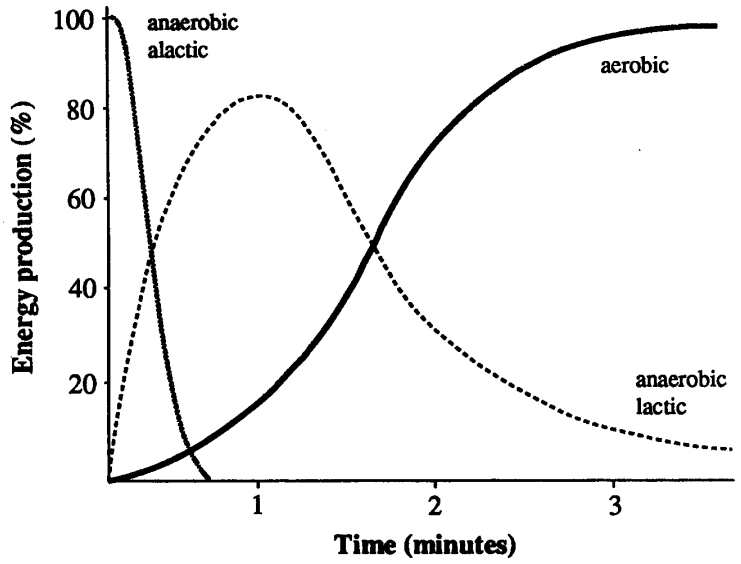

Figure 2 Schematic presentation of the various metabolic pathways during the first few minutes of exercise [3]

Metabolism of fat

Dietary fats

Various nutritional fats can be offered to the horse. However, they differ as to palatability [28]. In general, horses do not fancy animal fats. In a survey with 10 different nutritional fats a preference for corn oil was observed [2]. Other vegetable oils are also accepted, except for coconut oil [28]. Palatability can be a problem when fat is given for prolonged periods at high quantities [8]. Fat has to be added to the diet gradually, and has to be divided into several portions per day. Fat can be offered after spraying over the forage and as part of a concentrate. Performance horses are often fed a fat-supplemented ration with a fat content up to 130 g/kg dry matter (DM)[21].

The requirement for vitamin E is increased when a high amount of unsaturated fat is added to the diet; in that case it is recommended to double the amount of vitamin E so that 2 mg is present per kg body weight (BW) per day [32].
A disadvantage of feeding a diet with a high fat content is the limited tenability because unsaturated fatty acids oxidize rapidly. To prevent oxidation, antioxidants can be added to the diet, but after these compounds have been oxidized the unsaturated fatty acids will still be oxidized [28].

Digestion and transport of fat
Feed-derived fat is transported in the body as component of lipoproteins [25]. Lipoproteins are classified according to their density; there are chylomicrons, VLDL, low-density-lipoproteins (LDL) and high-density-lipoproteins (HDL). Fatty acids can be bound to albumin and transported as such. For energy supply, VLDL and free fatty acids are important.

In brief, the uptake of fat from the diet is as follows. Dietary fat reaches the small intestine essentially in the form of TAG. A monoacylglycerol (MAG) remains after pancreatic lipase has liberated two fatty acids from a TAG molecule. MAG and NEFA's together with bile acids form water soluble micelles. Following uptake of the micelles by mucosa cells, TAG is resynthesised and, together with protein and phospholipids, chylomicrons are formed. Chylomicrons are secreted into the lymph and via the ductus thoracicus they reach the circulation. Short-chain fatty acids are taken up by the mucosa cells as such and carried via the portal system to the liver. Through the action of LPL, chylomicrons are rid off their esterified fatty acids which are taken up by the adipose tissue. In case of energy shortage, *e.g.* during exercise or fasting, fatty acids are released from the adipose stores for utilization by liver or muscle. The primary role of the liver is to esterify the fatty acids into TAG and to assemble them into VLDL which in turn deliver their fatty acids to tissues with a high activity of LPL [1,22]. LPL is bound to endothelial cells, but is released into the circulation after intravenous administration of heparin [48], which makes it possible to study LPL activity.

Fat and fibre digestibility
Up to levels of 20-25% of the DM of the total ration, fat is efficiently digested by the horse with an apparent digestibility of 70-90% [18]. Results of studies concerning effects of fat intake on apparent digestibility of fibre in horses are conflicting. Several researchers reported that addition of fat to the ration did not affect the digestibility of fibre [47], whereas others found an increase [20,40]. However, in those studies fat was not the only variable. Recently, the effect of replacing nonstructural carbohydrates with fat on fibre digestibility was studied in

horses. A fat-rich diet significantly decreased the apparent digestibility of fibre [21]. It has been suggested that with a fat-rich diet more fatty acids will reach the cecum, inhibiting fermentation by bacteria [21].

Fat and energy demand

Exercise is a major determinant of the nutrient requirements of a horse. For example, an adult horse of 600 kg will need for maintenance 44.5 MJ net energy. In case the horse is very intensely exercised for 1½ hour per day, it needs 110 MJ . The energy requirements are guidelines only as the energy needs of individual horses can vary greatly [13].

Two factors are important when estimating the energy requirement of a horse. On the one hand, a horse can only take up 2 to 2.5% of its BW in the form of DM per day [46]. This amounts to 12-15 kg for a 600 kg horse. On the other hand, for optimal fermentation in colon and cecum the ration has to have a minimum amount of fibre. However, there is no official minimal requirement the amount of fibre. Meyer [32] advised that a ration should provide a minimum of 0.5 kg of forage/100 kg of BW. Lewis [28] recommended 1-1.5 kg roughage/100 kg BW. On a weight basis, fibre contains a limited amount of energy. Therefore, an exercising horse fed roughage solely will lack sufficient energy.

Thus, intensively exercised horses have to consume relatively large amounts of concentrates. However, not every horse is willing to consume a lot of concentrates and therefore, uptake of sufficient feed during periods of heavy training can be a problem. So that the uptake of energy becomes lower than the energy need. Replacement of a large portion of the dietary roughage by a carbohydrate-rich concentrate increases the risk of digestion disorders and founder [15]. To increase the energy density of a ration, fat can be used as substitute for nonstructural carbohydrates. On a weight basis fat contains 2.5 times as much energy as carbohydrate [39]. With a fat-rich diet the horse can consume a relatively low amount of feed to satisfy its energy needs [20]. For example, for each 100 g of soybean oil (24.4 MJ net energy/kg) 300 g of concentrate (8 MJ net energy/kg) less has to be offered so that 200 g of DM less has to be taken up. A reduction of total feed intake will decrease the weight of the intestinal content, which is considered an advantage in some disciplines of horse sports. In addition, extra dietary fat may increase the amount of available energy [28], because replacement of carbohydrate by fat reduces fermentation in cecum and colon which, in turn, diminishes loss of energy as heat [39,42]. A fat-rich ration reduces the gross energy demand because horses utilize fat efficiently [42]. Potter *et al.* [39] indicate that with a fat-rich ration, depending on body condition of the horse and environmental temperature, 5 to 25% less gross energy in the total ration is needed.

Metabolic changes due to training

Training of horses causes adaptations not only in the cardiovascular and respiratory systems, but also in their metabolism [9,26]. A profitable effect of training is the observed increase in the contribution of fat to the energy supply so that glycogen will be spared and fatigue postponed [9,31]. The etiology of fatigue during heavy exercise is very complex and is considered to be associated with high levels of lactate in blood and muscle. However, it has also been postulated that a low concentration of glycogen in muscle plays an important role [5]. Factors also considered are a low level of blood glucose as well as metabolic acidosis and accumulation of NH_3 [17]. During prolonged exercise, trained horses have lower blood lactate concentrations compared to their non-trained counterparts because production is less and probably because lactate is removed faster [44]. Topliff *et al.* [45] and Hambleton [14] have reported a higher concentration of muscle glycogen in trained versus non-trained horses.

A higher contribution of fat to the energy supply should result in a lower RQ. Indeed, in trained horses, lower RQ values are observed during submaximal exercise [9,31]. At very high rates of exercise there is no difference in RQ for trained versus non-trained horses because they will all switch completely to glycolysis [9,31]. The mechanism underlying the shift from carbohydrate to fat combustion during prolonged exercise as evoked by training is not yet clear. It could be attributed to adaptations on the cellular level [19,26].

- Training affects mobilization, transport and oxidation of fatty acids; higher mobilization of fatty acids has been observed in trained horses during exercise when compared to untrained horses [30].
- Uptake by muscle of fatty acids, from circulating TAG, is stimulated by training. Hodgson *et al.* [19] demonstrated an enhanced activity of LPL in response to training in the horse.
- The oxidative capacity of muscle increases as a result of training; this is evidenced by enhanced activity of citrate synthase, a key enzyme of the Krebs cycle [19].
- Training induces an increased activity of 3-hydroxy-acyl-CoA dehydrogenase [19], an enzyme participating in the breakdown of fatty acids prior to combustion in the Krebs cycle.

Metabolic changes due to diet

Apart from the diet, factors such as body condition, natural ability and health of the animal, as well as the ability of the rider or driver, all affect the performance of the horse. Therefore, it is almost impossible to unequivocally determine the effect of dietary changes on performance. The ideal experiment would be one with large groups of horses of comparable level of training studied under exactly the same conditions, except for their diet. In practice such an experiment is not feasible. Based on the reported effects of nutrition on muscle metabolism, it should be possible to estimate the effect of nutrition on performance. Therefore, the effects of dietary fat supplementation on the following metabolic parameters have been studied extensively: concentration of glycogen in muscle, utilization of glycogen during exercise and concentration of circulating lactate. In quite a number of studies, a fat-rich ration with fat contents between 8 and 13 % of DM caused an increase in muscle glycogen in horses [6,16,20,23,24,35,41]. These observations have been explained by a glycogen-sparing effect of fatty acid combustion by the resting horse. Thus, during exercise more glycogen is available for metabolism and this could be favourable for aerobic [6] as well as for anaerobic [16] metabolism. Harkins *et al.* [16] observed an improvement of horse racing time after dietary fat supplementation and Eaton *et al.* [6] has described a lengthening of the time before exhaustion sets in. Other studies with fat-supplemented horses have shown decreased plasma lactate concentrations during prolonged exercise [11,33]. However, higher plasma lactate concentrations were found during sprint racing in horses fed on a fat-rich ration [23,35,41].

Adaptation to high fat intakes appears to involve a sparing of glucose and glycogen during an aerobic workload and promoting of glycolysis during anaerobic exercise. Analogously to training, the contribution of fat to the energy supply during exercise increases on a fat-rich diet. Addition of fat to the diet decreases the RQ during submaximal exercise [9,37]. Horses trotting for 75 min had a reduction of the RQ with increasing work load, indicating a shift towards greater utilization of fat [17]. The shift occurred earlier in fat-supplemented horses [9,37].
Further metabolic adaptations may contribute to the stimulation of fat oxidation. Supplementation with fat affects both the transport through blood and the oxidation of fatty acids. In horses fed for 10 weeks a ration containing 20% of total energy as fat, a higher activity of LPL was found when compared to a control group [36]. Geelen *et al.* [10] also reported an increased LPL activity in horses to which 15% of the total energy requirement was provided as fat; the increase had occurred already after two weeks [10]. Concomitantly, the plasma TAG level was decreased markedly [10]. In addition, Orme *et al.* [36] observed an enhanced activity of

citrate synthase in the gluteus muscle in horses fed a fat-rich diet, but Geelen *et al.* [10] were unable to confirm this.

Both humans and rats are able to sustain submaximal exercise for longer periods of time when given a fat-rich diet [34,43]. The percentage of fat in the fat-supplemented diets used for humans and rats is rather high, *i.e.* 60-70% of the total energy uptake. In experiments with horses fed supplementary fat, the amounts of fat were much lower, *i.e.* 5-35% of the total energy intake. In absolute terms, the amounts of fat provided in experiments with horses were lower than those in experiments with humans and rats, but the experimental horses did receive 4 to 5 times the amount of fat given to the control group. In experiments with humans, 70% of energy is somewhat less than double the amount present in a normal diet [9].

The kind of fat added to the ration should be taken into account. Pagan *et al.* [38] demonstrated that horses fed on a ration with 10% coconut oil, an oil type not preferred by horses, had lower blood lactate and higher NEFA levels after a standard exercise test than horses fed on a low-fat control ration or fed a ration with 10% soybean oil. The authors concluded that both the amount and type of fat, *i.e.* chain length and degree of saturation, play a role in the metabolic changes occurring as a result of fat feeding.

The results described above indicate that the contribution of fatty acids to the energy supply of horses increases with the addition of fat to the ration. Thus, metabolic adaptation to fat supplementation appears to proceed in a manner similar to the metabolic adaptation due to training.

Conclusion

Fat supplementation to the ration of horses is quite possible and does not appear to be detrimental. Fat is digested efficiently by horses. The feeding of fat is a practical method of providing a concentrated form of energy. This is especially important for horses in which the feed uptake does not meet the energy requirement. Feeding of extra fat to horses has distinct effects on various metabolic parameters. Feeding a fat-rich ration triggers metabolic adaptations on the cellular level which are comparable to those induced by training. The adaptations appear to be beneficial for the energy supply during prolonged exercise. The contribution of fat to the energy supply increases so that glycogen is spared and more glycogen is available for combustion during exercise, which is advantageous for both aerobic and

anaerobic exercise. The potential of performance by horses may improve by extra dietary fat. The question whether or not the performance actually improves needs further investigation. A disadvantage of feeding a fat-rich ration in the long term is a decreased acceptance of the diet by certain horses.

Aim and scope of this study

High fat diets have attained considerable interest as a potential tool to improve performance. The enhanced performance seen after fat feeding has been attributed to fat-induced changes in the metabolic pathways of fat utilization. Thus, initially attention was focussed on investigating the effect of dietary fat supplementation on equine lipid metabolism in the resting state.

The aim of the study described in Chapter 2 was to investigate the effect of fat supplementation on various lipid variables in plasma. It was hypothesized that feeding a fat-rich diet enhances the flux of fatty acids, in the form of TAG, through the circulation into skeletal muscle. The hypothesis was tested indirectly by measuring the concentration of plasma TAG and the activity of LPL in post-heparin plasma. Plasma total TAG concentrations decreased following fat supplementation, whereas plasma total cholesterol concentrations increased. These changes were accompanied by an increase in LPL activity as determined in post-heparin plasma. It would appear that fat feeding raises LPL activity, which in turn lowers plasma TAG, being consistent with an increase in fatty acid turnover in the form of TAG.

The study described in Chapter 3 is an extension of the first experiment in that it concerns the dose-response relationship between fat intake and plasma LPL activity. Fat feeding was found to raise post heparin plasma LPL activity in a linear, dose-dependent fashion.

The aim of the investigation described in Chapter 4 was to unravel the mechanism underlying the dietary fat-induced decrease of VLDL-TAG found earlier. The hypothesis tested was that extra fat intake would stimulate lipolysis in adipose tissue. As mentioned above, a high-fat diet is expected to lead to an increase in the turnover of fatty acids in the form of TAG. As plasma free fatty acids are major substrates for the synthesis of VLDL-TAG in the liver, there should be enhanced mobilisation of fatty acids from adipose tissue after fat feeding. In addition, the fatty-acid oxidative capacity in skeletal muscle was assessed by measuring the activities of key oxidative enzymes in muscle biopsies. Fat supplementation did

neither affect the *in vitro* lipolytic rate nor the activity of key oxidative enzymes in muscle tissue.

Because fat feeding has been shown to stimulate fatty acid oxidation by muscle in species other than the horse, Chapter 5 presents the effect of fat supplementation on energy metabolism in different muscle types of the pony. It was hypothesized that fat supplementation would increase the transport of fatty acids into mitochondria. Furthermore, hepatic lipogenesis was investigated to establish whether a diminished production of TAG-rich lipoprotein particles could contribute to the dietary fat-induced decrease in the plasma TAG concentration. It was found that fat feeding enhanced both the transport of fatty acids into the mitochondria and the oxidative capacity in highly aerobic muscle. In addition, the results indicated that a dietary fat-induced reduction of plasma TAG may be caused, at least in part, by a decrease in the rate of hepatic *de novo* fatty acid synthesis.

Chapter 6 describes studies concerned with the metabolism of HDL cholesteryl esters (HDL CE). It was examened whether in equines, animal species that lack cholesteryl ester transfer protein (CETP), transfer of HDL CE to LDL takes place and to shed light on the mechanism underlying the observed increase in plasma HDL cholesterol (HDLC) following the consumption of a fat-supplemented diet. The results showed that transfer of HDL CE to LDL does take place, despite the absence of CETP, and that fat feeding lowered the transfer rate which was associated with an increase in HDL CE levels.

In Chapter 7, rates of TAG secretion into the circulation are described. An increased LPL activity must be accompanied by an accelerated turnover of TAG and thus enhanced production of VLDL particles. The hypothesis was tested indirectly by measuring the VLDL-TAG production rate after administration of the nonionic detergent, Triton WR 1339. The results point at a lower hepatic TAG secretion in the animals given the high-fat diet.

Finally, in Chapter 8 the General Conclusions of the studies presented are listed.

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Chapter 2

DIETARY FAT SUPPLEMENTATION AND EQUINE PLASMA LIPID METABOLISM

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Summary

Feeding of a fat-rich diet to horses may enhance the flux of fatty acids, in the form of triacylglycerols (TAG), through the circulation into skeletal muscle. This hypothesis was tested indirectly by measuring the concentration of plasma TAG and the activity of lipoprotein lipase (LPL) in post-heparin plasma. Six adult horses were fed a high-fat or a control diet according to a cross-over design with feeding periods of 6 weeks. The control diet contained 1.5% fat in the dry matter and the high-fat diet 11.8%. The high-fat diet was formulated by adding soybean oil to the control diet at the expense of an isoenergetic amount of corn starch plus glucose. Both diets consisted of hay and concentrate and were given on a restricted basis. Nine hours after feeding, whole-plasma TAG concentration decreased significantly by 84% following fat supplementation, whereas the whole-plasma concentrations of cholesterol and phospholipids were significantly increased by 53 and 26%, respectively. The level of HDL-cholesterol was raised by 54%. The changes in plasma lipids were accompanied by a 79% increase in LPL activity in post-heparin plasma. These results indicate that in the fasting state a high-fat diet raises the flux of fatty acids, in the form of TAG, into skeletal muscles as illustrated by the observed decrease in plasma TAG concentrations and increase in LPL activity. It is speculated that the increased flux of fatty acids is associated with an increased oxidative capacity of skeletal muscle which might be advantageous to exercising horses.

Introduction

Exercise performance in horses has been suggested to improve following a period of feeding a fat-supplemented diet (Eaton *et al.* 1995, Harkins *et al.* 1992, Oldham *et al.* 1990), but the mechanisms involved are still obscure. Equine muscle has a high aerobic capacity, resulting in a significant ability to use fatty acids as an energy source during high intensity exercise (Snow *et al.* 1983). The feeding of a high-fat diet could produce a substantial flux of fatty acids which will increase their use as fuel for aerobic metabolism. This may facilitate oxidation of fatty acids during exercise. In humans and rats, a lowering of plasma concentrations of triacylglycerols (TAG) and an increase in the activity of lipoprotein lipase (LPL) from the luminal surface of capillary endothelial cells of skeletal muscle are seen after fat feeding (Jacobs *et al.* 1982, Kiens and Lithell 1989, Delorme and Harris 1975). Since LPL hydrolyses TAG in chylomicrons and very-low-density lipoproteins (VLDL) so that fatty acids can be taken up by muscle tisssue, an increase in LPL activity and a decrease in TAG concentrations may indicate an accelerated turnover of fatty acids. Interestingly, similar metabolic changes are induced by exercise (Jacobs 1981, Meyers *et al.* 1987). It appears that exercise leads to a specific adaptation of metabolism in order to use fatty acids efficiently when energy demands increase. In this light the feeding of a high-fat diet might be a suitable adjunct to training.

Orme et al. (1997) have demonstrated an increase in the total lipase activity of post-heparin plasma and a decrease in plasma TAG concentrations in trained horses given a fat-supplemented diet. LPL is released from the endothelial membranes into the circulation by intravenous administration of heparin (Watson *et al*. 1993). Thus, in horses, fat feeding may also increase the uptake of fatty acids by skeletal muscle which in turn activates fatty acid oxidation. High activities of LPL are generally associated with high levels of high-density lipoprotein (HDL) cholesterol (Kantor *et al.* 1987, Stanley *et al.* 1986). When TAG in VLDL are hydrolysed by LPL, surface material of VLDL particles, including apoproteins, cholesterol and phospholipids, is transferred to HDL (Stanley *et al.* 1986), explaining why LPL activity and HDL concentrations are directly correlated. Thus, it could be suggested that fat feeding raises HDL cholesterol in horses. Total lipase activity in post-heparin plasma includes hepatic triacylglycerol lipase (HTGL) which is located on hepatic endothelial cells, and thought to be involved in the hepatic uptake of cholesterol and phospholipids from HDL (Janssen *et al.* 1980, Bamberg *et al.* 1983, Watson *et al.* 1993). If fat feeding raises HDL cholesterol in

horses, then the activity of HTGL may also be increased. Orme et al. (1997) did not measure HDL cholesterol and HTGL activity.
The aim of the present study was to investigate in a cross-over design the effect of fat supplementation to the diet on a number of lipid variables in plasma which may form part of an adaptive response.

