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High fat intake lowers hepatic fatty acid synthesis and raises fatty acid oxidation in aerobic muscle in Shetland ponies

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The metabolic effects of feeding soyabean oil instead of an isoenergetic amount of maize starch plus glucose were studied in ponies. Twelve adult Shetland ponies were given a control diet (15 g fat/kg DM) or a high-fat diet (118 g fat/kg DM) 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 reduced glycogen concentrations significantly by up to 65 % in masseter, gluteus and semitendinosus muscles ($P < 0{\cdot}05$, $P < 0{\cdot}01$ and $P < 0{\cdot}01$ respectively). The high-fat diet significantly increased the TAG content of semitendinosus muscle by 80 % ($P < 0{\cdot}05$). Hepatic acetyl-CoA carboxylase and fatty acid synthase activities were 53 % ($P < 0{\cdot}01$) and 56 % ($P < 0{\cdot}01$) lower respectively 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 effect of diet on the activity of phosphofructokinase in the different muscles. The activity of citrate synthase was raised significantly (by 25 %; $P < 0{\cdot}05$) in the masseter muscle of fat-fed ponies, as was CPT-I activity (by 46 %; $P < 0{\cdot}01$). We conclude that fat feeding enhances both the transport of fatty acids through the mitochondrial inner membrane and the oxidative capacity of 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.

Equine: Dietary fat: Hepatic lipogenesis: Muscle fat metabolism: Fatty acid oxidation

High-fat diets have been shown to stimulate fatty acid oxidation by muscle in rats and human subjects. Fat feeding increases the activities of muscle 3-hydroxy-acyl-CoA dehydrogenase (3-HAD) and muscle citrate synthase (CS), key enzymes in the β-oxidation pathway and in Krebs' cycle, respectively (Miller *et al.* 1984; Simi *et al.* 1991; Helge & Kiens' 1997). Furthermore, a 44 % increase in carnitine palmitoyltransferase-I (CPT-I) activity, the enzyme responsible for the transport of fatty acyl-CoA through the mitochondrial inner 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) in muscle of 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 human subjects consuming a high-fat diet muscle glycogen levels decreased (Hultman & Bergstrom, 1967; Bergstrom *et al.* 1987). In contrast, an increase in resting muscle glycogen concentrations has been found in fat-supplemented horses (Oldham *et al.* 1990; Jones *et al.* 1992; Scott *et al.* 1992), whereas other authors have failed to show such an effect (Hodgson *et al.* 1986; Greiwe *et al.* 1989; Essen-Gustavson *et al.* 1991).

The previously mentioned discrepancy in the effect of fat

Abbreviations: ACC, acetyl-CoA carboxylase; CS, citrate synthase; CPT-I, carnitine palmitoyltransferase-I; DGAT, diacylglycerol acyltransferase; FAS, fatty acid synthase; 3-HAD, 3-hydroxy-acyl-CoA dehydrogenase; PFK, phosphofructokinase; TAG, triacylglycerol.

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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 fibre composition of the biopsy samples. Fibres are most commonly divided into slow-twitch, type I fibres with a high oxidative ability, type IIA fibres with an intermediate oxidative ability and fast-twitch, type IIB fibres with a low oxidative ability. Type I fibres have high concentrations of mitochondrial enzymes such as 3-HAD and type IIB fibres have a high concentration of glycolytic enzymes such as phosphofructokinase (PFK). Type I fibres depend largely on aerobic metabolism of glucose and fatty acids for their energy requirement. The type II fibres 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 predominantly of highly-oxidative type I fibres (Kayar *et al.* 1988; Barrey *et al.* 1995) and are of interest in the study of the effects of fat supplementation, especially when compared and contrasted with the semitendinosus, a muscle that contains predominantly glycolytic, type IIB fibres (Barrey *et al.* 1995).

In a previous study with horses, a high-fat diet led to 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 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 decrease in plasma TAG 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) activities.

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 to: (1) determine the muscle TAG and glycogen concentrations and the activities of key oxidative and glycolytic enzymes in different muscles; (2) measure the CPT-I activity in different tissues; and (3) measure the activities of FAS, DGAT and ACC in liver.

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 were aged 3–13 years (5·8 (SD 3·9) and 6·4 (SD 4·7) for high-fat and control groups respectively). During a 3-week pre-experimental period the ponies had free access to hay. The ponies were then allocated to two groups of six ponies; one group was fed a fat-rich diet and the other a control diet. Pairs of a control and a test pony entered the experiment at intervals of 1–5 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 hours concentrate as well as hay were provided. 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 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 supplied daily with 0·88 (SD 0·09) kg hay (25 % of the net energy) and 1·41 (SD 0·17) kg test concentrate or 1·62 (SD 0·11) kg control concentrate (75 % of the net energy). The control diet contained 15 g fat/kg DM and the high-fat diet contained 118 g fat/kg DM. The ponies were housed individually in ventilated stables. All animals walked daily for 15 min on a mechanical horse walker at a speed of 80 m/min.

