

## **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.

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## 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  $\beta$ -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°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°C until homogenization. Then, adipose tissue was sampled from the perineal 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 xxxxx

Table 1  
Composition of the experimental concentrates

Ingredients (g)	Dietary variables		
	High fat	Starch	Glucose
Corn starch	-	966	-
Glucose	-	-	702
Soybean oil	375 <sup>1</sup>	-	-
Constant components <sup>2</sup>	625	625	625
Total	1000	1591	1327
Nutrients <sup>3</sup> (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

<sup>1</sup> Including the 183 ml of soybean oil that was given each meal on top of the concentrate (see text).

<sup>2</sup> 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<sub>4</sub>·7H<sub>2</sub>O, 0.66; Na<sub>2</sub>SeO<sub>3</sub>·5H<sub>2</sub>O, 0.76; KIO<sub>3</sub>, 0.32; MnSO<sub>4</sub>·H<sub>2</sub>O, 172.4; CuSO<sub>4</sub>·5H<sub>2</sub>O, 27.2; ZnSO<sub>4</sub>·H<sub>2</sub>O, 192.4; vitamin A, 12.0 (500.000 IU/g); vitamin D<sub>3</sub>, 5.2 (100.000 IU/g); vitamin E, 240.0 (500 IU/g); vitamin B<sub>1</sub>, 1.8 (purity 100%); vitamin B<sub>2</sub> (purity 100%), 1.8; vitamin B<sub>12</sub> (purity 0.1%), 1.8; biotin (purity 100%), 0.4; corn starch (carrier), 343.26.

<sup>3</sup> Calculated using 1996 CVB tables (Centraal Veevoederbureau, Lelystad, the Netherlands)

ADF = Acid-detergent fibre, NDF = Neutral-detergent fibre

in a thermostated (37°C) container. The incision was sutured with number 1 ethicon (Mersilene<sup>R</sup>).

#### *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 37°C, 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 37°C under an atmosphere of O<sub>2</sub>:CO<sub>2</sub> (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<sup>-5</sup> M). All incubations were carried out at 37°C 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 -20°C 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<sup>R</sup> 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 β-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 -80°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), hexokinase (EC 2.7.1.2; HK) and

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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.

Measure	Dietary variables		
	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 ( $5 \cdot 10^{-5}$  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 Lipolysis	Dietary variables		
	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.

Measure	Dietary variables		
	High fat	Starch	Glucose
TAG	86 ± 16	57 ± 12	67 ± 18
Glycogen	368 ± 31	378 ± 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 availability 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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