Materials and Methods

Animals and diets
Six horses (2 mares, 4 geldings) weighing 397-473 kg and aged 4-12 years were fed a high-fat or a control diet according to a cross-over design (2x2 Latin square) with feeding periods of 6 weeks. The horses were randomly allocated to the order of the diets. Thus, 3 horses received the low-fat diet followed by the high-fat diet and 3 horses had the opposite order. The diets consisted of hay and either a control or high-fat concentrate. At 08.00h and 20.00h the concentrates were offered, and at 10.00h and 22.00h the hay was provided. The high-fat concentrate was formulated by adding soybean oil to the control concentrate at the expense of an isoenergetic amount of starch plus glucose (Table 1). The diets were given on a restricted basis (*i.e.* at a level equivalent to 90% of the calculated amount of energy needed for maintenance of their initial body weight) to ensure that all feed was consumed. On average, the horses received 0.8 kg hay and 1.4 and 1.2 kg of the control and high-fat concentrates per meal, respectively. The control diet contained 1.5% fat in the dry matter and the high-fat diet 11.8%. The horses were individually housed in stands which were located in a ventilated stable. All animals walked each day for 60 min in a mechanical horse walker at a speed of 100 m/min.

Sampling and assay procedures
Blood samples were collected in heparinized tubes by jugular venepuncture at 07.00h each week. The samples were analysed for whole-plasma TAG, phospholipids and cholesterol concentrations and for their lipoprotein profile. Serum lipoproteins were isolated by density gradient ultracentrifugation (Terpstra *et al.* 1981) at the following densities (*d*, g/mL): VLDL, $d<1.006$; low-density lipoproteins (LDL), $1.019<d<1.063$ and HDL, $1.063<d<1.210$. Isolated lipoprotein samples were frozen and stored at -20^0C until analysis. Whole plasma and lipoprotein lipid concentrations were measured enzymatically with an autoanalyser (COBAS-BIO, Hoffmann-La Roche, Mijdrecht, The Netherlands) and test combinations purchased from Boehringer, Mannheim, Germany.
Prior to feeding, but after regular sampling, blood samples were also obtained every 2 weeks for the analysis of LPL and HTGL at 5, 10, 20 and 60 min after intravenous injection of heparin (70 IU/kg body weight). Following plasma

preparation, samples were stored at -80^{0}C until analysis. Total and hepatic lipase activities were determined according to Nilsson-Ehle and Schotz (1976) in the presence of a low and high concentration of NaCl, respectively. LPL activity was calculated as the difference.

Statistical analyses

Dietary effects on the plasma variables were sought using repeated measurements, two-way analysis of variance (ANOVA) with period, horse and experimental treatment as factors. The outcome is given in the legends to the figures. As the data were not significantly affected by period they were also evaluated with Student's paired *t*-test, using the values pooled per dietary treatment at the end of each experimental period. The results of this test are described in the text. The level of statistical significance was pre-set at $P<0.05$.

Table 1 Composition of the experimental concentrates (g)

Ingredient	Low-fat concentrate	High-fat concentrate
Corn starch	193	-
Glucose	140	-
Soybean oil	-	150
Constant components *	850	850
Total	1183	1000

* The constant components consisted of the following (g): Alfalfa meal, dehydrated, 342.4; Corn starch, 150; Glucose, 150; Soya beans, extracted, 100; Molasses, beet, 50; Linseed expeller, 20, Ca_2PO_3, 15; NaCl, 15; MgO, 3.4, $CaCO_3$, 1.7; Premix **, 2.5

** The premix consisted of the following (g/kg): $CoSO_4.7H_2O$, 0.66; $Na_2SeO_3.5H_2O$, 0.76; KIO_3, 0.32; $MnSO_4.H_2O$, 172.4; $CuSO_4.5H_2O$, 27.2; $ZnSO_4.H_2O$, 192.4; Vitamin A, 12.0 (500.000 IU/gr); Vitamin D3, 5.2 (100.000 IU/ gr); Vitamin E, 240.0 (500 IU/ gr); Vitamin B1, 1.8 (purity 100%); Vitamin B2 (purity 100%), 1.8; Vitamin B12 (purity 0.1%), 1.8; Biotin (purity 100%), 0.4; Corn starch (Carrier), 343.26.

Results

Feed intake and body weight. The horses consumed all feed supplied. Due to the restricted feeding regimen all horses lost some weight during the course of the experiment. During the first period, weight loss averaged 7 and 6% and during the second period it was 1 and 4% for the control and high-fat diet, respectively. There was no significant diet effect on weight change.

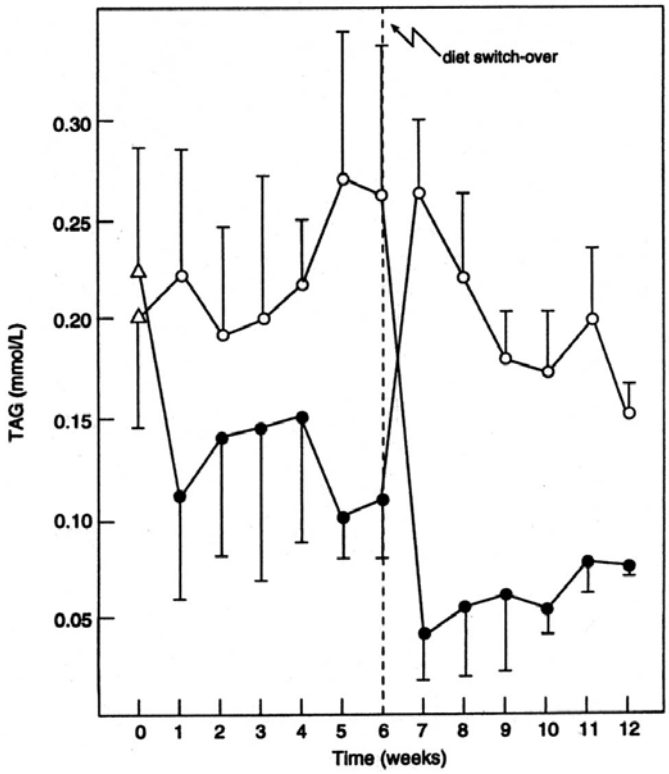

Fig. 1. Time course of triacylglycerol (TAG) concentrations in whole plasma when the horses were fed the high-fat (●) or control (o) diet. The effect was statistically significant (P=0.008, ANOVA). Each point and bar represents the mean ± SEM for three horses. The triangular symbol depicts the starting values.

Plasma triacylglycerols. Concentrations of whole plasma TAG (Fig. 1) did not show a significant period effect. Compared with the control diet, the high-fat diet produced significantly lower whole plasma TAG (P=0.047, Student's paired t-test).

Following the diet switch-over, the values decreased when the control diet was replaced by the high-fat diet and increased after substitution of the control diet for the high-fat diet. On average, 54% of whole plasma TAG was associated with VLDL particles.

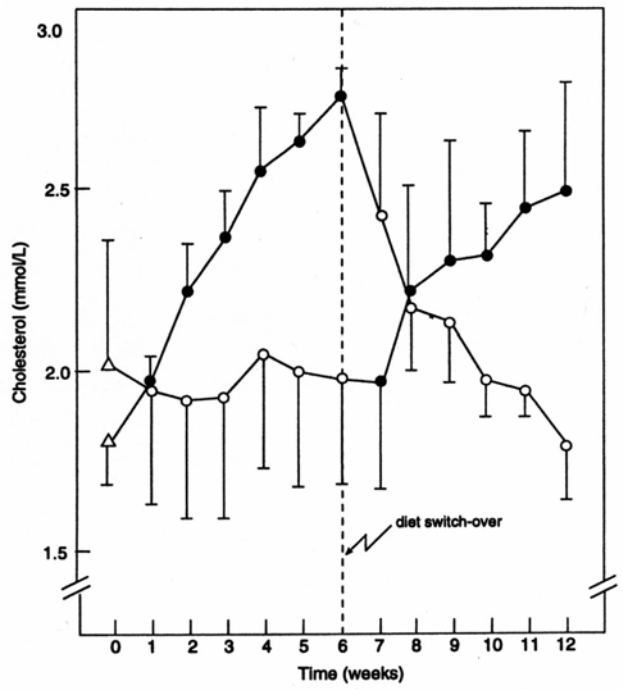

Fig. 2. Time course of cholesterol concentrations in whole plasma when the horses were fed the high-fat (●) or control (o) diet. The effect of diet was statistically significant (P=0.009, ANOVA). Each point and bar represents the mean ± SEM for three horses. The triangular symbol depicts the starting values.

Whole-plasma and lipoprotein cholesterol. Diet had a significant effect on whole-plasma cholesterol concentrations (Fig. 2). The level increased gradually during the first 6 weeks of fat feeding and then decreased gradually towards the baseline value after the diet switch-over. In the horses that were first given the control diet, whole plasma cholesterol concentration was stable until the diet switch-over and then rose when the high-fat diet was supplied. Student's paired t-test revealed a significant diet effect (P=0.002). Changes in whole plasma cholesterol parallelled those in HDL cholesterol (Fig. 2 and 3A). LDL cholesterol concentrations did not systematically respond to the diet changes (Fig. 3B).

Student's paired *t*-test showed a significant diet effect on HDL cholesterol ($P<0.0001$), but not on LDL cholesterol ($P=0.508$). On average, 91% of the increase in whole plasma cholesterol for the high-fat diet, was located in HDL cholesterol.

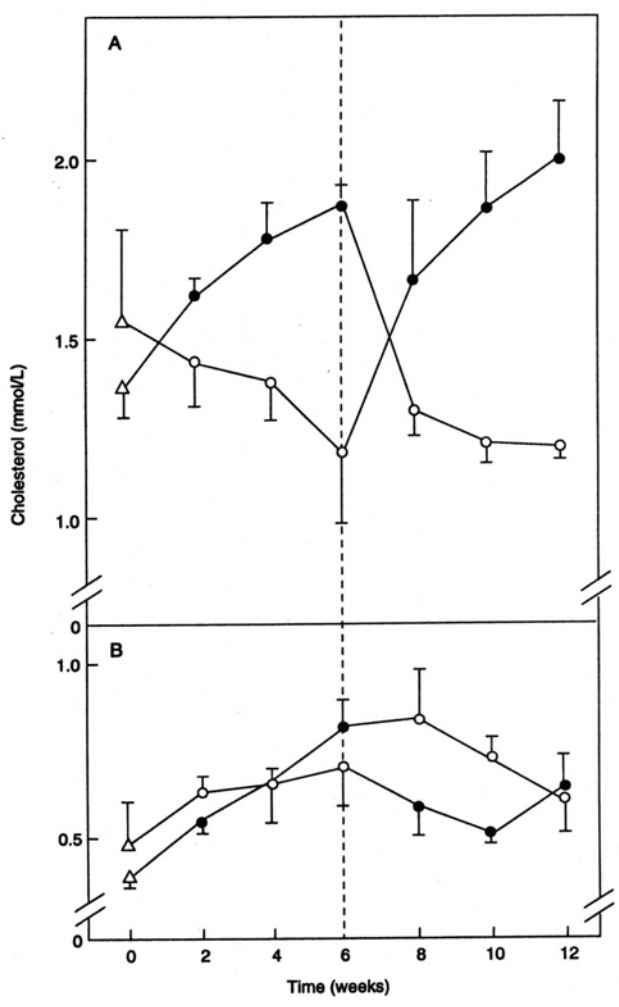

Fig. 3. Time course of cholesterol concentrations in HDL (*panel A*) and in LDL (*panel B*) isolated from plasma of the horses when they were fed the high-fat (●) or control (o) diet according to a cross-over design. ANOVA showed a significant effect of diet on HDL cholesterol ($P<0.0001$), but not on LDL cholesterol ($P=0.198$). Each point and bar represents the mean ± SEM for three horses. The triangular symbol depicts the starting values.

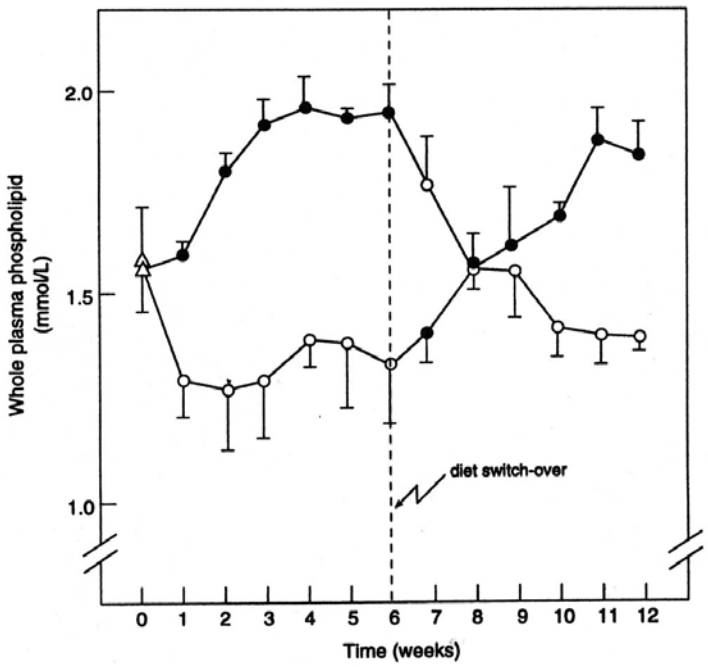

Fig. 4. Time course of phospholipid concentrations in whole plasma when the horses were fed the high-fat (●) or control (o) diet. The effect of diet was statistically significant ($P<0.0001$, ANOVA). Each point and bar represents the mean ± SEM for three horses. The triangular symbol depicts the starting values.

Plasma phospholipids. After fat loading, whole plasma phospholipids increased ($P<0.0001$, Student's paired *t*-test). The time course of whole plasma phospholipids (Fig. 4) resembled that of whole plasma cholesterol.

Post-heparin lipase activity. Total lipase activity showed no significant differences between sampling times at 5, 10 and 20 min after heparin injection. HTGL and LPL activities at 60 min post-heparin administration were on average 30 and 10% lower than those collected at 5 min after heparin injection.
There was a significant diet effect on LPL ($P<0.0001$, Student's paired *t*-test) and HTGL ($P=0.042$, Student's paired *t*-test) activities. Fat loading produced a rapid increase in LPL activity which also fell rapidly after switching over to the control diet (Fig. 5A). A similar pattern was found for HTGL activity, although the diet effect was less pronounced (Fig. 5B).

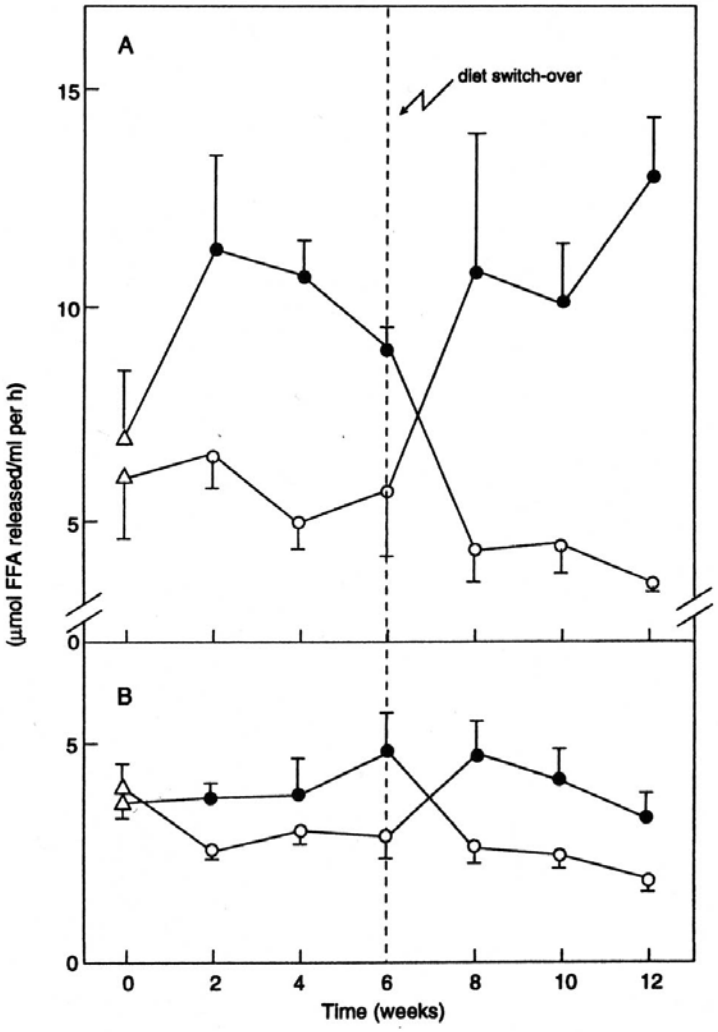

Fig. 5. Time course of LPL (*panel A*) and HTGL (*panel B*) activities in post-heparin plasma prepared from blood collected 5 min following heparin injection in the horses when they were fed the high-fat (●) or control (o) diet according to a cross-over design. ANOVA showed a significant effect of diet on LPL (P=0.002) and on HTGL (P=0.009). Each point and bar represents the mean ± SEM for three horses. The triangular symbol depicts the starting values.

Discussion

This study shows that feeding a high-fat diet to horses caused pronounced changes in lipid metabolism. Fat loading lowered the concentration of plasma TAG and raised LPL activity in plasma collected 9 hours after feeding. These observations support those of Orme et al. (1997) and indicate that lipid metabolism in horses responds to fat feeding in a fashion similar to that in humans (Jacobs *et al.* 1982, Kiens and Lithell 1989) and rats (Delorme and Harris 1975). The observed lowering of plasma TAG may be secondary to a fat-feeding-induced increase in LPL activity. It would follow that fat feeding raises the flux of fatty acids, at least in the form of TAG, which could facilitate the oxidation of fatty acids during exercise. Various new findings emerged from this study. Fat feeding produced an increase in both plasma concentrations of cholesterol and phospholipids. These effects probably are secondary to an increase in HDL which is the major carrier of cholesterol and phospholipids in horse plasma (Watson *et al* 1993). The observed increase in HDL cholesterol is explained by the increased generation of VLDL surface material as a result of the increase in LPL activity. Fat feeding also raised the activity of HTGL so that the increased transfer of cholesterol and phospholipids to HDL may reach a new equilibrium with their removal.
In conclusion, fat feeding in horses increased post-heparin plasma LPL activity and decreased the concentration of circulating TAG, indicating an increased flux of fatty acids. The increased activity of LPL in post-heparin plasma could reflect an increase in muscle LPL activity because the samples were taken in the fasting state when only little LPL is derived from adipose tissue (Mackie *et al.* 1980, Terjung *et al.* 1982), but this needs to be demonstrated in horses fed a high-fat diet. An increase in muscle LPL could be associated with an increase in the oxidative capacity of the muscle which may be advantageous to exercising horses.

Acknowledgement

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Chapter 3

Fat Feeding Increases Equine Heparin-released Lipoprotein Lipase Activity

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Summary

The aim of this study was to establish the dose-response relationship between fat intake and heparin-released plasma lipoprotein lipase (LPL) activity in horses. Eight mature trotters were fed four rations with different fat levels (3.0, 5.0, 7.7 or 10.8% fat in the dry matter) according to a 4 X 4 Latin square design. The experimental rations consisted of hay and different concentrates; the concentrates and hay were given in a 3:1 ratio on an energy basis. Soybean oil was added to the concentrates at the expense of isoenergetic amounts of glucose. Blood samples were taken at the end of each dietary period, which lasted three weeks. Fat feeding was found to increase heparin-released plasma LPL activity in a dose-dependent fashion. When the data from this study and previous studies were combined it was calculated that an increase in fat intake by 1 g/kg dry matter is associated with an increase in LPL activity by 0.98 μmol fatty acid released/ml per h. Fat feeding raised the plasma concentrations of total cholesterol, high-density lipoprotein cholesterol and phospholipids. Diet did not have a statisticallly significant effect on plasma triacylglycerol concentrations. The results are discussed in the light of the possible enhancing effect of fat feeding on the oxidative capacity of skeletal muscle.

Introduction

There is some evidence that exercise performance in horses will improve following a period of feeding a fat supplemented diet.[1-3] High fat diets enhance the capacity of human athletes to oxidize fatty acids.[4,5] A greater oxidative capacity may be beneficial to equine muscle during aerobic exercise. The feeding of extra fat to horses has been shown to increase heparin-released lipoprotein lipase (LPL) activity in plasma and to decrease plasma triacylglycerol (TAG) concentrations.[6,7] Lipoprotein lipase hydrolyses TAG transported by very-low density lipoproteins (VLDL) so that the fatty acid constituents can be taken up by muscle tissue. Thus, a high-fat diet may stimulate the uptake of fatty acids by skeletal muscles with an associated increased oxidative capacity of the muscle.
The fat-induced increase in LPL activity has been demonstrated in horses fed a high-fat diet containing 9.5 % fat in the dry matter versus a control diet containing 2.8 %, and for a high-fat diet with 11.8 % fat as opposed to a control diet with 1.5 % of fat.[6,7] The objective of the present study was to establish the dose-response relationship between dietary fat content and LPL activity. Four levels of fat were fed to 8 mature horses according to a 4 X 4 Latin-square design, and the heparin-released LPL activity in plasma was measured. In addition, the concentrations of TAG, phospholipids and cholesterol in whole plasma were determined.

Material and methods

Animal and diets
The trial had a cross-over design with four dietary treatments and feeding periods of 3 weeks each. Eight mature trotters (two mares and six geldings), weighing 373-473 kg and aged 4-12 years, were used. Before the start of the experiment the horses had been fed a ration consisting of hay only. Baseline plasma values were determined in samples obtained before starting the experimental feeding period. These samples were collected under the same conditions as the samples during the experimental feeding period. During the first three days of each period, the horses were gradually transferred from the previous to the next diet. Two horses were randomly allocated to the order of the diets. The experimental diets consisted of hay and concentrates so that the whole rations contained either 3.0, 5.0, 7.7 or 10.8% fat in the dry matter. Soybean oil was added to the concentrates at the expense of isoenergetic amounts of glucose (Table 1). To formulate the

Table 1. Composition of the experimental concentrate rations

	Concentrate			
Ingredient	**1**	**2**	**3**	**4**
	g as fed (% net energy)			
Glucose	259 (23 %)	173 (16 %)	86 (8 %)	0 (0 %)
Soybean oil	0 (0 %)	50 (5 %)	100 (10%)	150 (15%)
Constant components*	850 (77 %)	850 (79%)	850 (82%)	850 (85 %)
Total	1109 (100 %)	1073 (100 %)	1036 (100 %)	1000 (100 %)

* The constant components consisted of the following (g): alfalfa meal, dehydrated, 342.4; corn starch, 150; glucose, 150; soya beans, extracted, 100; beet, molasses, 50; linseed expeller, 20; Ca_2PO_3, 15; NaCl, 15; MgO, 3.4, $CaCO_3$, 1.7; premix **, 2.5.

** The premix consisted of the following (g/kg): $CoSO_4.7H_2O$, 0.66; $Na_2SeO_3.5H_2O$, 0.76; KIO_3, 0.32; $MnSO_4.H_2O$, 172.4; $CuSO_4.5H_2O$, 27.2; $ZnSO_4.H_2O$, 192.4; vitamin A, 12.0 (500.000 IU/r); vitamin D3, 5.2 (100.000 IU/ g); vitamin E, 240.0 (500 IU/ g); vitamin B1, 1.8 (purity 100%); vitamin B2 (purity 100%), 1.8; vitamin B12 (purity 0.1%), 1.8; biotin (purity 100%), 0.4; corn starch (carrier), 343.26.

concentrates, the following net energy values (MJ/kg product) were used: soybean oil, 24.96 and glucose, 14.45. The composition of the hay was as follows (g/kg dry matter): crude protein, 142; crude fat, 31; crude fibre, 294; neutral-detergent fibre, 617; acid-detergent fibre, 330; cellulose, 287; crude ash, 119. The horses were fed an amount of energy that was equivalent to the calculated amount of energy needed for maintenance of their initial body weight, 351 kJ net energy/$kg^{0.75}$.[8] The concentrates and hay were given in a 3:1 ratio on an energy basis. At 09.30 and 21.30 h the concentrates were offered, and at 10.00 and 22.00 h the hay was provided. The average amounts of concentrates fed were: Diet A – 3.42 kg; Diet B – 3.27 kg; Diet C – 3.14 kg and Diet D – 2.98 kg. All horses were fed 2.1 kg of hay per day. The analysed composition of the whole rations is given in Table 2. All animals walked each day for 60 min in a mechanical horse walker at a speed of 100 m/min.

Table 2. Analysed composition and calculated energy density of the four experimental rations

	Experimental diet			
	A	B	C	D
		g/kg dm		
Crude fat	30	50	77	108
Crude protein	118	119	122	126
Crude fiber	164	168	172	181
		MJ/kg dm		
Net energy	7.1	7.5	7.9	8.3

Energy density was calculated according to the Dutch net energy (NE) system for horses.[1]

Sampling and assay procedures

Blood samples were collected in heparinized tubes by jugular venipuncture at 08.00 h at the end of each trial period. The samples were analysed for whole-plasma TAG, phospholipids and total cholesterol and high-density lipoprotein (HDL) cholesterol concentrations with the use of an autoanalyser (COBAS-BIO, Hoffmann-La Roche, Mijdrecht, The Netherlands) and test combinations purchased from Boehringer, Mannheim, Germany. Also prior to feeding, but after regular sampling, blood samples were obtained at the end of each trial period for the analysis of LPL and hepatic triacylglycerol lipase (HTGL). The samples were taken 5 min after intravenous injection of heparin (70 IU/kg body weight). Plasma was collected after centrifugation of EDTA-treated blood samples. These samples were stored at -80^{0}C until analysis. Total and hepatic lipase activities were determined according to Nilsson-Ehle and Schotz in the presence of a low and high concentration of NaCl, respectively.[9] LPL activity was calculated as the difference between total and hepatic lipase activities. In brief, the reaction mixture contained in a Tris buffer (pH 8.6) radiolabelled triolein as a substrate. Sonicated triolein emulsions were incubated at 37^{0}C with plasma samples as the enzyme source. At the end of the incubation period, extraction solution was added for separation of the liberated fatty acids from non-hydrolysed triolein by using a two-phase system. The radioactivity of liberated fatty acids was determined by liquid scintillation counting. To determine intra- and inter assay coefficients of variance, a pooled horse plasma was used: the values were 3.4 (n=20) and 12% (n=8), respectively.

Statistical analyses
The data were subjected to ANOVA. Horse, experimental period, and dietary treatment were factors.[10] When the influence of dietary treatment reached statistical significance, Bonferroni's *t* test was used to compare treatment means. For the data from each horse (n=8) and for each diet (n=4), linear correlations were calculated between blood variables and fat intake. The calculations were done under the assumption that the 32 data points were independent.[10] The level of statistical significance was preset at $P< 0.05$ or at $P< 0.017$ according to Bonferroni's adaptation.

Results

The horses consumed all feed supplied and maintained their body weight throughout the study. There was no effect of diet on body weight.
Baseline plasma values were as follows: LPL, 6.9 ± 0.8 μmol fatty acid released/ml per h (mean ± SEM, n=8); HTGL, 5.1 ± 0.6 μmol fatty acid released/ml per h; TAG, 0.24 ± 0.037 mmol/l; cholesterol, 2.02 ± 0.10 mmo/l; HDL cholesterol, 1.26 ± 0.42 mmo/l; phospholipids, 1.42 ± 0.46 mmol/l.
Increasing fat intakes were accompanied by statistically significant increases in LPL and HTGL activities and plasma concentrations of total cholesterol, HDL cholesterol and total phospholipids (Table 3). In general, the highest versus lowest fat intake produced statistically significant increases in the plasma values. There was no significant effect of fat intake onTAG concentrations. There was a linear relationship between dietary fat and LPL and HTGL as well as total plasma cholesterol, HDL cholesterol and phospholipids, but not TAG (Table 4).

Table 3. Plasma LPL activity and lipid concentrations in horses fed the eperimental diets.

	Experimental diet					
	A	**B**	**C**	**D**	Pooled SEM	ANOVA P value
LPL	5.7 [b]	6.6 [b]	9.8 [ab]	12.6 [a]	1.1	< 0.001
HTGL	5.6 [b]	6.0 [b]	7.5 [ab]	8.8 [a]	0.5	0.002
TAG	0.20 [a]	0.21 [a]	0.19 [a]	0.17 [a]	0.02	0.744
Cholesterol	1.98 [c]	2.23 [b]	2.47 [a]	2.55 [a]	0.05	< 0.001
HDLcholesterol	1.20 [b]	1.35 [a]	1.45 [a]	1.48 [a]	0.04	< 0.001
Phospholipids	1.37 [c]	1.56 [b]	1.71 [a]	1.78 [a]	0.03	< 0.001

Means and pooled SEM for 8 horses. Lipase values are expressed as µmol fatty acid released/ml per h. TAG, cholesterol,
HDL cholesterol and phospholipid values are expressed as mmol/l.
[a, b, c] Values in the same line with different superscript differ significantly ($P < 0.017$: Bonferroni's *t* test).

Table 4. Linear regression models for either plasma LPL activity or plasma lipid concentrations as dependent variables and the dietary concentration (g/kg dry matter) as independent variable in horses fed the experimental diets.

Dependent variables	Constant ± SD	Slope ± SD	r*	P_{model}
LPL	2.53 ± 1.44	0.09 ± 0.02	0.65	<0.001
HTGL	4.11 ± 1.16	0.04 ± 0.01	0.44	0.012
TAG	214 ± 33	-0.32 ± 0.46	0.13	0.488
Cholesterol	1823 ± 166	7.33 ± 2.29	0.51	0.003
HDLcholesterol	1141 ± 79	3.52 ± 1.09	0.51	0.003
Phospholipids	1266 ± 78	5.11 ± 1.07	0.66	<0.001

* Pearson correlation coefficient
Lipase values are expressed as µmol fatty acid released/ml per h. TAG, cholesterol,
HDL cholesterol and phospholipid values are expressed as µmol/l.

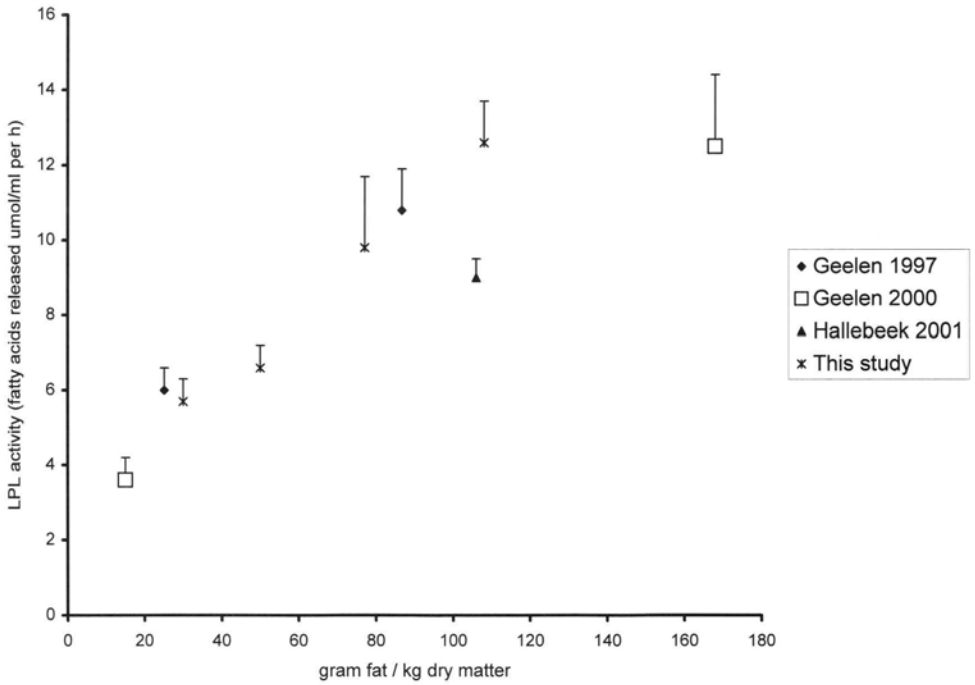

Fig. 1. Heparin-released LPL activity in plasma prepared from blood collected 5 min after heparin injection (70 IU/kg body weight) in horses fed different amounts of fat. The fat content of the whole rations used in all these studies was analysed. The data points correspond with group means ± SD for 6-8 horses and were derived from different studies. The linear regression equation based on the group mean data is $y = 0.95x + 4.184$ ($R^2 = 0.819$, P=0.001) .

Discussion

This study confirms previous work in that fat feeding to horses increases heparin-released LPL activity.[6,7,11] The relationship between fat intake and LPL activity appears to be linear. The data from this study combined with those from previous studies (Fig. 1) show that an increase in fat intake by 1 g/kg dry matter is associated with an increase in LPL activity by 0.98 µmol fatty acid released/ml plasma per h. The type of fat may not play an important role. Hallebeek and Beynen fed horses either palm oil or medium-chain triacylglycerols at a level of 106 g/kg dry matter and found no effect of fat type on the increase in LPL activity.[12]

Fat supplementation had no effect on plasma TAG concentration. Earlier studies have shown a convincing lowering of plasma TAG after feeding extra fat to horses.[6,7,13] The fat-induced increase in LPL activity is most likely responsible for the decrease in TAG observed in these studies. During LPL-mediated hydrolysis of VLDL-TAG there is formation of HDL cholesterol[14,15], which explains the observed increase in HDL cholesterol after fat feeding. Phospholipids in plasma are primarily transported by HDL so that an increase in HDL will cause an increase in plasma phospholipids.[14] Thus, it seems that fat feeding triggers changes in lipid metabolism through activation of LPL, but the underlying mechanism is not known. As mentioned above, the increase in LPL activity as induced by high fat diets leads to an increase in the turnover of fatty acids in the form of TAG. We speculate that this could be potentially beneficial to equine muscle during aerobic exercise.

It is clear from this study that supplementation of horse rations with fat may cause a dose-dependent increase in LPL activity and thus most likely also in the turnover of plasma fatty acids. Within the range of fat intakes tested, the dose-response relationship did not apparently reach a plateau. Negative effects of fat feeding have not been reported[16]. Fat digestibility in horses is efficient and does not limit dietary fat utilization.[16]

In summary, an important adaptation to increasing amounts of dietary fat is an increase in plasma LPL activity. As discussed above, this alteration in LPL activity might occur independently of the type of dietary fat. There is suggestive evidence[1-3] that fat feeding would enhance aerobic exercise in horses due to a glycogen-sparing effect associated with enhanced utilization of fat for energy. Therefore, it may be justified to feed extra fat to exercising horses and to tailor the type of fat to its acceptance by individual animals.

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Chapter 4

LIPID METABOLISM IN EQUINES FED A FAT-RICH DIET

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Summary

The hypothesis tested was that dietary fat, when compared with an isoenergetic amount of non-structural carbohydrates, stimulates lipolysis in adipose tissue and also stimulates the fatty-acid oxidative capacity in skeletal muscle from horses. Six adult horses were fed a high-fat, glucose or starch containing diet according to a 3 x 3 Latin square design with feeding periods of three weeks. The diets were formulated so that the intake of soybean oil versus either glucose or corn starch were the only variables. In accordance with previous work, whole plasma triacylglycerol (TAG) concentration decreased significantly by 58 % following fat supplementation. This fat effect was accompanied by a 247 % increase in lipoprotein lipase (LPL) activity in post-heparin plasma. The dietary variables did neither significantly affect the basal in-vitro lipolytic rate nor the lipolytic rate after adding noradrenaline. There was no significant diet effect on the activities of hexokinase and phosphofructokinase as indicators of glycolytic flux and citrate synthase and 3-hydroxy-acyl-CoA dehydrogenase as indicators of fatty-acid oxidative capacity. The concentrations of muscle glycogen and TAG were not affected by fat supplementation. It is concluded that our hypothesis is not supported by the present results.

Introduction

High-fat diets have attained considerable interest as a potential tool to improve performance of athletic horses [1, 2] because such diets may enhance the capacity to oxidize fatty acids [2, 3]. A greater availability of fatty acids in combination with increased oxidative capacity may be beneficial to equine muscle during aerobic exercise. In horses, exercise-induced fatty acid oxidation is further increased after dietary fat supplementation [4, 5]. A high-fat diet has been demonstrated to raise plasma post-heparin lipoprotein lipase (LPL) activity [6]. It was suggested that the increase in LPL activity reflects the enzyme as derived from the luminal surface of capillary endothelial cells of skeletal muscle. Fat feeding also produced a decrease in the concentration of plasma triacylglycerols (TAG) in the fasted state [6, 7]. LPL hydrolyses TAG transported by very-low density lipoproteins (VLDL) so that the fatty acid constituents can be taken up by muscle tissue. It was thus reasoned that a high-fat diet leads to an increase in the turnover of fatty acids in the form of TAG. This should be associated with enhanced mobilisation of fatty acids from adipose tissue, these fatty acids being major substrates for the synthesis of VLDL-TAG in the liver. To our knowledge there are no data available as to the effect of fat supplementation on the mobilization of fatty acids from adipose tissue in horses.

If fat feeding indeed stimulates fatty acid oxidation by equine muscle, there will be metabolic adaptations. The information in horses is limited [7]. However, in rats and humans, high-fat diets have been shown to increase the activities of muscle 3-hydroxy-acyl-CoA dehydrogenase (3-HAD), a key enzyme in the ß-oxidative pathway, and of muscle citrate synthase (CS), a key enzyme in the Krebs cycle [8, 9, 10]. Kiens et al. [11] and Conlee et al. [12] reported an increase in the concentration of TAG of muscle in human subjects and in rats, respectively, in response to a high-fat diet. In addition, fat feeding has been shown to depress the glycolytic flux in muscle from man [4, 13] which may be reflected by lower activities of the key enzymes hexokinase (HK) and phosphofructokinase (PFK).

The purpose of the present experiment with horses was to investigate the effect of fat supplementation on fat metabolism. Specifically, the experiment was aimed at (*i*) determining the in-vitro lipolytic rate in biopsies of adipose tissue, (*ii*) determining the muscle TAG and glycogen concentrations, and (*iii*) measuring the activities of key oxidative and glycolytic enzymes in muscle biopsies. The influence of extra intake of soybean oil was compared with isoenergetic amounts of either glucose or starch. It was anticipated that the source of dietary carbohydrate would affect lipid metabolism as has been shown in rats [14].

Material and methods

Animal and diets
Six Standardbred horses (2 mares, 4 geldings) weighing 373-473 kg and aged 6-14 years were fed three diets according to a 3 x 3 Latin square design with feeding periods of 3 weeks. During the first three days of each period, the horses were gradually transferred from the previous to the next diet. There were two horses per treatment which were randomly allocated to the order of the diets. The diets consisted of hay and concentrates rich in either glucose, starch or fat. The composition of the grass hay was as follows (g/kg dm): crude protein, 145; crude fat, 31; crude fibre, 279; Neutral-detergent fibre, 588; Acid-detergent fibre, 339; cellulose, 305; crude ash, 110. The fat-rich concentrate contained soybean oil (Table 1). To formulate the other two concentrates, fat was replaced by either glucose or starch in amounts equal to 63 % of the net energy represented by the concentrate. The diets were given to the horses at a level equivalent to the calculated amount of energy needed for maintenance of their initial body weight. Due to the inter-individual variation in body weight, the horses were fed different amounts of energy. The animals were housed in individual tie-up stalls. Each meal, the horses received on average 1.2 kg hay and either 1 kg of the glucose concentrate, 1.2 kg of the starch concentrate or 0.6 kg of the fat concentrate topped with 183 ml of soybean oil. The concentrates and hay were given in a 2.2:1 ratio on an energy basis. At 08.00 h and 20.00 h the concentrates were offered, and at 10.00 h and 22.00 h the hay was provided. All animals walked each day for 60 min in a mechanical horse walker at a speed of 100 m/min.

Sampling procedures
Prior to feeding in the morning, blood samples for determination of whole-plasma TAG were collected in heparinized tubes by jugular venepuncture. Subsequently, blood samples were obtained for the analysis of LPL and hepatic lipase (HTGL) at 5 min after intravenous injection of heparin (70 IU/kg body weight). The samples were stored at -80^{0}C until analysis. The blood samples were collected at the end of each feeding period, but one day before tissue samples were taken. Prior to feeding, muscle samples were obtained from the middle gluteal muscle, according to the method of Snow and Guy [15] using a 6 mm Bergström biopsy needle. The samples were snap frozen in liquid nitrogen and stored at -80^{0}C until homogenization. Then, adipose tissue was sampled from the perinial region according to the following procedure. Animals were prepared for aseptic surgery and local anesthesia was performed using lidocaine hydrochloride (2%). An incision approximately 2 cm long was made and about 600 mg of adipose tissue was removed and submerged in Krebs-Henseleit buffer (pH 7.4) supplemented with 5% (w/v) defatted and dialysed serum albumin xxxx

Table 1
Composition of the experimental concentrates

	Dietary variables		
	High fat	Starch	Glucose
Ingredients (g)			
Corn starch	-	966	-
Glucose	-	-	702
Soybean oil	375[1]	-	-
Constant components[2]	625	625	625
Total	1000	1591	1327
Nutrients[3](g/kg DM)			
Crude protein	81	53	61
Crude fat	377	3	3
Crude fiber	12	8	9
NDF	30	19	22
ADF	13	8	10
Cellulose	15	10	11
Crude ash	12	8	10

[1] Including the 183 ml of soybean oil that was given each meal on top of the concentrate (see text).
[2] The constant components consisted of the following (g): corn starch, 376.15; wheat, 76; soybean extract, 155; NaCl, 8.79; MgO, 5.04, premix, 4.02. The premix consisted of the following (g/kg): $CoSO_4.7H_2O$, 0.66; $Na_2SeO_3.5H_2O$, 0.76; KIO_3, 0.32; $MnSO_4.H_2O$, 172.4; $CuSO_4.5H_2O$, 27.2; $ZnSO_4.H_2O$, 192.4; vitamin A, 12.0 (500.000 IU/g); vitamin D3, 5.2 (100.000 IU/g); vitamin E, 240.0 (500 IU/g); vitamin B1, 1.8 (purity 100%); vitamin B2 (purity 100%), 1.8; vitamin B12 (purity 0.1%), 1.8; biotin (purity 100%), 0.4; corn starch (carrier), 343.26.
[3] Calculated using 1996 CVB tables (Centraal Veevoederbureau, Lelystad, the Netherlands)
ADF = Acid-detergent fibre, NDF = Neutral-detergent fibre

in a thermostated (37ºC) container. The incision was sutured with number 1 ethicon (Mersilene^{R}).

Incubation of adipose tissue

Immediately after removal from the horse, the adipose tissue sample was transported to the laboratory, freed as much as possible from vascular and connective tissue on a dissecting table maintained at 37ºC, cut into pieces of 10 to 30 mg, and incubated in portions of about 100 mg in 3 ml of Krebs-Henseleit buffer (pH 7.4) supplemented with 10 mM glucose plus 5% (w/v) defatted and dialysed serum albumin at 37ºC under an atmosphere of O_2:CO_2 (19:1). After about 15 min, an aliquot of 1 ml of the incubation medium was withdrawn for zero-time analysis, and agonist was added. The agonist used in this experiment was noradrenaline (5.10^{-5} M). All incubations were carried out at 37ºC in 25-ml Erlenmeyer flasks in a metabolic shaker at about 100 oscillations per min. After 120 min, the incubations were stopped by placing the flasks on ice. The tissue was filtered from the media, and the media were stored at -20ºC until analysis.

Assay Procedures

Whole plasma TAG concentration was measured enzymatically with an autoanalyser (COBAS-BIO, Hoffmann-La Roche, Mijdrecht, The Netherlands) and a test combination purchased from Boehringer, Mannheim, Germany. Total and HTGL lipase activities were determined according to Nilsson-Ehle and Schotz [16] in the presence of a low and a high concentration of NaCl, respectively. LPL activity was calculated as the difference.

Lipolysis in adipose tissue was monitored by following the release of glycerol. Glycerol was measured in a coupled assay using glycerol kinase and glycerol 3-phosphate dehydrogenase as described by Wieland [17]. The measurements were performed in triplicate, and the data were pooled prior to statistical analysis.

The muscle samples were homogenized with the IKA-Ultra Turrax^{R} T5-FU tissue homogenizer (Janke and Knukel GmbH and Co. KG, Staufen, Germany) in 9 volumes of a buffer (pH 8.0) containing 25 mM HEPES and 5 mM ß-mercapto-ethanol. Aliquots of this homogenate were used to measure the levels of TAG [18] and glycogen [19]. To ensure full release of mitochondrial enzymes in the remaining homogenate, Triton X-100 (final concentration 0.5%) was added to the strong hypotonic preparation. The activities of the enzymes measured in this study were not affected by the concentration of detergent used. The Triton X-100-treated homogenate was centrifuged at 48,000 x g for 30 min. The supernatant was snap frozen in liquid nitrogen and stored at -80ºC until analysed a few days later for enzyme activities. The activities of CS (EC 4.1.3.7), 3-HAD (EC 1.1.1.35), hexokinase (EC 2.7.1.2; HK) and

phosphofructokinase (EC 2.7.1.11; PFK) were determined spectrophotometrically as described by Stitt [20], Passonneau and Lowry [21], Tielens et al. [22] and Ishikawa et al. [23], respectively.

Statistical Analysis
The data were subjected to ANOVA. Horse, period and experimental treatment were factors. When the influence of dietary treatment reached statistical significance, Fischer's *t* test was used to compare means for two treatments. The level of statistical significance was pre-set at $P<0.05$.

Results

Feed intake and body weight. The horses consumed all feed supplied and maintained their body weight throughout the study. There was no effect of diet on body weight.

Plasma triacylglycerols. Compared with the glucose and starch containing diets, the fat-rich diet produced significantly lower whole plasma TAG (Table 2). There was no difference between the glucose and starch diet.

Post-heparin lipase activity. Fat loading produced increases in plasma LPL and HTGL activities (Table 2). There was no effect of dietary carbohydrate source on LPL and HTGL activities.

In-vitro lipolytic rate. The mean basal lipolytic rate in biopsies of adipose tissue as measured by glycerol production did not significantly differ between the three diets (Table 3). The lipolytic rate after the addition of noradrenaline, expressed as percentage of the basal lipolytic rate, tended to be higher when the horses were fed the fat-rich diet, but the difference did not reach statistical significance.

Triacylglycerol and glycogen concentrations in muscle. There was no significant effect of diet on either TAG or glycogen concentrations in muscle (Table 4).

Enzyme activities in muscle. In homogenates of muscle biopsies, the activities of HK and PFK as indicators of glycolytic flux and the activities of CS and 3-HAD as indicators of fatty acid oxidation were determined. There were no significant diet effects on these enzyme activities (Table 4).

Table 2
Triacylglycerol (TAG) concentrations, post-heparin lipase (LPL) and hepatic lipase (HTGL) activities in plasma of horses that were fed a high-fat diet, a starch diet or a glucose diet according to a Latin-square design.

	Dietary variables		
Measure	High fat	Starch	Glucose
TAG	86.2 ± 12.6*	207.5 ± 39.0	201.3 ± 28.1
LPL	12.5 ± 1.9*	3.5 ± 0.6	3.7 ± 0.6
HTGL	6.9 ± 1.2*	3.8 ± 0.5	4.0 ± 0.6

Data are means ± SEM for 6 horses. TAG values are expressed as μmol/l. Lipase values are expressed as μmol fatty acid released/ml per h. Asterisks indicate a significant difference (P<0.001) between the high-fat diet and either one of the carbohydrate-containing diets.

Table 3
Basal and noradrenaline (5.10^{-5} M)-affected lipolytic rates in adipose tissue removed from horses that were fed a high-fat diet, a starch diet or a glucose diet according to a Latin-square design.

In-vitro	Dietary variables		
Lipolysis	High fat	Starch	Glucose
Basal	0.80 ± 0.09	1.09 ± 0.17	0.93 ± 0.10
Noradrenaline	1.41 ± 0.14	1.74 ± 0.18	1.48 ± 0.13

Data are means ± SEM from 6 horses and expressed as μmol glycerol released/g per 2 h.

Table 4
Triacylglycerol (TAG) and glycogen concentrations and activities of citrate synthase (CS), 3-hydroxy-acyl-CoA dehydrogenase (3-HAD), hexokinase (HK) and phosphofructokinase (PFK) in muscle biopsies from horses that were fed a high-fat diet, a starch diet or a glucose diet according to a Latin-square design.

	Dietary variables		
Measure	High fat	Starch	Glucose
TAG	86 ± 16	57 ± 12	67 ± 18
Glycogen	368 ± 31	378 ±0 35	329 ± 21
CS	190 ± 9	168 ± 21	170 ± 26
3-HAD	370 ± 6	337 ± 36	359 ± 38
HK	0.56 ± 0.07	0.51 ± 0.07	0.51 ± 0.08
PFK	830 ± 59	726 ± 91	781 ± 106

Data are means ± SEM for 6 horses. TAG and glycogen values are expressed as nmol/mg protein. Enzyme values are expressed as nmol/min.mg protein.

Discussion

In accordance with previous studies [6, 7], fat feeding to horses caused an increase in post-heparin plasma LPL activity and a decrease in plasma TAG concentration, pointing at an increased uptake of fatty acids by muscle which in turn may activate fatty acid oxidation. Orme et al. [7] showed an increased muscle CS activity in horses following 10 weeks of fat supplementation to the diet. Our study showed a 12%-

higher group mean activity of CS when the horses were fed the fat-supplemented diet for 3 weeks, when compared to the feeding of either glucose or starch. Both Orme et al. [7] and we ourselves found a lack of effect of fat feeding on the activity of 3-HAD. This could relate to the fact that 3-HAD activity is much higher than that of CS and thus would not limit the oxidative capacity.

An increase in uptake of VLDL-TAG and subsequent oxidation of fatty acids, as probably occurs after fat feeding, could spare muscle TAG. The TAG levels in muscle when the horses were given the high-fat diet were not significantly increased, which confirms the findings of Orme et al. [7] and Essen-Gustavson et al. [24]. In contrast, in humans there is an increase in muscle TAG associated with increased availibility of lipids from the diet [11]. Extra oxidation of exogenous fatty acids could also spare glycogen stores and reduce glycolytic activity. However, no significant increase in muscle glycogen concentration was observed in response to fat feeding. Likewise, Hodgson et al. [25] and Essen-Gustavson et al. [24] reported no significant change in muscle glycogen in response to fat supplementation in non-exercising horses, but others [26, 27, 28] have reported an increase. Fat feeding did not affect glycolytic flux in skeletal muscle as indicated by the unchanged activities of HK and PFK.

Our results confirm that noradrenaline increases the in vitro lipolytic rate in adipose tissue of horses [29]. However, there was no significant diet effect on either the basal rate or after that induced by noradrenaline. Percentage-wise, the noradrenaline-affected stimulation of the lipolytic rate was higher in adipose tissue collected when the horses were given the high-fat diet. This suggests that feeding a fat-rich diet renders lipolysis more sensitive to stimulation, which could be beneficial for performance. Further work is necessary to substantiate or refute this suggestion. At present, it can not be concluded whether the observed tendency that fat feeding raised noradrenaline-induced lipolysis has biological relevance and will reach significance in an experiment with ample statistical power.

In conclusion, fat feeding in horses increased post-heparin plasma LPL activity and decreased the concentration of circulating TAG, indicating an increased flux of fatty acids to extra-hepatic tissues. However, it could not be demonstrated that this increased flux influenced the concentrations of TAG and glycogen and the activities of HK, PFK, 3-HAD and CS in muscle. Noradrenaline-determined lipolysis in adipose tissue tended to be higher after fat feeding. Thus, under the assumption that any diet-induced effects on enzyme activities would survive pre-assay procedures, this study does not support the idea that fat feeding to horses stimulates the fatty-acid oxidative capacity and reduces that of glycolysis in muscle.

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Chapter 5

HIGH FAT INTAKE LOWERS HEPATIC FATTY ACID SYNTHESIS AND RAISES FATTY ACID OXIDATION IN AEROBIC MUSCLE IN SHETLAND PONIES

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Synopsis

The metabolic effects of soybean-oil feeding instead of an isoenergetic amount of corn starch plus glucose were studied in ponies. Twelve adult shetland ponies were given a control diet (15 g/kg fat in dry matter) or a high-fat diet (118 g/kg fat in dry matter) according to a parallel design. The diets were fed for 45 d. Plasma triacylglycerol (TAG) concentrations decreased by 55% following fat supplementation. Fat feeding also significantly reduced glycogen concentrations by up to 65% in masseter, gluteus and semitendinosus. The high-fat diet significantly raised the TAG content of semitendinosus muscle by 80%. Hepatic acetyl-CoA carboxylase and fatty acid synthase activities were 53% and 56% lower in the high-fat group, but diacylglycerol acyltransferase activity was unaffected. Although carnitine palmitoyltransferase-I (CPT-I) activity in liver mitochondria was not influenced, fat supplementation did render CPT-I less sensitive to inhibition by malonyl-CoA. There was no significant diet effect on the activity of phosphofructokinase in the various muscles. The activity of citrate synthase was significantly raised (by 25%) in the masseter muscle of fat-fed ponies and so was CPT-I activity (by 46%). We conclude that fat feeding enhances both the transport of fatty acids through the mitochondrial inner membrane and the oxidative capacity in highly aerobic muscles. The higher oxidative ability together with the depressed rate of *de novo* fatty acid synthesis in liver may contribute to the dietary-fat-induced decrease in plasma TAG concentrations in equines.

Introduction

High-fat diets have been shown to stimulate fatty acid oxidation by muscle in rats and humans. Fat feeding increases the activities of muscle 3-hydroxy-acyl-CoA dehydrogenase (3-HAD) and muscle citrate synthase (CS), key enzymes in the ß-oxidation pathway and in the Krebs cycle, respectively (Helge & Kiens 1997, Miller *et al.* 1984, Simi *et al.* 1991). Furthermore, a 44 %-increase of carnitine palmitoyltransferase-I (CPT-I) activity, the enzyme responsible for the transport of fatty acyl-CoA through the mitochondrial outer membrane, was reported in human subjects fed a high-fat diet (Phinney *et al.* 1983). Kiens *et al.* (1987) and Conlee *et al.* (1990) reported an increase in the concentration of triacylglycerols (TAG) of muscle in human subjects and in rats in response to a high-fat diet. However, Orme *et al.* (1997) and Geelen *et al.* (1999) could not confirm these fat-induced changes in horses. It has been reported that in humans consuming a high-fat diet muscle glycogen levels decreased (Bergstrom *et al.* 1987, Hultman & Bergstrom 1967). In contrast, an increase in resting muscle glycogen concentrations has been found in fat-supplemented horses (Jones *et al.* 1992, Oldham *et al.* 1990, Scott *et al.* 1992), whereas other authors have failed to show such an effect (Essen-Gustavson *et al.*1991, Greiwe *et al.* 1989, Hodgson *et al.* 1986).

The above-mentioned discrepancy in the effect of fat feeding on muscle TAG and glycogen concentrations in the different studies could reflect differences in biopsy site which may, at least in part, be attributed to variation in the fiber composition of the biopsy samples. Fibers are most commonly divided into slow twitch, type I fibers with a high oxidative ability, type IIA fibers with an intermediate oxidative ability and fast twitch, type IIB fibers with a low oxidative ability. Type I fibers have high concentrations of mitochondrial enzymes such as 3-HAD and type II B fibers have a high concentration of glycolytic enzymes such as phosphofructokinase (PFK)). Type I fibers tend to depend largely on aerobic metabolism of glucose and fatty acids for their energy requirement. The type II fibers derive energy mainly from anaerobic glycolysis with glycogen as the main substrate (Snow 1983, Vusse & Reneman 1996). The masseter and the heart are muscles that are composed nearly completely of highly oxidative, type I fibers (Kayar *et al.*1988, Barrey *et al.* 1995). To study effects of fat supplementation these muscle tissues are interesting, especially when compared and contrasted with the semitendinosus, a muscle which contains predominantly glycolytic, type IIB fibers (Barrey *et al.* 1995).

In a previous study with horses, a high-fat diet produced a decrease in the concentration of plasma TAG in the fasted state (Geelen *et al.*1999). The primary mechanism by which a high-fat diet reduces plasma TAG may include increased removal and/or diminished production of TAG-rich lipoprotein particles. Our study

indicated that the decreased concentration of plasma TAG could be attributed to an increased removal through an increase in lipoprotein lipase activity. However, decreased esterification of fatty acids, decreased *de novo* fatty acid synthesis and enhanced fatty acid oxidation within hepatocytes could contribute to the observed TAG-lowering in fat-fed equines. In terms of enzyme activities, these changes would be associated with stimulation of CPT-I and reduction of diacylglycerol acyltransferase (DGAT), acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS).

The aim of the present study was to investigate the effect of dietary fat supplementation on fatty acid esterification, *de novo* fatty acid synthesis and fatty acid oxidation in equines. Specifically, the experiment aimed at (*i)* determining the muscle TAG and glycogen concentrations and the activities of key oxidative and glycolytic enzymes in different muscles, (*ii*) measuring the CPT-I activity in different tissues, and (*iii*) measuring the activities of FAS, DGAT and ACC in liver.

Experimental methods

Animal, diets and experimental design

The experimental design was approved by the animal experiments committee of the Utrecht Faculty of Veterinary Medicine. Twelve shetland ponies (all stallions) weighing 120-220 kg were fed a high-fat or a control diet according to a parallel design. The animals aged 3-13 years (5.8 ± 3.9 and 6.4 ± 4.7 for high-fat and control groups, respectively). During a three week pre-experimental period the ponies had free access to hay. The ponies were allocated to two groups of six ponies each. One group was fed a fat-rich diet and the other a control diet. Pairs of a control and test pony entered the experiment with intervals of 1 to 5 d. The experimental period lasted 45 d for each pair. The diets consisted of hay and either a control or high-fat concentrate. At 10.00 h and 22.00 h concentrate as well as hay were provided. The high-fat concentrate was formulated by adding soybean oil to the control concentrate at the expense of an isoenergetic amount of starch plus glucose (Table 1). The diets were given to the ponies at a level equivalent to the calculated amount of energy needed for maintenance of their initial body weight. On average, the ponies were daily supplied with 0.88 ± 0.09 kg of hay (25 % of net energy) and 1.41 ± 0.17 kg of the test concentrate or 1.62 ± 0.11 kg of the control concentrate (75 % of net energy). The control diet contained 1.5% fat in the dry matter and the high-fat diet 11.8%. The ponies were housed individually in ventilated stables. All animals walked daily 15 min in a mechanical horse walker at a speed of 80 m/min.

Table 1: Composition of the experimental concentrates (g)

Ingredient	Control concentrate	High fat concentrate
Corn starch	193	-
Glucose	140	-
Soybean oil	-	150
Constant components*	850	850
Total	1183	1000

* The constant components consisted of the following (g): Alfalfa meal, dehydrated, 342.4; Corn starch, 150; Glucose, 150; Soya beans, extracted, 100; Molasses, beet, 50; Linseed expeller, 20, $Ca_3(PO_4)_2$, 15; NaCl, 15; MgO, 3.4, $CaCO_3$, 1.7; Premix **, 2.5

** The premix consisted of the following (g/kg): $CoSO_4.7H_2O$, 0.66; $Na_2SeO_3.5H_2O$, 0.76; KIO_3, 0.32; $MnSO_4.H_2O$, 172.4; $CuSO_4.5H_2O$, 27.2; $ZnSO_4.H_2O$, 192.4; Vitamin A, 12.0 (500 000 IU/g); Vitamin D3, 5.2 (100 000 IU/g); Vitamin E, 240.0 (500 IU/g); Vitamin B1, 1.8 (purity 100%); Vitamin B2 (purity 100%), 1.8; Vitamin B12 (purity 0.1%), 1.8; Biotin (purity 100%), 0.4; Corn starch (Carrier), 343.26.

Sampling procedures

At the end of the experiment, at 09.00 h after an overnight fast, blood samples were collected in heparinised tubes by jugular venepuncture. Directly after blood sampling, the shetland ponies were killed by stunning and exsanguination. Tissue samples (2-4 g) were always taken at the same site for each horse to minimize sampling error. Samples were taken from heart, musculus masseter, musculus semitendinosus, musculus gluteus medius and left liver lobulus. Muscle samples were quickly trimmed of visible fat and connective tissue. Then, samples of muscle or liver were frozen in less than 10 min post exsanguination in liquid N_2 and stored at -80^0C for subsequent analyses. One part of the liver sample was immediately homogenized with a loose-fitting Dounce homogenizer (five strokes) in a medium containing 50 mmol/L

Hepes (pH 7.5), 0.25 mol/L mannitol, 4.0 mmol/L citrate, 6.16 mmol/L EDTA and 5 mmol/L ß-mercaptoethanol. The crude homogenate was centrifuged at 12,000 *x* g for 5 min and the supernatant was quickly frozen in liquid N_2 and stored at -80^{0}C until analysis for the activity of FAS and ACC and for mass measurements of ACC. A second part of the liver sample was homogenized with 5 strokes of a glass-Teflon Potter-Elvehjem tissue homogenizer in 4 volumes of a buffer containing 0.25mol/L sucrose, 20 mmol/L Tris-HCl (pH 7.4) and 1 mmol/L EDTA. The homogenate was centrifuged at 600 *x* g for 5 min. The supernatant was recentrifuged at 10,000 *x* g for 15 min. From the supernatant a microsomal pellet was obtained by centrifugation at 105,000 *x* g for 65 min. The final supernatant was termed cytosol.

Assay procedures

Whole plasma TAG concentration was measured enzymatically with an autoanalyser (COBAS-BIO, Hoffmann-La Roche, Mijdrecht, The Netherlands) and a test combination purchased from Boehringer, Mannheim, Germany.

The muscle samples were homogenized with the IKA-Ultra Turrax^R T5-FU tissue homogenizer (Janke and Knukel GmbH and Co. KG, Staufen, Germany) in 9 volumes of a buffer (pH 8.0) containing 25 mmol/L HEPES and 5 mmol/L ß-mercapto-ethanol. Aliquots of this homogenate were used to measure the levels of TAG (Sundler et al.1974) and glycogen (Hassid and Abraham, 1957). To ensure full release of mitochondrial enzymes in the remaining homogenate, Triton X-100 (final concentration 0.5%) was added to the strong hypotonic preparation. The activities of the enzymes measured in this study were not affected by the concentration of detergent used. The Triton X-100-treated homogenate was centrifuged at 48,000 *x* g for 30 min. The supernatant was snap frozen in liquid nitrogen and stored at -80^{o}C until analysed a few days later for enzyme activities. The activities of CS (EC 4.1.3.7), 3-HAD (EC 1.1.1.35), and PFK (EC 2.7.1.11) were determined spectrophotometrically as described by Stitt (1983), by Passonneau & Lowry (1993) and by Ishikawa *et al.* (1990), respectively. Measurement of ACC (EC 6.4.1.2) and FAS (EC 2.3.1.85) activities was performed as described previously (Tijburg *et al.* 1988). The activity of DGAT (EC 2.3.1.20) was evaluated as described before (Tijburg *et al.* 1988). For measuring CPT-I (EC 2.3.1.21) activity, mitochondria were isolated (Guzmán *et al.* 1995). The assay for CPT-I activity was exactly as described (Guzmán & Geelen, 1992).

Mass measurement of ACC was performed by avidin-based ELISA using as the probing antibody a primary antiserum against rat-liver ACC exactly as described before (Geelen *et al.* 1997).

Statistical analysis
Statistical analysis was performed by the Student's *t*-test. The level of statistical significance was pre-set at $P < 0.05$. Values are means ± S.D.

Results

Feed intake and body weight
The ponies consumed all feed supplied, except for two ponies that were given the high-fat diet. The two animals occasionally refused some concentrate, but this was not substantial. The daily feed intake in g/kg body weight (mean ± SD) was 5.52 ± 0.40 of hay and 8.89 ± 0.84 of the test concentrate or 5.46 ± 0.52 of hay and 10.14 ± 0.58 of the control concentrate. The carbohydrate intake in g/kg body weight was 4.81 ± 0.40 and 6.07 ± 0.42 for the test group and control group, respectively. The values for fat intake were 1.30 ± 0.11 and 0.20 ± 0.01 for test and control group, respectively. The ponies maintained their body weight throughout the study. There was no effect of diet on body weight. Initial and final body weights were 158 ± 18 kg and 160 ± 19 kg for the control group and 165 ± 30 kg and 162 ± 30 kg for the test group.

Enzyme activities in liver
The influence of 45 d of fat feeding on the activity in liver of a number of key enzymes of lipid metabolism is presented in Table 2. The specific activity of DGAT was unaffected, but the activities of ACC and FAS were significantly diminished in fat-treated animals. The measurement of the activity of ACC is subject to modification by several factors (Geelen *et al.* 1997). Therefore, mass measurement of ACC was performed by ELISA. The results showed that livers of fat-treated ponies contained substantially less (2.37 ± 0.84 times) ACC protein than those of control ponies. The activity of CPT-I was not affected by fat feeding. However, the process of hepatic fatty acid oxidation is controlled by the specific activity and/or the sensitivity to malonyl-CoA of CPT-I (Guzmán & Geelen, 1993). Thus, hepatic CPT-I sensitivity to inhibition by malonyl-CoA was determined. As indicated in Fig. 1, the fat feeding resulted in loss of sensitivity of hepatic CPT-I to malonyl-CoA.

Table 2: Activities of acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), diacylglycerol acyltransferase (DGAT) and carnitine palmitoyltransferase-I (CPT-I) in liver of ponies fed a low-fat or a high-fat diet.

Enzyme	Diet	
	Control	High-fat
	(nmol/min . mg protein)	
ACC	0.015 ± 0.003	0.007 ± 0.002[a]
FAS	0.192 ± 0.024	0.084 ± 0.019[b]
DGAT	0.163 ± 0.021	0.158 ± 0.068
CPT-I	8.01 ± 1.77*	6.26 ± 1.56

Data are means ± S.D. of six ponies, except for CPT-I where * indicates the mean ± S.D. of five ponies. Significantly different from the control: [a]$P<0.01$, [b]$P<0.001$.

Enzyme activities in muscle

In homogenates of the different muscle samples, the activity of PFK as indicator of glycolytic flux and the activities of CS and 3-HAD as indicators of the capacity for fatty acid oxidation were determined. The activity of PFK was higher in the muscles with a high glycolytic capacity and those of 3-HAD and CS were highest in the aerobic muscles. There were no significant diet effects on the activities of PFK and 3-HAD (Table 3). The activity of CS was significantly enhanced in the masseter muscle of ponies fed the high-fat diet. The activity of CPT-I, a regulatory enzyme of fatty acid oxidation, was significantly higher in mitochondria of the masseter muscle from fat-supplemented ponies.

Table 3: Activities of phosphofructokinase (PFK), 3-hydroxy-acyl-CoA dehydrogenase (3-HAD), citrate synthase (CS) and carnitine palmitoyltransferase-I (CPT-I) in different muscle types of ponies fed a low-fat or a high-fat diet.

Tissue Enzyme	Control diet	High-fat diet
	(nmol/min.mg protein)	
Heart		
PFK	1009 ± 260	1007 ± 137
3-HAD	997 ± 195	1145 ± 322
CS	343 ± 50	335 ± 31
CPT-I	-	-
Masseter		
PFK	701 ± 175	682 ± 109
3-HAD	669 ± 167	784 ± 93
CS	148 ± 24	186 ± 25[a]
CPT-I	4.60 ± 0.67	6.73 ± 0.63[b*]
Gluteus		
PFK	2231 ± 307	2478 ± 608
3-HAD	304 ± 92	253 ± 48
CS	85 ± 19	65 ± 10
CPT-I	-	-
Semitendinosus		
PFK	1918 ± 381	1846 ± 450
3-HAD	264 ± 84	252 ± 66
CS	71 ± 12	65 ± 11
CPT-I	4.99 ± 1.89[*]	4.36 ± 1.37

Data are means ± S.D. of six horses, except for CPT-I where the * indicates means ± S.D. of five horses. Significantly different from control: [a]$P<0.05$, [b]$P<0.001$.

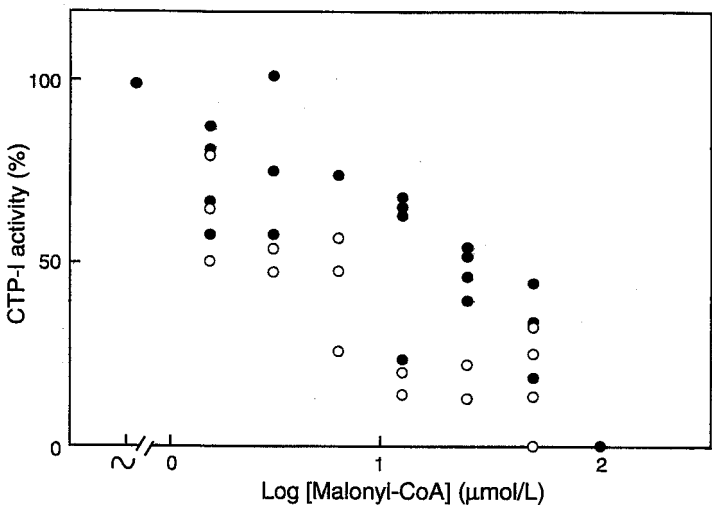

Fig. 1: Effect of dietary fat on the sensitivity of hepatic carnitine palmitoyltransferase-I (CPT-I) to inhibition by malonyl-CoA. Enzyme activity was determined in mitochondria isolated from ponies fed a high-fat (●) or control diet (○) in the presence of increasing concentrations of malonyl-CoA. The regression lines for the test and control diets were y = -0.85x + 79.1 (n = 21, r = -0.94) and y = -0.70x + 57.6 (n = 17, r = -0.72), respectively.

Table 4: Triacylglycerol (TAG) and glycogen contents in different muscle tissues of ponies fed a low-fat or a high-fat diet.

Muscle	Diet			
	Control		High-fat	
	TAG	Glycogen	TAG	Glycogen
	(nmoles/mg protein)			
Heart	24.9 ± 15.9	295 ± 98.1	41.3 ± 29.9	195.0 ± 86.9
Masseter	34.7 ± 15.2	161.7 ± 89.4	26.9 ± 3.7	56.7 ± 44.3[a]
Gluteus	156.3 ± 62.3	583.8 ± 218.6	165.0 ± 59.9	285.8 ± 65.1[b]
Semitendinosus	74.2 ± 26.3	978.4 ± 376.0	134.4 ± 52.6[a]	390.8 ± 140.7[b]

Data are means ± S.D. of 6 ponies. Significantly different from the control: [a]$P<0.05$, [b]$P<0.01$.

Triacylglycerol and glycogen concentrations in muscle
There was no significant effect of diet on the TAG concentration in the heart, masseter or the gluteus muscle. However, dietary fat induced a significantly higher TAG concentration in the semitendinosus muscle. The glycogen concentration was lower in all 4 muscles of the fat-supplemented ponies, this effect reached statistical significance in the masseter, gluteus and semitendinosus muscle (Table 4).

Discussion

Consistent with previous observations (Duren *et al*. 1987, Geelen *et al.* 1999, Orme *et al.* 1997), the ponies fed the high-fat diet had lower plasma concentrations of TAG than did their counterparts fed the control diet. On d 45 of the experiment, the values were 123 ± 70 and 274 ± 107 μmol/L, respectively (P<0.016). This study indicates that the fat-induced reduction of plasma TAG was caused, at least in part, by a decrease in *de novo* fatty acid synthesis as evidenced by the decreased activities of ACC and FAS in liver. Rates of hepatic *de novo* fatty acid synthesis are associated with rates of secretion of TAG-rich very low density lipoproteins (Beynen *et al.* 1981). Thus, we conclude that the decreased plasma levels of TAG reported in fat-supplemented equines is not only due to increased removal of lipoprotein particles (Geelen *et al.* 1999) but to diminished production rates as well.
The activity of CPT-I plays a central role in the control of hepatic fatty acid oxidation (McGarry & Brown, 1997). Its activity is potently inhibited by malonyl-CoA, which is the product of the reaction catalyzed by ACC. The hepatic concentration of malonyl-CoA is directly related with the rate of *de novo* fatty acid synthesis (Beynen *et al.* 1979). Thus, the concentration of malonyl-CoA is crucial in the coordinate control of fatty acid oxidation and fatty acid synthesis. Although CPT-I activity in the liver was not significantly different between groups, the sensitivity of CPT-I for inhibition by malonyl-CoA was lower after fat feeding. In addition, the fat-induced drop in the activity of hepatic ACC, will lower the concentration of malonyl-CoA. Thus, in fat-supplemented animals the effect of a decrease in hepatic malonyl-CoA is amplified through the desensitization of CPT-I to inhibition, thereby allowing an increased rate of oxidation of fatty acids as a source of energy for hepatic requirement. This reasoning would imply that the *in situ* activity of CPT-I was higher in the ponies fed the high-fat diet.
Another new finding of the present study is the 46%-increase in CPT-I activity in the masseter, a muscle with a high aerobic energy metabolism, as a result of the extra dietary fat. This finding is in good agreement with that reported by Phinney *et al.*

(1983) for humans and Boyadjiev (1996) for rats fed a high-fat diet. The increase points at a substantial enhancement of the capacity for fatty acid oxidation in this muscle. Comparison of the specific activities of 3-HAD, CS and CPT-I shows that the former two activities are in large excess and that an increase in the activity of the enzyme that shuttles fatty acids into mitochondria, *i.e.* CPT-I, will be most effective in upgrading the capacity for fatty acid oxidation and thus of energy production. In accordance with earlier results (Geelen *et al.* 2000, Orme *et al.* 1997), dietary fat induced an increase in masseter CS activity, while 3-HAD activity was not significantly affeced. This may relate to the fact that the activity of 3-HAD is much higher than that of CS and CPT- I and thus will not limit the oxidative capacity.

In line with observations by Snow (1983), we observed that highly aerobic muscles (heart and masseter) contain less glycogen than low aerobic muscle (semitendinosus). In all 4 muscle types dietary fat reduced the glycogen content markedly. The reduction was statistically significant in masseter, gluteus and semitendinosus. This result is in agreement with data reported for horses by Greiwe *et al.* (1989) and Pagan *et al.* (1987). However, others showed an increased muscle glycogen concentration in response to a comparable level of fat supplementation (Harkins *et al.* 1992, Jones *et al.* 1991, Oldham *et al.* 1990). The variability in results between the different studies could be due to many factors. Some of these include, breed, age, differences in diet composition, variations in the duration of feeding the fat-supplemented diet, sampling sites, body condition, and training status.

In contrast to results reported by Snow (1983) and Essen-Gustavson *et al.* (1984), the TAG content in highly aerobic muscle was less than in low aerobic muscle. The semitendinosus muscle accumulated significantly more TAG in the ponies subjected to the fat-supplemented diet. Accordingly, in humans there is an increase in muscle TAG after high fat intake (Kiens *et al.* 1987). However, the TAG contents of other muscle-types examined in our study were unaffected by the high-fat diet. We have no explanation for this lack of effect.

It is clear that the lowering of plasma TAG in horses seen after fat feeding relates to an increase in the activity of lipoprotein lipase (Geelen *et al*.1999, Orme *et al.* 1997). The results of this study show that a decrease in *de novo* fatty acid synthesis may contribute to the dietary-fat-induced decrease in plasma TAG concentration. Furthermore, fat supplementation rendered hepatic CPT-I less sensitive to inhibition by malonyl-CoA, which could result in an increased fatty acid oxidation rate. This can also contribute to the plasma TAG-lowering effect of high fat intake. In addition, metabolic adaptation to fat supplementation could be demonstrated in a highly oxidative muscle, the masseter muscle, which showed enhancement of CPT-I and CS activity. If these changes extend to other highly oxidative muscles, there would be an increase in the capacity for fatty acid oxidation which may be advantagous to exercising horses.

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Chapter 6

HIGH DENSITY LIPOPROTEIN CHOLESTERYL ESTER METABOLISM IN THE PONY, AN ANIMAL SPECIES WITHOUT PLASMA CHOLESTERYL ESTER TRANSFER PROTEIN ACTIVITY: TRANSFER OF HIGH DENSITY LIPOPROTEIN CHOLESTERYL ESTERS TO LOWER DENSITY LIPOPROTEINS AND THE EFFECT OF THE AMOUNT OF FAT IN THE DIET.

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Abbreviations
ACAT, acylCoA: cholesterol acyltransferase;
CE, cholesteryl esters;
CETP, cholesteryl ester transfer protein;
FFA, free fatty acids;
HDL, high density lipoproteins (d > 1.090 kg/L);
HTGL, hepatic triacyl glycerol lipase;
LCAT, lecithin: cholesterol acyl transferase;
LDL, low density lipoproteins (1.019 < d < 1.09 kg/L);
LPDS, lipoprotein deficient serum;
LPL, lipoprotein lipase;
TAG, triacylglycerol;
VLDL, very low density lipoproteins (d < 1.019 kg/L).

Abstract

1. We studied the metabolism of high density lipoprotein cholesteryl esters (HDL CE) in the pony, an animal species without plasma cholesteryl ester transfer protein (CETP) activity. Studies were done in ponies fed a low (1.5% fat, w/w) and a high fat diet (11.5%, w/w fat).

2. The ponies fed the high fat diet had higher plasma HDL CE concentrations (1.08 ± 0.15 vs 0.84 ± 0.11 mmol/L, mean ± SD, $n = 6$, $P < 0.01$) and plasma lipoprotein lipase (LPL) activities (14.3 ± 4.0 vs 5.7 ± 3.4 mmol free fatty acids (FFA)/mL/hr, $P < 0.05$) than those on the low fat diet. Plasma triacylglycerol (TAG) concentrations were lower on the high fat diets (0.129 ± 0.043 vs 0.180 ± 0.050 mmol/L), but these differences were not statistically significant. There was a negative correlation between the levels of plasma TAG ($r = 0.598$, $P < 0.05$) and VLDL CE ($r = 0.658$, $P < 0.05$) on one hand and the HDL CE concentrations on the other hand.

3. The transport rates of HDL CE tended to be higher in the ponies on the high fat diet (0.029 ± 0.008 mmol HDL CE/hr/L plasma) than in those on the low fat diets (0.024 ± 0.004).

4. HDL CE were transferred to low density lipoproteins (LDL) and we calculated that the percentage of LDL CE derived from HDL was 0.69 ± 0.13 in the ponies fed the low fat diet and 0.53 ± 0.05 in the ponies fed the high fat diet ($P < 0.05$).

5. The results of these *in vivo* studies suggest that in ponies, similarly as reported in rats and pigs, HDL CE can be transferred to LDL despite the absence of plasma CETP activity, and that the magnitude of this transfer is related to the levels of HDL CE as induced by the amount of fat in the diet.

Indexing Keywords:
Ponies, Lipoproteins, Cholesterol, Cholesteryl Ester Transfer Protein Activity, Dietary Fat, Lipoprotein lipase, Triacyglycerols, Hepatic triaglycerol lipase

Introduction

High density lipoproteins (HDL) play an important role in reverse cholesterol transport, i.e. the transport of cholesterol from the peripheral tissues to the liver (Swenson, 1992). Unesterified cholesterol can move freely from peripheral tissues to HDL and be esterified by the enzyme lecithin: cholesterol acyltransferase (LCAT). Humans and most animal species have plasma cholesteryl ester transfer protein (CETP) activity (Ha and Barter, 1979) and the LCAT generated HDL cholesteryl esters can be tranferred to lower density lipoproteins and be cleared from the plasma by the liver. Because of this transfer, HDL cholesteryl esters are metabolized at a considerably higher rate than HDL apolipoproteins (Ha *et al*., 1981)

Several animal species like the rat and the pig (Ha and Barter, 1979) lack plasma CETP activity. Kinetic studies, however, have shown that in the rat (Terpstra, 1993, Glass *et al*., 1985, Van 't Hooft *et al*.,1981) and pig (Ha *et al*., 1981) HDL cholesteryl esters are also metabolized at a much higher rate than HDL apoproteins and it has been suggested that the LCAT generated HDL cholesteryl esters in these animal species are directly cleared from the HDL (Glass *et al*., 1985 van 't Hooft *et al*., 1981). There are, however, also studies that have indicated that HDL cholesteryl esters can be transferred to low density lipoproteins (LDL) despite the absence of plasma CETP activity. Metabolic studies in pigs (Terpstra *et al*., 1993) and rats (Terpstra, 1993) have shown that radiolabeled HDL cholesteryl esters are transferred to LDL and it was calculated that 36% of the cholesteryl esters in pig LDL were derived from HDL. There was no transfer to very low density lipoproteins (VLDL) and it has been speculated that the VLDL remnants may be the acceptors of the HDL cholesteryl esters and that subsequently these HDL cholesteryl ester enriched remnants are converted into LDL (Terpstra *et al*., 1993).

This observed transfer of HDL cholesteryl esters to LDL in the rat and the pig suggests that animal species that lack plasma CETP activity may have a alternative pathway or mechanism to tranfer LCAT generated HDL cholesteryl esters to lower density lipoproteins. The objective of the present study was to test further this hypothesis. Ponies also lack plasma CETP activity (Watson *et al*., 1993) and we examined whether there was also transfer of HDL cholesteryl esters to LDL in this CETP deficient animal species. HDL radiolabeled in the cholesteryl ester moiety were intravenously administered to ponies and their metabolic fate was followed.

We did these studies in ponies fed a high and a low fat diet. Feeding high fat diets to horses results in an increase of HDL cholesterol concentrations (Geelen *et al.*, 1999) and other studies in mice (Hayek *et al*., 1993) and pigs (Terpstra *et al.*, 1993) have

indicated that high fat diets also result in increased HDL CE transport rates. In the present study we also wanted to examine whether modulations in HDL CE concentrations induced by the amount of fat in the diet, also affects the magnitude of this possible transfer of HDL CE to lower density lipoproteins.

Materials and methods

Animals and diets

The experimental protocol was approved by the animal experiments committee of the Faculty of Veterinary Medicine, Utrecht University, The Netherlands. Twelve Shetland ponies (stallions) weighing 120-220 kg and of age 3-13 years were fed a high fat or a control diet according to a parallel design. During the pre-experimental period, the ponies had free excess to hay. The ponies were at random allocated to three groups of four animals. In each group of four ponies, two ponies were fed a fat rich diet and two ponies were fed a controle diet and were assigned to the diets on the basis of their body weight. The three groups of four ponies were staggered and started their experimental protocol with an interval of one week.

The diets consisted of hay and either a control or high fat concentrate. Concentrate as well as hay were provided every day at 10.00 hr and 22.00 hr.

Table 1. Composition of the experimental concentrates.

Ingredient	Low fat concentrate	High fat concentrate
Corn starch	193	---
Glucose	140	---
Soybean oil	---	150
Constant components	850	850
Total	1183	1000

The ingredients are expressed in grams. The constant components consisted of the following (g): alfalfa meal, dehydrated, 342.4; corn starch, 150; glucose, 150; soya beans, extracted, 100; molasses, beet, 50; linseed expeller, 20, Ca_2PO_3, 15; NaCl, 15; MgO, 3.4, $CaCO_3$, 1.7; premix, 2.5. The premix contained (g/kg premix): $CoSO_4.7H_2O$, 0.66; $Na_2SeO_3.5H_2O$, 0.76; KIO_3, 0.32; $MnSO_4.H_2O$, 172.4; $CuSO_4.5H_2O$, 27.2; $ZnSO_4.H_2O$, 192.4; vitamin A, 12.0 (500.000 IU/gr); vitamin D_3, 5.2 (100.000 IU/ gr); vitamin E, 240.0 (500 IU/ gr); vitamin B_1, 1.8 (purity 100%); vitamin B_2 (purity 100%), 1.8; vitamin B_{12} (purity 0.1%), 1.8; biotine (purity 100%), 0.4; corn starch (carrier), 343.26.

The high fat concentrate was formulated by adding soyabean oil to the control concentrate at the expense of an isoenergetic amount of starch plus glucose (Table 1). The estimated energy density of the control concentrate was 8783 kJ/kg dm and that of the high-fat concentrate was 10393 kJ/kg dm. The diets were given to the ponies at a level equivalent to the calculated amount of energy needed for maintenance of their initial body weight. On average, the ponies received 0.7 kg hay and 1.3 and 0.7 kg of the control and high fat concentrates per meal, respectively. The control diet contained 1.5% fat (w/w) in the dry matter and the high fat diet 11.8% fat. The experimental period for each group of four ponies lasted 33 days. The ponies were housed individually in ventilated stables. All animals walked 15 min in a mechanical horse walker at a speed of 80 m/min.

Blood sampling procedures
After two weeks on the experimental diets, blood samples were taken from the jugular vein in heparinized syringes for the preparation of radiolabeled lipoproteins. After 3 weeks on the experimental diets, blood samples were obtained for the analysis of lipoprotein lipase (LPL) activity and hepatic triacylglycerol lipase (HTGL) activity. After 4 weeks on the experimental diets, blood was sampled in the morning prior to feeding for the analysis of plasma triacylglycerol and lipoprotein cholesterol concentrations and then the kinetic studies were done.

Lipoprotein lipase activity
Plasma samples were drawn in EDTA-containing syringes 5 min after injection of heparin (70 IU/kg body weight, Watson *et al.,* 1993) and the samples were stored at – 80 ^{0}C until analysis. Post-heparin lipase activities were measured according to the method of Nilsson-Ehle and Schotz (1976) with all the modifications added by Herbert *et al*. (1984). HTGL (E.C. 3.1.1.3) activity was measured in the presence of 1 M sodium chloride and the LPL (E.C. 3.1.1.34) activity was calculated as the difference between total lipase activity and HTGL activity.

Preparation of Radiolabeled Lipoproteins
HDL ($1.09 < d < 1.21$ kg/L) were isolated by a combination of differential and density gradient ultracentrifugation (Terpstra and Pels, 1988). Cholesteryl (1-^{14}C)oleate (specific activity of 2.04 TBq/mol; Amersham, Buckinghamshire, England) was incorporated into pony HDL as previously described (Terpstra *et al.*, 1989). Briefly, radiolabeled CE were dissolved in 50 ml absolute ethanol and mixed with pony HDL and heat inactivated (10 min at 60 ^{o}C) lipoprotein deficient serum (LPDS) of cholesterol fed rabbits. LPDS from cholesterol fed rabbits has a high plasma CETP activity (Son and Zilversmit, 1989) that facilitates the incorporation of radiolabeled CE into

lipoproteins. The mixture was incubated in a shaking waterbath at 37°C for 24 hours, and the radiolabeled HDL were reisolated by density gradient ultracentrifugation.

Kinetic Studies
The radiolabeled lipoproteins were administered through i.v. injection into the jugular vein. Blood samples were collected into heparinized tubes by jugular venepuncture after 10 minutes and at appropiate time points for a perid of 70-90 hours. Plasma volume was calculated from the isotopic dilution in the ten minute (zero time) plasma sample. VLDL + LDL in the plasma samples were precipitated with a phosphotungstic acid $MgCl_2$ solution (Assman *et al*.,1983, Sigma Diagnostics, St Louis, MO, catalogue number 352-4) and the supernatant (HDL) was assayed for radioactivity. Radioactivity in whole serum, HDL, and VLDL+LDL were expressed as a fraction of the radioactivity in the plasma sample obtained at 10 minutes after tracer administration. Radioactivity die-away curves were constructed and extrapolated to infinite time. The precursor-product curves could be described by biexponential functions, and the areas under the curves were determined by integrating these functions. The area under the VLDL+LDL product curve was calculated as the area under the total plasma die-away curve minus the area under the HDL die-away curve. A non-compartmental model was used to analyze the kinetic data (Shipley and Clark, 1975, Terpstra, 1989), and the various kinetic parameters were calculated. The residence time (hr) or the average life span of the HDL CE was calculated as the area under the die-away curve and the fractional catabolic rate (FCR, hr^{-1}) was calculated as the reciprocal of the area under the curve. The fraction of LDL CE derived from HDL was calculated as the area under the LDL specific activity product curve divided by the area under the HDL specific activity precursor curve. The transport rate (mmol CE /hr/L plasma) was calculated as FCR x HDL CE concentration.

We also calculated the fraction of total plasma CE that was derived from the LCAT reaction. Plasma CE are derived from the esterification of free cholesterol by the LCAT and the acylCoA: cholesterol acyltransferase (ACAT) enzyme (Glomset, 1970, Terpstra *et al*.,1995). The LCAT enzyme acts predominantly on HDL whereas the ACAT enzyme is active in the liver. The ACAT generated CE in the liver are secreted into plasma VLDL which can subsequently be converted into LDL. Ponies do not have plasma CETP activity, and as a consequence, the CE can not move back and forward between the various lipoprotein fractions. As reported previously in studies with pigs (Terpstra *et al*., 1993) and rats (Terpstra, 1993) which also lack CETP activity and confirmed in the present study with ponies, there is, however, transfer of HDL CE to LDL despite the absence of plasma CETP activity. Thus, the HDL CE can only be derived from the LCAT reaction. The VLDL CE, on the other hand have to be derived from the ACAT reaction, whereas the LDL CE can originate from both the LCAT and

ACAT reaction. The fraction of plasma CE generated by LCAT was calculated as {(fraction LDL derived from HDL x LDL CE concentration) + HDL CE concentration} / total plasma CE concentration.

Analytical Methods
Plasma and lipoprotein cholesterol (Buculo *et al*., 1973) and triglyceride (Allain *et al.*, 1974) concentrations were measured enzymatically on an autoanalyzer (Cobas Bio, Roche, Switzerland). After 4 weeks on the diets, the concentrations of total and esterified cholesterol in whole plasma and the lipoprotein fractions were measured. HDL cholesterol levels were measured after precipitation of the VLDL and LDL with a phosphotungstic acid $MgCl_2$ solution (Assman *et al*., 1983, Sigma Diagnostics, St Louis, MO, catalogue number 352-4). VLDL cholesterol was measured in the VLDL lipoprotein fraction ($d < 1.019$ kg/L) isolated by density gradient ultracentrifugation (Terpstra *et al*.) and the levels of total LDL cholesterol was calculated as the difference between whole plasma cholesterol and HDL + VLDL cholesterol. The ratio of CE to total cholesterol in the HDL and LDL was measured in the HDL and LDL fractions isolated by density gradient ultracentrifugation and the concentrations of HDL and LDL cholesteryl esters were calculated as the concentrations of total cholesterol in the HDL and LDL fraction (measured as described above) multiplied by this ratio. Total plasma proteins concentrations were measured with the biuret method (Gornall *et al.*, 1949) to monitor changes in plasma volume during the kinetic studies and radioactivity in plasma and lipoproteins was corrected for these changes.

The procedure to determine HDL cholesterol concentrations in pony plasma with the phosphotungstic acid / $MgCl_2$ precicpitation method was first validated and compared with the measurement of HDL cholesterol concentrations by ultracentrifugation. HDL cholesterol levels were measured in 6 horse plasma samples and 4 pony plasma samples by using the precipitation method (Assman *et al.,* 1983) and the density gradient ultracentrifugation procedure (Terpstra *et al.*, 1981). The HDL fraction was isolated at $d > 1.09$ kg/L, since horses and ponies have LDL and HDL fractions with densities slightly different from those in humans (Terpstra *et al*., 1982, Papadopulo *et al.*, 1987).

Statistics:
The results in the ponies fed the high and low fat diet were statistically analyzed with an unpaired t-test and correlations between parameters were analyzed with the Pearson Product Moment correlation coefficient. The SigmaStat® statistical software package (Jandel Corporation, San Rafael, CA) was used for all the statistical analyses.

Results

Feed intake and body weight
The ponies consumed all feed supplied, except for two ponies. Two ponies that were fat fed, occasionally left over some concentrate but this was not substantial. The ponies maintained their body weight throughout the study (Table 2). There was no effect of diet on body weight.

Table 2. Plasma lipoprotein lipase and hepatic triacylglycerol lipase activity and kinetic parameters of HDL cholesteryl ester metabolism in ponies fed a low or high fat diet.

	Low fat diet	High fat diet
Lipoprotein lipase activity		
(mmol FFA/mL/hr)	6.02 ± 3.63	15.33 ± 4.35*
Hepatic triacylglycerol lipase activity		
(mmol FFA/mL/h)	4.05 ± 2.55	4.92 ± 1.11
Body weights		
Initial	158 ±18	165 ±30
At 28 days	156 ± 17	160 ± 35
Dose administered (kBq)	391 ± 64	377 ± 65
Plasma volume (% of body weight)	4.48 ± 0.15	4.30 ± 0.31
FCR HDL (hr^{-1})	0.030 ± 0.009	0.025 ± 0.004
Residence time HDL (hr)	35.7 ± 9.7	40.9 ± 6.8
Transport rate (mmol HDL CE/h/L plasma)	0.024 ± 0.004	0.029 ± 0.008
Fraction of LDL CE derived from HDL	0.69 ± 0.13	0.53 ± 0.05*
Fraction of plasma CE generated by LCAT	0.90 ± 0.05	0.90 ± 0.03

Values are means ± SD of six ponies. The lipase activities were measured after 3 weeks on the diets whereas the other kinetic parameters were measured after 4 weeks on the diets. The data were analysed with an unpaired Student's t-test: *$P < 0.05$.

Plasma lipids and lipase activities

In order to validate the phosphotungstic acid / $MgCl_2$ precipitation method to estimate HDL cholesterol concentrations in horse plasma, HDL cholesterol levels in 10 plasma samples was measured by either the precipitation or ultracentrifugation method. The two methods gave comparable results (Table 3). Horse LDL and HDL have densities somewhat different from those in humans and therefore horse HDL should be separated at a density d > 1.09 - 1.10 (Terpstra *et al.*, 1982, Papadopulo *et al.*, 1987). Horse HDL isolated at at d > 1.063 kg/L are contaminated with b-migrating lipoproteins (Robie *et al.*, 1975).

Table 3. HDL cholesterol concentrations in 6 horse and 4 pony samples measured by the precipitation method and the ultracentrifugation method.

	Plasma HDL cholesterol concentration (mmol/L)	
	Precipitation method*	Ultracentrifugation method†
Horse 1	1.82	1.95
Horse 2	1.91	1.90
Horse 3	1.71	1.82
Horse 4	1.79	1.73
Horse 5	1.74	1.69
Horse 6	1.63	1.63
Pony 1	1.45	1.50
Pony 2	1.52	1.67
Pony 3	1.65	1.77
Pony 4	1.30	1.33
Mean (n = 10)	1.65	1.70
SD	0.18	0.18

* VLDL and LDL in plasma samples were precipitated with aphosphotungstic acid /$MgCl_2$ solution and the cholesterol concentration in the supernatant (HDL) was measured.

† HDL (d > 1.09 kg/L) were isolated by density gradient ultracentrifugation and the HDL cholesterol concentration in the HDL fraction was measured.

Plasma triacylglycerol concentrations were higher in the low fat group compared with the high fat group although this effect was not statistically significant (Table 4). In both dietary groups, most of the plasma cholesterol was carried in the HDL fraction whereas only a very small proportion was measured in the VLDL fraction. Feeding ponies a high fat diet resulted in a increase of plasma cholesterol concentrations and a significant increase in HDL total cholesterol and cholesteryl ester concentrations. VLDL cholesterol levels, on the other hand, were significant higher in the low fat group. LDL total cholesterol and CE levels also tended to be lower in the high fat group. There was a negative correlation between the levels of plasma triglyceride ($r = 0.598$, $P < 0.050$) and VLDL CE ($r = 0.658$, $P < 0.05$) on one hand and the HDL CE concentrations on the other hand.

Table 4. Plasma triacylglycerol concentrations and cholesterol and cholesteryl ester concentrations (mmol/L) in lipoprotein fractions in ponies fed a low or high fat diet.

	Low Fat Diet	High Fat Diet
Triacylglycerol concentration	0.180 ± 0.050	0.129 ± 0.043
Total cholesterol concentration		
VLDL	0.06 ± 0.02	0.02 ± 0.01*
LDL	0.55 ± 0.17	0.46 ± 0.15
HDL	1.10 ± 0.16	1.46 ± 0.18*
Whole plasma	1.71 ± 0.19	1.94 ± 0.26
Cholesteryl ester concentration		
VLDL	n.d.	n.d.
LDL	0.40 ± 0.12	0.31 ± 0.11
HDL	0.84 ± 0.11	1.08 ± 0.15*
Whole plasma	1.24 ± 0.12	1.38 ± 0.20

Values are means ± SD of six ponies; n.d., not detectable. The ponies had been fed the diets for a period of 4 weeks. The data were analysed with an unpaired Student's t-test: *$P < 0.01$.

Feeding a high fat diet resulted in a significant increase in LPL activity (Table 2). HTGL activity, on the other hand, was not significantly affected by the amount of fat in the diet.

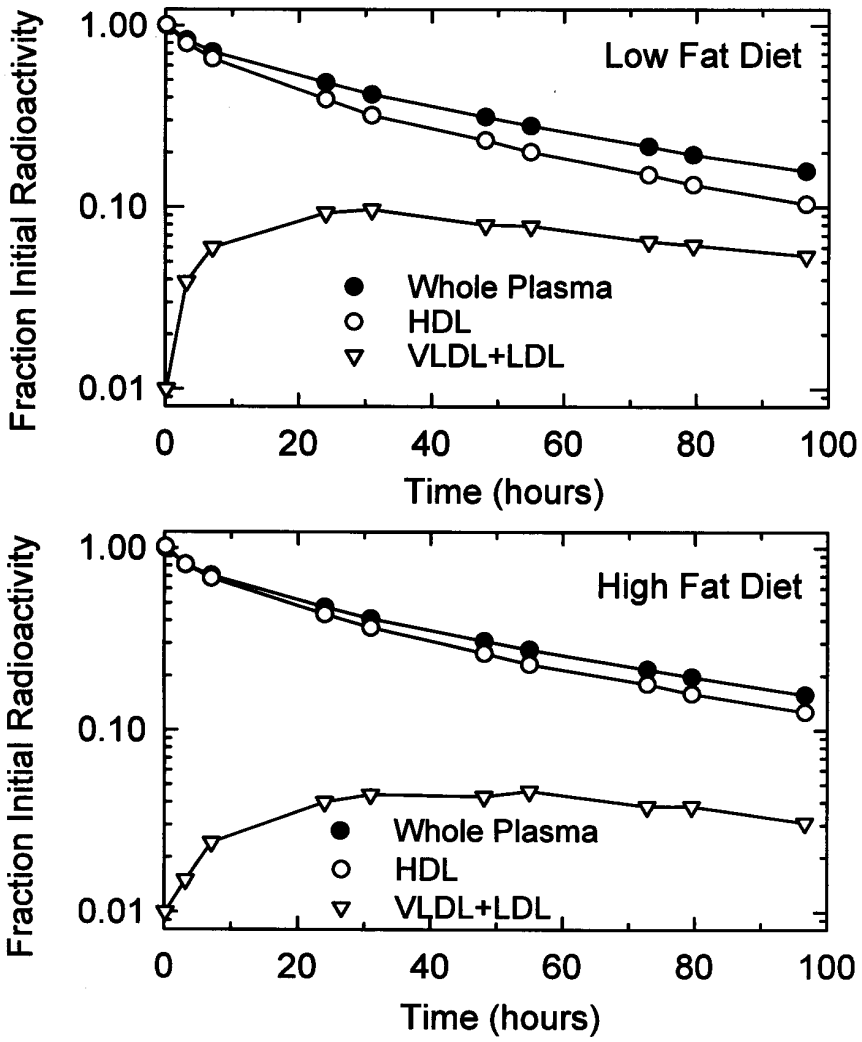

Figure 1. Plasma radioactivity curves of cholesteryl (1-^{14}C)oleate in ponies fed a low and high fat diet after administration of cholesteryl (1-^{14}C)oleate labeled HDL. Values are the means of six ponies.

HDL CE kinetics

Radiolabeled HDL were administered to the ponies and the plasma disappearance rate of the tracer was measured. The VLDL+LDL of plasma samples taken after tracer administration were precipitated with phosphotungstic acid / $MgCl_2$ and the supernatant was assayed for radioactivity. Tracer became precipitable during the course of the kinetic studies which suggests that transfer of HDL CE to lower density lipoproteins had occurred *in vivo* (Fig. 1). We also incubated pony plasma with a tracer amount of radolabeled HDL at 37 °C overnight, but no transfer of HDL cholesteryl esters to lower density lipoproteins was observed *in vitro* which confirms that pony plasma lacks CETP activity (Watson *et al.,* 1993).

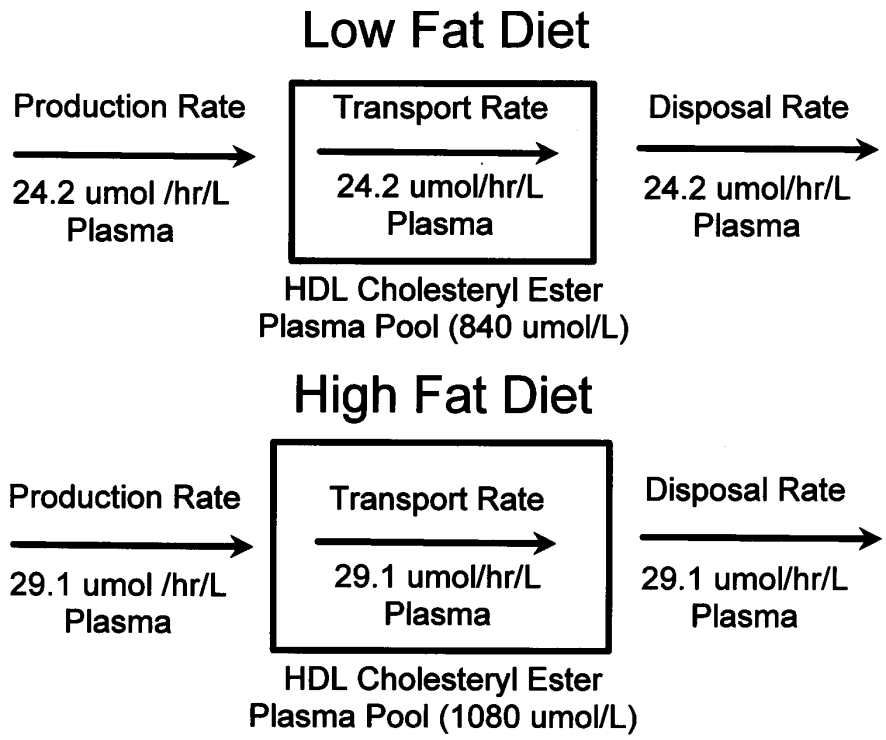

Figure 2. Production, transport , and disposal rates in ponies fed a low fat and high fat diet. The values are the means of 6 ponies.

We calculated that in the ponies fed the low fat diet, 69% of the LDL cholesteryl esters were derived from the HDL and in the fat fed ponies, 53% of the LDL cholesteryl esters originated from the HDL fraction ($P < 0.05$). Fig. 1 also shows that less HDL cholesteryl esters were transferred to the LDL fraction in the ponies fed the high fat diet compared with the low fat group. The proportion of plasma cholesteryl esters that were generated by the LCAT enzyme was similar for both dietary groups (90%). The residence time and the transport rates (Fig. 2) of the HDL CE in the high fat group were higher than in the low fat group although these differences were not statistically significant (Table 2).

Discussion

The results of this study indicates that in the pony, an animal species without plasma CETP activity, *in vivo* transfer takes place of HDL CE to LDL. Similar finding have been reported in the rat (Terpstra, 1993) and the pig (Terpstra *et al.*, 1993), animal species that also lack plasma CETP activity. Thus, it appears that species that do not have plasma CETP activity, have an alternative pathway for transfer of LCAT generated HDL cholesteryl esters to the lower density lipoproteins and reverse cholesterol transport can take place despite the absence of plasma CETP activity. In the rat and the pig, we have demonstrated that there was only transfer of HDL to LDL and not to VLDL and also that there was no transfer back of LDL cholesteryl esters to HDL. We have not examined whether this was also true for the pony, but it seems unlikely that the metabolic pathways of lipoprotein CE in the pony would substantially differ from that in the rat and the pig.
The ponies were infused with HDL that had been labeled with radioactive CE and that were isolated by ultracentrifugation. Adding a tracer amount of these radiolabeled HDL to carrier pony plasma and subsequent precipitation of the VLDL + LDL with phosphotungstic acid / $MgCl_2$ showed that more than 99% of the radioactivity in the HDL preparations remained in the supernatant fraction. This indicates that all the radiolabeled CE were localized in the HDL since all the apo B containing lipoproteins (VLDL + LDL) are precipitated with phosphotungstic acid / $MgCl_2$ (Assmann et al. 1983). During the kinetic studies, tracer became precipitable, which indicates that transfer of HDL CE to apo B containing lipoproteins had occurred, since it has been shown that all the apo B containing lipoproteins are precipitated with phosphotungstic acid / $MgCl_2$ and no co-precipitation of HDL_1, HDL_2, and HDL_3 takes place (Assmann et al. 1983).

It has been postulated that the transfer of HDL CE to LDL may be mediated by initial transfer of HDL CE to VLDL remnants that are subsequently converted into LDL

(Terpstra *et al.,* 1993, Terpstra, 1993). As discussed previously (Terpstra, 1993), in animal species deficient in CETP activity such as pigs, rats, gerbils and dogs, the HDL CE are generated by the LCAT reaction whereas the VLDL CE originate from the ACAT reaction in the liver. HDL CE are rich in polyunsaturated fatty acids such as linoleate or arachidonate, whereas VLDL CE are rich in oleate (Goodman, 1965). LDL CE, on the other hand, have a fatty acid composition intermediate between that of the HDL and VLDL, which supports the hypothesis that HDL CE are transferred to VLDL remnants and subsequently are converted into LDL. Moreover, *in vitro* incubation of horse VLDL with lipase resulted in LDL particles that contained a considerable lower proportion of CE than native LDL (Stachel and Weik, 1995) which is in line with our observation that *in vivo* transfer of HDL CE to LDL takes place.
In the pig fed a low fat commercial diet, only 36% of the LDL CE were derived from the HDL (Terpstra *et al.*, 1993) whereas we calculated that in the pony, 53 - 69% of the LDL CE were derived from the HDL. Further, the proportion of plasma CE derived from the LCAT reaction in the pig was 68% (Terpstra *et al*., 1993) but in the pony this proportion was 90%. Studies by Yamamoto *et al.* (1979b) similarly suggested that the majority of the plasma CE in the horse were produced by the LCAT reaction. The high proportion of LDL CE derived from HDL (53 - 69%) and the high fraction of plasma CE generated by LCAT (90%) in the horse may explain that in the horse as opposed to the pig (Terpstra, 1993), the fatty acid composition of the LDL CE does not much differ from that of the HDL CE (Yamamoto *et al.*, 1979b). Further, there is also evidence that in humans, most of the plasma CE are generated by the LCAT reaction (Glomset, 1972) and similar findings have been reported for rabbits (Goldberg *et al.,* 1991). Feeding cholesterol, however, appears to reduce the contribution of the LCAT reaction in the production of plasma CE and to increase the proportion of plasma CE generated by the ACAT reaction (Terpstra *et al.*, 1993, Rose, 1972).

Fat feeding resulted in a significant increase of plasma LPL activity. This increase may be an adaptation to deal with the high dietary fat load and to expedite the clearance of the increased flux of postprandial chylomicrons originating from the intestine (Hayek *et al.,* 1993, Orme *et al.,* 1997, Wolf, 1996). Because of this increased LPL activity, VLDL particles generated in the liver will also be cleared more rapidly which may explain the low VLDL CE and triglyceride levels in the ponies fed a high fat diet. Studies in humans (Parks *et al.,* 1999) have indicated that the transport rate of VLDL apoproteins and triglycerides is not affected by fat feeding, whereas the levels of VLDL CE and triglyceride are lower. Thus, fat feeding results in a lower number of VLDL particles with a shorter residence time or lifespan. As a consequence, less VLDL and VLDL remnants particles will be available and also their lifespan may be too short to serve as effective acceptors for HDL CE. This may explain the significantly lower transfer rate of HDL CE to LDL together with the significantly higher HDL CE levels

in the fat fed ponies compared with the low fat fed ponies. In addition, this possible mechanism is in line with the observation that there was a significant negative correlation between the plasma triglyceride and VLDL CE levels on one hand and HDL CE concentrations on the other hand.

Other studies with pigs (Terpstra et al, 1993), humans (Brinton *et al.*, 1990, Wolf, 1996), and horses (Geelen *et al.,* 2000) have also reported that high fat diets increase HDL cholesterol levels. Further, increasing the amount of fat in the diet has also been found to be associated with elevated transport rates of HDL CE in rats (Hayek *et al.*, 1993) and pigs Terpstra *et al.*, 1993) and with increased transport rates of the HDL apoproteins in humans (Brinton *et al.,* 1990, Wolf, 1996) and rabbits (Quig and Zilversmit, 1989). A similar trend was observed in our studies with ponies; fat feeding resulted in a significant increase in HDL CE levels together with an increase in the HDL CE transport rate.

As discussed previously (Terpstra *et al.,* 1995), in species without CETP activity, the plasma HDL cholesteryl esters have to be entirely derived from the LCAT reaction. Thus, the production rate of HDL CE by LCAT should reflect the HDL CE transport rate. In pigs (Terpstra *et al.,* 1993), we found an *in vivo* HDL CE transport rate of 54 mmol / h / L plasma which is comparable to the LCAT activity found in other studies by Stokke (1974) (42 - 52 mmol / h / L), Lacko *et al*. (1974) (43.6 mmol / h / L), and Forsythe *et al.* (1981) (46.4 mmol / h / L). In ponies, we measured an HDL CE transport rate of 24 - 29 mmol / h / L which is also more or less comparable to the production rate of HDL CE by the LCAT reaction (30 - 90 mmol / h / L) as reported in other studies (Yamamoto *et al.*, 1979a)

In conclusion, the results of this study indicate that in the pony, an animal species without CETP activity, transfer of HDLCE to LDL takes place which is not mediated by CETP. Thus, despite the absence of CETP activity, reverse cholesterol transport can take place, i.e. esterification of tissue derived cholesterol by HDL, transfer of LCAT generated HDL CE to lower density lipoproteins, and subsequent uptake of these lower density lipoproteins by the liver. Feeding fat appears to lower this transfer rate together with an increase in HDL CE levels.

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Chapter 7

INDIRECT MEASUREMENT OF THE PRODUCTION OF PLASMA TRIACYLGLYCEROLS BY HORSES GIVEN A HIGH-FAT DIET

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Abstract

The hypothesis tested was that the feeding of extra fat to horses would raise the production of plasma triacylglycerols (TAG). To measure TAG secretion, the indirect Triton method was used. Six adult horses were given a low-fat control or a high-fat diet according to a cross-over design. In keeping with our earlier work, the high-fat diet lowered fasting plasma TAG concentrations by on average 42 % and raised post-heparin total lipoprotein lipase activity by 79 %. The rate of TAG appearance in plasma after Triton administration was 49 % lower when the horses were fed the high-fat diet instead of the low-fat control diet. Thus, the hypothesis would be rejected. It is suggested that the dose of Triton used might have been too low to fully depress lipoprotein lipase activity, leading to an outcome of the study that was opposite to that expected.

Introduction

In previous studies (1, 2), the feeding of extra fat to horses raised lipoprotein lipase (LPL) activity in post-heparin plasma and lowered plasma triacylglycerol (TAG) concentrations. LPL hydrolyses TAG in chylomicrons and very-low-density lipoproteins (VLDL) so that the fatty acid constituents can be taken up by tissues. The observed low TAG concentration and high LPL activity in fasting horses fed a high-fat diet points at a high TAG turnover which should be associated with an enhanced production of VLDL by the liver. VLDL-TAG production rate can be measured indirectly by using a non-ionic detergent such as Triton WR 1339 (oxyethylated t-octyl-phenol polymethylene polymer). Intravenous administration of Triton to experimental animals including rats, rabbits and mice, produces hyperlipemia which is explained by a considerable decrease in LPL activity in post-heparin plasma (3, 4). The Triton-induced block of lipoprotein lipase causes TAG accumulation in plasma which has been used as an index of TAG secretion rates in rats (3, 5), gerbils (6) and sheep (7). As far as we know, the method has not yet been used in horses. Here we report on the use of Triton WR 1339 to measure plasma TAG production in horses, with the specific aim to test our hypothesis that fat feeding raises TAG production.

Experimental methods

Animals, diets and experimental design

Six Standardbred horses (3 mares, 3 geldings) weighing 432-556 kg and aged 6-13 years were fed a high-fat or a control diet according to a cross-over design (2 X 2 Latin square) with feeding periods of 5 weeks. During a two week pre-experimental period the horses had free access to hay. Pairs of horses fed either the control or test diet entered the experiment with intervals of 1 day. The experimental diets consisted of hay and either a control or high-fat concentrate. At 10.00 and 22.00 h concentrate as well as hay were provided. The high-fat concentrate was formulated by adding soybean oil to the control concentrate at the expense of an isoenergetic amount of cornstarch plus glucose (Table 1). The diets were given at a level equivalent to the calculated amount of energy needed for maintenance of the horses' initial body weight. On average, the horses received 1.1 kg hay and 1.7 and 1.5 kg of the control and high-fat concentrates per meal, respectively. The whole control ration contained 1.5 % fat in the dry matter and the high-fat diet 11.8 % fat. The horses were housed individually in stands which were located in a ventilated stable. All animals walked each day for 60 min in a mechanical horse walker at a speed of 100 m/min.

Table 1 Composition of the experimental concentrates.

Ingredient	Control	High fat
Corn starch, g	193	---
Glucose, g	140	---
Soybean oil, g	---	150
Constant components, g	850	850
Total, g	1183	1000

The constant components consisted of the following (g): alfalfa meal, dehydrated, 342.4; corn starch, 150; glucose, 150; soya beans, extracted, 100; molasses, beet, 50; linseed expeller, 20, Ca_2PO_3, 15; NaCl, 15; MgO, 3.4, $CaCO_3$, 1.7; premix, 2.5. The composition of the premix has been described elsewhere (1).

Sampling procedures

After 2 weeks on the experimental diets, prior to feeding in the morning, blood samples for determination of whole-plasma TAG were collected in heparinized tubes by jugular vene puncture. Subsequently, blood samples were obtained in EDTA-containing syringes at 5 min after injection of heparin for the analysis of LPL and hepatic lipase (HTGL). The dose of heparin was 70 IU/kg body weight as described by Watson et al(8). The samples were stored at –80ºC until analysis. After three weeks on the experimental diets, at 8.30 h, Triton WR-1339 was administered through intravenous injection into the jugular vein over a period of 30 min. The detergent was infused at a dose of 100 mg/kg body weight in 500 ml of phosphate-buffered saline solution. Blood samples for determination of whole plasma TAG were collected into heparinized tubes at the end of the infusion and at various time points for a period of 72 hours. According to the the original protocol, we also attempted to determine the clearance of intravenously administered TAG at rest and during exercise. As described below, the samples from the clearance studies could not be used due to a persisting effect of the Triton administration. For evaluation of this unexpected effect of Triton, the full design of the study is given. After four weeks on the experimental diets, at 8.30 h, Intralipid® (10% TAG emulsion) was administered through intravenous injection into the jugular vein. The amount infused was 0.3 ml of Intralipid® /kg body weight which was administered within 3 min. Blood samples were collected into heparinized tubes at the end of the infusion and at various points for a subsequent period of 80 min. The samples were analysed for whole-plasma TAG. After five weeks on the experimental diets, prior to feeding, the horses were exercised at 5 m/s for 22 min on a treadmill. After 9 min of exercise, Intralipid® (0.3 ml/kg body weight) was administered within 3 min. Blood samples were obtained from an indwelling

jugular catheter immediately prior to exercise, every 2 min during exercise, 5 times every 2 min immediately after exercise, followed by 4 times every subsequent 5 min and 2 times every 10 min.

Assay procedures
Total and HTGL lipase activities were determined according to Nilsson-Ehle and Schotz (9) in the presence of a low and high concentration of NaCl, respectively. LPL activity was calculated as the difference. Whole-plasma TAG concentrations were measured enzymatically with an autoanalyser (COBAS-BIO, Hoffmann-La Roche, Mijdrecht, The Netherlands) and a test combination purchased from Boehringer, Mannheim, Germany.

Statistical analysis
Post-Triton plasma TAG concentrations for each horse and each diet were subjected to linear regression analysis. As the data were not affected by period (ANOVA), statistical analysis was performed to evaluate diet effects with the Student's paired t-test. The level of statistical significance was preset at $P<0.05$.

Results

The horses consumed all feed supplied and maintained their body weight throughout the study. There was no effect of diet on body weight.

Table 2 Plasma triacylglycerol (TAG) concentrations and post-heparin lipase (LPL) and hepatic lipase activity (HTGL) in horses that were either fed a high-fat or control diet according to a cross-over design.

Measure	Control diet	High-fat diet
TAG	251 ± 75	145 ± 52*
LPL	4200 ± 1400	8600 ± 2500*
HTGL	4800 ± 1100	7500 ± 800*

Data are means ± SD for 6 horses. TAG values are expressed as μmol/l. Lipase values are expressed as μmol fatty acid released/l per h.
* Statistically significant ($P<0.05$) versus control diet.

Plasma TAG values are given in Table 2. After two weeks of consuming the experimental diets, the plasma TAG values were significantly lower when the horses were given the high-fat diet instead of the control diet. The high-fat diet produced

significant increases in plasma LPL and HTGL activities (Table 2).
Figure 1 shows for a typical horse the increment of plasma TAG concentration following Triton injection. The data for both the control and the high-fat diet are given, illustrating that the increase in TAG was less pronounced when the high-fat diet was fed. The slopes of the regression lines for post-Triton plasma TAG concentrations, which represent the TAG secretion rate, are given for the two diets and for all six horses in Table 3. The slopes for the high-fat diet were significantly lower than those for the control diet. The linear correlation coefficients for the two treatments ranged between 0.6 and 0.9.

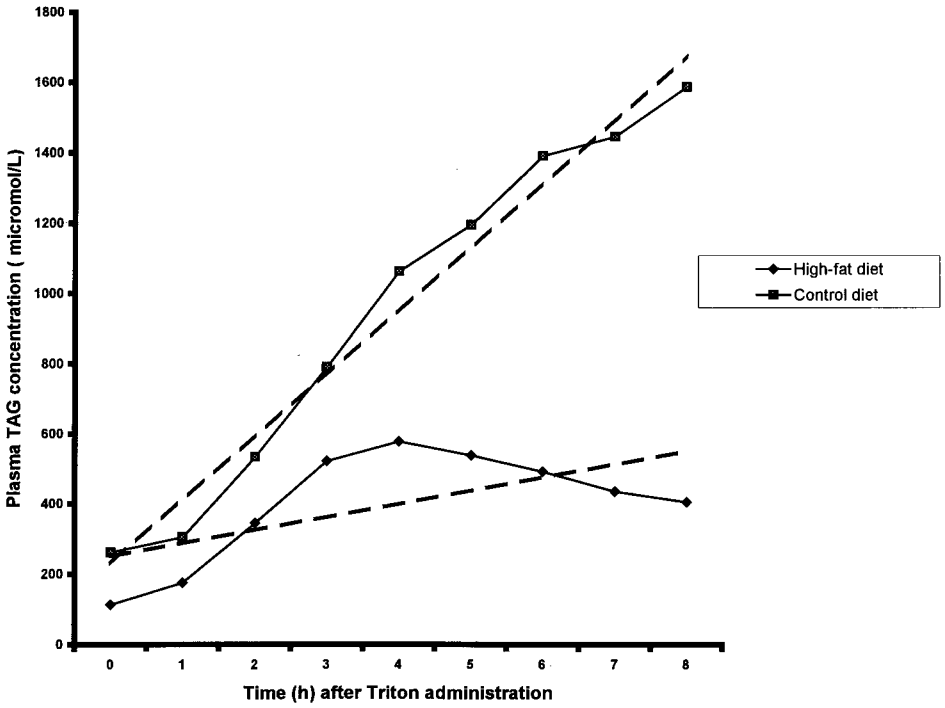

Figure 1 Plasma TAG accumulation after Triton administration in a horse when fed either the high-fat or the control diet. The dashed lines are based on linear regression analysis. The data correspond with those for horse 1 in Table 3.

Table 3 TAG secretion rates following Triton injection in 6 horses when fed either a control or high-fat diet.

Horse	Control diet	High-fat diet
1	542	113
2	197	38
3	711	622
4	190	22
5	279	258
6	308	87
Mean ± SD	372 ± 210	190 ± 228*

TAG secretion, μmol fatty acid released/l per h.
*Statistically significant ($P<0.05$) versus control diet.

A single Triton injection caused high levels of plasma TAG that had returned to baseline concentrations only after 7-14 days. However, 21 days after the second injection, plasma TAG levels were still high for 4 of the 6 horses; TAG levels in the 4 horses ranged from 4000 to 8000 μmol/l. The high TAG concentrations prevented the measurement of TAG clearance after Intralipid® administration.

Discussion

The present results are in agreement with previous studies showing that fat feeding to horses causes an increase in post-heparin plasma LPL activity and a decrease in plasma TAG concentration (1, 2, 10). We hypothesized that fat feeding would raise the rate of TAG appearance in the circulation and tested our hypothesis with the Triton method used earlier in other species (6, 7, 11). From an experiment with rats it was concluded that Triton-induced inhibition of TAG hydrolysis was due to coating of plasma lipoproteins by the detergent, thereby preventing the interaction of substrate and lipoprotein lipase (12). However, others observed that Triton has a direct inhibitory effect on lipoprotein lipase (3, 4). Intravenous administration of Triton WR 1339 will cause TAG accumulation which represent the secretion of lipoprotein-TAG by the gastrointestinal tract and the liver. Secretion by the gastrointestinal tract occurs in response to dietary fat. However, attempts to isolate chylomicrons from adult ponies following an oral fat load have been unsuccesful, indicating that post-prandial lipaemia is negligible (13). Hepatic TAG secretion is influenced by the amount of fatty acids delivered to the liver and by the rate of *de novo* fatty acid synthesis. Thus,

it is likely that in horses Triton-induced accumulation of TAG reflects net TAG secretion by the liver. When the horses were fed the high-fat diet they had significantly lower TAG secretion rates than when they were fed the control diet. The decreased TAG secretion is in agreement with the reduced de *novo* synthesis of fatty acids found previously in ponies given a high-fat diet (14). However, the outcome of this study is opposite to our hypothesis and is difficult to reconcile with the fat-induced increase in LPL activity. In the steady state, the rate of TAG input into plasma must equal TAG output. When assuming that the output is determined by LPL activity, then fat-feeding should raise TAG input and thus should lead to more TAG secretion as determined by the Triton method.

Table 4 TAG secretion rates following Triton WR 1339 injection in different species under various conditions.

Species (Reference)	Triton WR 1339 *mg/kg bw*	TAG secretion rate *µmol/h/kg bw*	Conditions
Sheep (7)	240	1.4 2.9 3.5	Fed state Fasted state Diabetic
Horse 1 (this study)	100	2.6 12.6	High-fat diet Control diet
Rat (5)	500	35.1 63.1	Treated with cafestol Treated with placebo
Gerbil (6)	225	126 72	Safflower oil-rich diet Coconut oil-rich diet

TAG secretion rates were calculated by multiplying the slope of the post-Triton plasma TAG regression line by plasma volume (7% of body weight), and expressed as µmol/h/kg body weight.

The measured rates of TAG secretion were 25 to 85 times smaller than the sum of LPL and HTGL activities in post-heparin plasma. This could relate to Triton not completely inhibiting lipoprotein lipase activity. In rats, gerbils and sheep, TAG secretion has been studied by using higher dosages of Triton WR 1339 than we used in our horses (Table 4). Thus, the amount of Triton WR 1339 used in this study might have been too low to completely inhibit LPL activity. In 2 horses not used in this experiment, we measured post-heparin LPL activity 5 hours after Triton administration: The activity was still 65% of the baseline activity. The unexpected results would thus be explained by Triton administration being less effective when the horses were given a high-fat diet because their LPL activity was increased. Thus, after

fat feeding the effect of Triton administration could have been relatively less than after feeding the low-fat control diet so that an apparant low TAG secretion was measured for the high-fat diet. The observation of having used not sufficient Triton would also be supported by the fact that TAG secretion rates in rats and gerbils were much higher than in our horses (Table 4). However, in sheep that received more than twice as much Triton as did our horses, TAG secretion rates were lower. In any event, there appear to be species differences with regard to the efficacy of Triton WR 1339. LPL in isolated adipose tissue from chickens was less sensitive to Triton-induced inhibition than that from rats (15). We avoided high dosages as it can be toxic. Triton WR 1339 given to dogs at a dose of 400 mg/kg body weight killed all 6 experimental animals with symptoms of an acute intravascular hemolytic episode (11). Two dogs that were given 250mg Triton/kg body weight every 3 days died following the fourth injection (11).

In conclusion, this study shows that contrary to our expectation TAG secretion, as measured with the Triton method, was lower when horses were fed a high-fat diet instead of a low-fat control diet. The Triton method deserves further study as to establishing the proper dose for horses. Our data indicate that twice versus single Triton administration may cause marked elevations of TAG lasting as long as 3 weeks. Such a sustained effect of Triton may be undesirable in cross-over studies when to be used as a tool to measure TAG secretion.

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Chapter 8

GENERAL CONCLUSIONS

General conclusions

In this summarizing chapter the main conclusions from this thesis are listed and briefly discussed. In all experiments, except for the experiment described in chapter 4, the diets with identical composition were used: a control diet that contained 1.5 % fat in the dry matter and a high-fat diet that contained 11.8 %. The high-fat diet was formulated by adding soybean oil to the concentrate at the expense of an isoenergetic amount of corn starch plus glucose. The control and high-fat diets consisted of hay with either the control or high-fat concentrate.

Chapter 2: Fat feeding in horses increases post-heparin plasma LPL activity and decreases the concentration of whole plasma TAG.

It is hypothesized that the increased activity of LPL in post-heparin plasma in the fasting state reflects an increase in muscle LPL activity. This would imply that a high-fat diet raises the flux of fatty acids, in the form of TAG, into skeletal muscle. The increased flux of fatty acids should be associated with an increased oxidative capacity of skeletal muscle. As training leads to comparable metabolic adaptations, the feeding of a high-fat diet might be a suitable adjunct to training.
The dietary-fat induced enhancement of LPL activity and the decrease in TAG concentration are confirmed by the experiments described in chapters 3, 4, 6 and 7.

Chapter 3: Fat feeding raises post-heparin plasma LPL activity in a dose-dependent fashion.

The fat-induced increase in LPL activity is linear in the range of 3-16 % of fat in the dry matter. It is suggested that not only under experimental conditions, but also in practice, high-fat diets can be a potential tool to improve performance of athletic horses.

Chapter 4: There is no evidence for fat-supplemented horses having an enhanced mobilisation of fatty acids from adipose tissue.
There is no evidence that fat feeding changes the fatty acid oxidation and glycolysis in muscle.

The assumed extra mobilisation of fatty acids from adipose tissue in fat-supplemented horses could not be proven. However, the noradrenaline-induced stimulation of the lipolytic rate tended to be higher when the horses were fed a fat-rich diet. It is not known whether this observation has biological relevance, but it suggests that feeding a fat-rich diet renders lipolysis more sensitive to stimulation, which may be advantageous to exercising horses.
In species other than the horse, high-fat diets have been shown to increase the oxidative capacity in muscle tissue. However, it could not be demonstrated that dietary fat enhances the activities of key oxidative enzymes in muscle. The biopsies were obtained from the gluteus muscle. This muscle is a "mixed muscle", containing approximately the same amount of type I and type II B fibers. If a muscle containing predominantly type I fibers, with high oxidative ability and thus high concentration of oxidative enzymes, would have been biopsied, an enhancement of oxidative capacity might have been observed. A similarly reasoning applies to the glycolytic activity. If a muscle containing predominantly type IIB fibers, with low oxidative ability and high concentration of glycolytic enzymes, would have been biopsied a reduced glycolytic activity might have been observed. Another explanation for the unexpected results can be that enzyme activities did not survive pre-assay procedures.

Chapter 5: Fat feeding enhances both the transport of fatty acids through the mitochondrial inner membrane and the oxidative capacity in highly aerobic muscles.
A depressed rate of *de novo* fatty acid synthesis in liver contributes to the dietary-fat induced decrease in plasma TAG.

Metabolic adaptations to fat supplementation can most easily be demonstrated in highly oxidative muscles which have a significant ability to use fatty acids as an energy source. The masseter muscle from ponies was used to study the effects of fat supplementation. This muscle obviously does not play a role during exercise but it has a very high percentage of type I fibers, that facilitate oxidation of fatty acids during endurance exercise.
The lowering of plasma TAG in horses seen after fat feeding (chapters 2, 3, 4, 6) certainly relates to the increase in the activity of LPL, but the observed decrease in *de novo* fatty acid synthesis may contribute to the dietary-fat induced decrease in plasma TAG concentration. Furthermore, fat supplementation renders hepatic CPT less sensitive to inhibition by malonyl-CoA, which can result in an increased fatty acid oxidation rate. It is suggested that the effect on CPT also contributes to the plasma

TAG-lowering effect of high fat intake.

Chapter 6: In the pony transfer of HDL CE to LDL takes place, but is not mediated by CETP.
Fat feeding lowers the transfer of HDL CE to LDL.

It is demonstrated that ponies, an animal species lacking plasma CETP activity, have an alternative pathway to transfer LCAT generated HDL CE to lower density lipoproteins. Thus, reverse cholesterol transport can take place.
HDL CE levels are significantly higher in ponies given a high-fat diet compared to ponies fed a low-fat diet. Low transfer rates of HDL CE to LDL are found after fat feeding. It is hypothesized that HDL CE are transferred to VLDL remnants and subsequently converted into LDL. Because fat feeding results in a lower number of VLDL particles with a shorter lifespan, less particles will be available to serve as effective acceptors for HDL CE. This may explain the lower transfer rates of HDL CE to LDL together with the higher HDL CE levels in ponies on a high-fat diet.

Chapter 7: TAG secretion as measured with the Triton method is lower when horses are fed a high-fat diet instead of a low-fat control diet.

It was hypothesized that the TAG secretion would be enhanced in fat-supplemented horses compared with horses fed a control diet. The outcome of the study was opposite to what was hypothesized. The Triton method to measure the production of plasma TAG has not been used in horses previously. The use of a too low dosage of Triton may explain why the measured rates of TAG secretion were much lower than could be expected on the basis of the sum of LPL and HTGL activities. Triton administration possibly was less effective in horses fed a high-fat diet because of their increased LPL activity, explaining the unexpected result.

Summary

Introduction

This thesis deals with the influence of fat feeding on lipid metabolism in horses. High-fat diets have attained considerable interest as a potential tool to improve performance. Many factors affect performance so that large numbers of horses are needed to unequivocally determine the effect of diet on performance. In practice, the execution of trials on diet in relation to performance is not possible. However, based on the reported effects of nutrition on metabolism, it should be possible to predict the effect of nutrition on performance. The aim of the studies described in this thesis was to demonstrate a rationale, or a lack of it, for feeding horses on diets with extra fat. Literature data show that, analogously to training, the contribution of fatty acids to the energy supply during exercise is increased on a fat-rich diet. The fat-induced changes in the metabolic machinery might lead to a sparing of glucose and glycogen during an aerobic workload and to the promoting of glycolysis during anaerobic exercise. Attention was focussed on investigating the effect of dietary fat supplementation on equine lipid metabolism in untrained horses in the fasting state.

Research

The first hypothesis tested (Chapter 2) was that the feeding of extra fat enhances the flux of fatty acids, in the form of triacylglycerols (TAG), through the circulation into skeletal muscle. This was tested indirectly by measuring the concentration of plasma TAG and the activity of lipoprotein lipase (LPL) in post-heparin plasma. In a cross-over trial, 6 horses were given either a low- or a high-fat diet. The low-fat diet contained 1.5% fat in the dry matter and the high-fat diet contained 11.8%. The high-fat diet was formulated by adding soybean oil to the control diet at the expense of an isoenergetic amount of corn starch plus glucose. Both diets consisted of hay and concentrate. In all experiments, except for the experiment described in Chapter 4, diets with identical composition were used. It appeared that fat feeding increased post-heparin plasma LPL activity and decreased the concentration of circulating TAG, indicating an increased flux of fatty acids. It was suggested that the increased activity of LPL in post-heparin plasma reflects an increase in muscle LPL activity because the samples were taken in the fasting state when only little LPL is derived from adipose tissue.

An attempt was made to establish a dose-response relationship between fat intake and post-heparin plasma LPL activity (Chapter 3). Eight horses were fed four rations with

different fat levels (3.0, 5.0, 7.7 or 10.8% fat in the dry matter) according to a 4 X 4 Latin square design. Fat feeding was found to raise post-heparin plasma LPL activity in a dose-dependent fashion. Within the range of fat intakes tested, the dose-response relationship did not reach a plateau. There was a decreased acceptance by certain horses of the diet with the highest fat level.

As mentioned above, a high-fat diet is expected to lead to an increase in the turnover of fatty acids in the form of TAG. This would imply that there is enhanced mobilisation of fatty acids from adipose tissue after fat feeding. Therefore, the in-vitro lipolytic rate in biopsies of adipose tissue was determined (Chapter 4). Six horses were fed diets either high in fat, glucose or corn starch. The diets were formulated so that the intake of soybean oil versus either glucose or corn starch were the only variables. The dietary variables did neither significantly affect the basal in-vitro lipolytic rate nor the lipolytic rate after adding noradrenaline. However, the percentage of noradrenaline-induced stimulation of the lipolytic rate was higher in adipose tissue sampled when the horses were given the high-fat diet. This observation suggests that feeding a fat-rich diet renders lipolysis more sensitive to stimulation, which could be beneficial for performance. It was speculated that the increased flux of fatty acids mentioned earlier is associated with an increased oxidative capacity of skeletal muscle. Therefore the fatty acid oxidative capacity in skeletal muscle was assessed by measuring the activities of key oxidative enzymes in muscle biopsies. Fat supplementation did not affect the activity of the key enzymes in muscle tissue. The biopsies were obtained from the gluteus muscle. This muscle is a “mixed muscle”, containing approximately the same amount of type I and type IIB fibers. If a muscle containing predominantly type I fibers, with high oxidative ability and thus a high concentration of oxidative enzymes, would have been biopsied, an enhancement of oxidative capacity might have been observed.

Thus, the effect of fat supplementation on energy metabolism in different muscle types was studied in ponies (Chapter 5). The masseter and the heart are muscles that are composed nearly completely of highly oxidative fibers. These muscle were compared and contrasted with the semitendinosus, a muscle which contains predominantly glycolytic fibers. Metabolic adaptation to fat supplementation could be demonstrated in the masseter muscle, which showed enhancement of carnitine palmitoyltranferase-I (CPT-I) and citrate synthase (CS) activity. However, there was no diet effect on glycolytic enzyme activities in the various muscles. In addition, key enzymes of hepatic lipogenesis were investigated to establish whether a diminished production of TAG-rich lipoprotein particles could contribute to the dietary fat-induced decrease in the plasma TAG concentration. The lowering of plasma TAG in horses seen after fat feeding certainly relates to the increase in the activity of LPL. However, the results

indicated that a dietary fat-induced reduction of plasma TAG is caused, at least in part, by a decrease in the rate of hepatic *de novo* fatty acid synthesis. Furthermore, fat supplementation rendered hepatic CPT-I less sensitive to inhibition by malonyl-CoA, which would result in an increased fatty acid oxidation rate. It was suggested that the effect on CPT-I also contributes to the plasma TAG-lowering effect of high fat intake.
In the experiment presented in Chapter 6 the metabolism of HDL cholesteryl esters (HDL CE) was studied. It was examined whether in the equine, an animal species that lacks cholesteryl ester transfer protein (CETP), transfer of HDL CE to LDL takes place. It was anticipated that the results obtained would shed light on the mechanism underlying the observed increase in plasma HDL cholesterol (HDL C) following the consumption of a fat-supplemented diet. Twelve ponies were either fed a fat-rich diet or a control diet according to a parallel design. Radiolabeled HDL was administered to the ponies and the plasma disappearance rate of the tracer was measured. It was demonstrated that ponies have an alternative pathway to transfer LCAT-generated HDL CE to lower density lipoproteins. It was suggested that HDL CE is transferred to VLDL remnants and subsequently converted into LDL. Fat feeding resulted in a significant increase in HDL CE levels together with an increase in the HDL CE transport rate.
The observed low TAG concentration and high LPL activity in post-heparin plasma in horses fed a high-fat diet points at a high TAG turnover which should be associated with an enhanced production of VLDL by the liver. This reasoning was tested indirectly by using the Triton method (Chapter 7). Triton WR 1339 blocks LPL activity which in turn causes TAG accumulation in plasma and may serve as an index of TAG secretion rates. Six horses were given either a low- or high-fat diet according to a cross-over design and were intravenously injected with Triton WR 1339. When the horses were fed the high-fat diet they had significantly lower TAG secretion rates than when they were fed the control diet. Thus, the outcome of the study was opposite to what was hypothesized. It was suggested that the use of a dosage of Triton that was too low is responsible for the measured rates of TAG secretion that were lower than could be expected on the basis of the sum of LPL and HTGL activities. Triton WR 1339 might have been insufficiently effective in horses fed a high-fat diet because of their increased LPL activity, which could also explain the unexpected results.
This thesis shows that feeding a fat-rich diet triggers metabolic adaptations on the cellular level which are comparable to those described as induced by training. Thus, feeding a high-fat diet might be advantagous to exercising horses.

Samenvatting

Inleiding

Dit proefschrift bevat de weerslag van mijn onderzoek naar het effect van extra dieetvet op het metabolisme van lipiden bij het paard. Vetrijke-diëten staan sterk in de belangstelling omdat het prestatieniveau van het dier hierdoor beinvloed zou kunnen worden. Aangezien vele factoren van invloed zijn op de prestatie zijn grote aantallen paarden nodig om eenduidig het effect van voeding op de prestatie vast te stellen. Dat betekent in feite dat het doen van voedingsproeven in relatie tot de prestatie niet goed mogelijk is.

Het doel van de in dit proefschrift beschreven studies was te onderzoeken of er al dan niet grond is voor het voeren van extra vet aan paarden. Literatuurgegevens laten zien dat, naar analogie met training, de bijdrage van vetzuren aan de energievoorziening gedurende arbeid toeneemt op een vetrijk rantsoen. Vet-geïnduceerde veranderingen in de metabole toerusting kunnen leiden tot het sparen van glucose en glycogeen tijdens aërobe inspanning en tot het bevorderen van de glycolyse tijdens anaërobe arbeid.

De aandacht was voornamelijk gericht op het onderzoek van het effect van extra dieetvet op de stofwisseling van lipiden van ongetrainde, nuchtere paarden.

Het onderzoek

Allereerst werd de hypothese getoetst (Hoofdstuk 2) of extra dieetvet een toename geeft van de flux van vetzuren, in de vorm van triacylglycerolen (TAG), van de circulatie naar de skeletspier. Dit werd indirect getest door de concentratie TAG en de activiteit van lipoproteinlipase (LPL) te meten. In een cross-over proef kregen 6 paarden een laag- of een hoog-vet rantsoen. Het lage-vet rantsoen bevatte 1,5% en het hoge-vet rantsoen 11,8% vet in de droge stof. Het hoge-vet rantsoen werd verkregen door aan het controle dieet soja-olie toe te voegen ten koste van een isoenergetische hoeveelheid maïszetmeel plus glucose. Beide diëten bestonden uit hooi en krachtvoer. Behalve voor de proef beschreven in Hoofdstuk 4, werden steeds diëten met een identieke samenstelling gebruikt. Na het voeren van extra vet was de post-heparine plasma LPL activiteit in het plasma verhoogd, hetgeen wijst op een verhoogde flux van vetzuren. De toegenomen activiteit van LPL in post-heparine plasma is waarschijnlijk een afspiegeling van een toegenomen LPL activiteit in de spier aangezien de monsters verzameld zijn in nuchtere toestand en er dan slechts weinig LPL van vetweefsel afkomstig is.

In Hoofdstuk 3 is geprobeerd een dosis-response relatie vast te stellen voor de opname van vet en de post-heparine plasma LPL activiteit. Aan 8 paarden werden volgens een 4 X 4 Latijnse vierkant 4 rantsoenen met verschillend vetgehalte (3,0; 5,0; 7,7 of 10,8% vet in de droge stof) gevoerd. Door de vetvoeding nam de post-heparine plasma LPL activiteit toe op een dosis-afhankelijke manier. In het geteste bereik van de vetopnames bereikte de dosis-response relatie geen plateau. De acceptatie van het dieet met het hoogste vetgehalte was bij sommige paarden verminderd.
Zoals eerder vermeld is de verwachting dat een hoog vetgehalte van het dieet de turnover van vetzuren uit TAG doet toenemen. Dat betekent dat er na vetvoedering verhoogde mobilisatie van vetzuren is, in nuchtere toestand, vanuit het vetweefsel. Om dat te onderzoeken werd de *in vitro* lipolysesnelheid bepaald in biopten van vetweefsel (Hoofdstuk 4). Zes paarden kregen daartoe diëten met hoog vet, glucose of maïszetmeel. De diëten werden zodanig samengesteld dat soja-olie versus of glucose of maïszetmeel de enige variabelen waren. De dieetvariabelen hadden geen significant effect op de basale *in vitro*-lipolysesnelheid of de lipolysesnelheid na toevoeging van noradrenaline. In verhouding nam de noradrenaline-gestimuleerde lipolysesnelheid sterker toe in biopten afkomstig van paarden op het hoge vetdieet. Deze waarneming suggereert dat het voeren van een vetrijk dieet de lipolyse gevoeliger maakt voor stimulering, hetgeen een gunstig effect kan hebben op de prestatie. Het is mogelijk dat de toegenomen flux van vetzuren, zoals eerder vermeld, samengaat met een toename in de oxidatieve capaciteit van de skeletspier. Om daar inzicht in te krijgen werd de oxidatieve capaciteit van skeletspier bepaald door in spierbiopten de activiteit te meten van oxidatieve sleutelenzymen. Extra voedingsvet bleek niet van invloed te zijn op de activiteit van de onderzochte sleutelenzymen in spierweefsel. De biopten werden verkregen uit de gluteusspier. Deze spier is een zogenaamde gemengde spier die ongeveer gelijke hoeveelheden type I en type IIB vezels bevat.
In biopten van een spier met voornamelijk type I vezels, met een hoog oxidatief vermogen en dus een hoge concentratie aan oxidatieve enzymen, zou een toegenomen oxidatieve capaciteit waargenomen kunnen zijn. Daarom is in Hoofdstuk 5 onderzocht wat het effect is van extra voedingsvet op het energiemetabolisme van verschillende spiertypen van de pony. De masseter en het hart zijn spieren die bijna volledig uit hoog-oxidatieve vezels bestaan. Deze spieren werden vergeleken met de semitendinosus, een spier met voornamelijk glycolytische vezels. Toegenomen activiteit van carnitine pamitoyltransferase-I (CPT-I) en citraatsynthase (CS) in de masseter laat metabole aanpassing aan vetsuppletie zien in deze spier. Er was echter geen effect van dieet op de activiteit

van glycolytische enzymen in de verschillende spieren. Tevens werden sleutelenzymen van de hepatische lipogenese onderzocht om na te gaan of verminderde productie van TAG-rijke lipoproteinen bij kan dragen aan de vet-geïnduceerde afname in de plasma TAG concentratie. De afname van de plasma TAG concentratie in paarden heeft zeker te maken met de toegenomen LPL activiteit. De resultaten laten zien dat de vet-geïnduceerde reductie in plasma TAG in ieder geval gedeeltelijk veroorzaakt wordt door een verlaagde *de novo* synthesesnelheid van vetzuren in de lever. Bovendien maakt vetsuppletie de hepatische CPT-I minder gevoelig voor remming door malonyl-CoA, hetgeen moet leiden tot een toegenomen snelheid van vetzuuroxidatie. Ook het effect op CPT-I lijkt zo bij te dragen aan de verlaging van de plasma TAG concentratie na vetvoeding.

In het experiment beschreven in Hoofdstuk 6 werd het metabolisme van HDL cholesterylesters (HDL CE) bestudeerd. Onderzocht werd of in het paard, een diersoort waarbij cholesterylester transferproein (CETP) ontbreekt, overdracht van HDL CE naar LDL plaats vindt. De verwachting was dat de resultaten inzicht zouden verschaffen omtrent het mechanisme dat ten grondslag ligt aan de waargenomen toename van plasma HDL cholesterol (HDL C) na het nuttigen van een vetrijk dieet. In een parallelle opzet werden twaalf ponies gevoerd met een controle dieet of een vetrijk dieet. Radioactief-gemerkt HDL werd aan de ponies toegediend en de verdwijnsnelheid van de radioactiviteit uit het plasma werd gemeten. Vastgesteld werd dat ponies een alternatief pad hebben voor het overdragen van LCAT-gegenereerde HDL CE naar lipoproteinen met een lagere dichtheid. Er wordt gesuggereerd dat HDL CE overgedragen wordt aan VLDL remnants en vervolgens omgezet in LDL. Het voeren van vet leidde to een significante toename van het gehalte aan HDL CE samen met een toename in de HDL transportsnelheid.

De waargenomen lage TAG concentratie en de hoge LPL activiteit in post-heparine plasma in vetgevoerde paarden duidt op een hoge turnover van TAG die gepaard zou moeten gaan met een toegenomen productie van VLDL door de lever. Deze hypothese werd getoetst op een indirecte manier met behulp van de Triton methode (Hoofdstuk 7). Blokkering van LPL door Triton WR 1339 veroorzaakt ophoping van TAG in het plasma en zou kunnen dienen als een index voor de snelheid waarmee TAG uitgescheiden wordt. Zes paarden kregen een laag- dan wel hoog-vet dieet volgens een cross-over opzet. Triton WR 1339 werd intraveneus toegediend. Vergeleken met het controle dieet hadden paarden op het hoog-vet dieet significant lagere TAG secretiesnelheden. Het resultaat van deze studie was dus tegenovergesteld aan de hypothese. Verondersteld werd dat gebruik

van een te lage dosis Triton verantwoordelijk is voor de gemeten secretiesnelheid van TAG die lager is dan verwacht mocht worden op grond van de gezamelijke activiteiten van LPL en HTGL. Het onverwachte resultaat zou ook verklaard kunnen worden als Triton WR 1339 minder effectief is in vetgevoerde paarden vanwege hun toegenomen LPL activiteit.
Het onderzoek dat in dit proefschrift beschreven is heeft laten zien dat een vetrijk dieet metabole veranderingen bewerkstelligt die te vergelijken zijn met veranderingen die beschreven zijn als gevolg van training. Het voeren van een vetrijk dieet kan dus een gunstig effect hebben op de prestatie van sportpaarden.

Curriculum vitae

Suzanne Geelen is geboren op 6 februari 1966 te Utrecht. Zij behaalde in 1984 haar VWO diploma aan het Bonefatius College in Utrecht. Aansluitend verbleef ze een jaar in Parijs als *au pair*. In 1986 behaalde ze het propedeuse scheikunde met genoegen. Datzelfde jaar werd aangevangen met de studie diergeneeskunde aan de Universiteit Utrecht, het propedeuse examen werd behaald met genoegen. In januari 1994 legde zij het dierenartsexamen "met genoegen" af. Vervolgens werkte ze in een Landbouwhuisdieren praktijk te Wognum. Sinds juni 1994 werkte ze als Specialist in Opleiding bij de Vakgroep Inwendige Ziekten en Voeding der Grote Huisdieren. In 1997 werd ze aangesteld als dierenarts-assistent/onderzoeker. In 1999 werd ze geregistreerd als specialist in de Inwendige Ziekten van het paard.

Suzanne Geelen was born on 6th February, 1966 in Utrecht. In 1984 she passed her final exams at the grammar school Bonefatius College in Utrecht. In 1985 she went to Paris for a year to learn french and to work as an *au pair*. In 1986 she studied chemistry for a year. Since 1987 she studied Veterinary Medicine at the Utrecht University and she graduated in January 1994. Than she worked in a large animal practice in Wognum. In June 1994 she started working at the Department of Large Animal Medicine and Nutrition, Utrecht University. In 1997 she started this thesis. In 1999 she was registered by the Royal Dutch Association of Veterinary Medicine as Diplomate in Equine Internal Medicine.

Voor iedereen die een bijdrage heeft geleverd aan dit proefschrift:

Bedankt,

Suzanne