Sampling procedures

At the end of the experiment, at 09.00 hours after an overnight fast, blood samples were collected in heparinized tubes by jugular venepuncture. Directly after blood sampling, the ponies were killed by stunning and exsanguination. Tissue samples (2–4 g) were always taken from the same site for each pony 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°C for subsequent analyses. One part of the liver sample was homogenized immediately with a loose-fitting Dounce homogenizer (five strokes) in a medium containing (mmol/l): 50 Hepes (pH 7·5), 0·25 mannitol, 4·0 citrate, 6·16 EDTA, 5 β-mercaptoethanol. The crude homogenate was centrifuged at 12 000 ***g*** for 5 min and the supernatant was frozen quickly in liquid N_2 and stored at −80°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

Table 1. Composition of the experimental concentrates (g)

Ingredient	Control concentrate	High-fat concentrate
Maize starch	193	–
Glucose	140	–
Soyabean oil	–	150
Constant components*	850	850
Total	1183	1000

* The constant components consisted of the following (g): lucerne (Medicago sativa) meal, dehydrated, 342·4; maize starch, 150; glucose, 150; soyabeans, 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): $CoSO_4{\cdot}7H_2O$ 0·66, $Na_2SeO_3{\cdot}5H_2O$ 0·76, KIO_3 0·32, $MnSO_4{\cdot}H_2O$ 172·4, $CuSO_4{\cdot}5H_2O$ 27·2, $ZnSO_4{\cdot}H_2O$ 192·4, Vitamin A, 12·0 (500 000 IU/g), Cholecalciferol 5·2 (100 000 IU/g), Vitamin E 240·0 (500 IU/g), thiamin, 1·8 (purity 100 %), riboflavin 1·8 (purity 100%), vitamin B_{12} 1·8 (purity 0·1 %), biotin 0·4 (purity 100 %), maize starch (carrier) 343·26.

with five strokes of a glass–Teflon Potter-Elvehjem tissue homogenizer in 4 vol. of a buffer containing 0·25 mol sucrose/l, 20 mmol Tris hydrochloride (pH 7·4)/l and 1 mmol EDTA/l. The homogenate was centrifuged at 600 ***g*** for 5 min. The supernatant was recentrifuged at 10 000 ***g*** for 15 min. From the supernatant a microsomal pellet was obtained by centrifugation at 105 000 ***g*** for 65 min. The final supernatant was termed cytosol.

Assay procedures

Whole-plasma TAG concentration was measured enzymically 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 ®T5-FU tissue homogenizer (Janke and Knukel GmbH and Co. KG, Staufen, Germany) in 9 vol. of a buffer (pH 8·0) containing 25 mmol Hepes/l and 5 mmol β-mercaptoethanol/l. Aliquots of this homogenate were used to measure the levels of TAG (Sundler *et al.* 1974) and glycogen (Hassid & Abraham, 1957). To ensure full release of mitochondrial enzymes in the remaining homogenate, Triton X-100 (final concentration 0·5 % (v/v)) was added to the strong hypotonic preparation. The activities of the enzymes measured in the present study were not affected by the concentration of detergent used. The Triton X-100-treated homogenate was centrifuged at 48 000 ***g*** for 30 min. The supernatant was snap frozen in liquid N_2 and stored at −80°C until analysed a few days later for enzyme activities. The activities of CS, 3-HAD, and PFK were determined spectrophotometrically as described by Stitt, (1983), by Passonneau & Lowry (1993) and by Ishikawa *et al.* (1990) respectively. Measurement of ACC, FAS and DGAT was performed as described by Tijburg *et al.* (1988). Mitochondria were isolated (Guzmán *et al.* 1995) for the measurement of activity as described previously (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, as described previously (Geelen *et al.* 1997).

Statistical analysis

Statistical analysis was performed by using the Student's *t* test. The level of statistical significance was preset at $P < 0·05$. Values are means and standard deviations.

Results

Feed intake and body weight

The ponies consumed all feed supplied, except for two ponies given the high-fat diet; these animals occasionally refused some concentrate, but the amount was not substantial. The daily feed intake (g/kg body weight) was 5·52 (SD 0·40) hay and 8·89 (SD 0·84) test concentrate or 5·46 (SD 0·52) hay and 10·14 (SD 0·58) control concentrate. The carbohydrate intake (g/kg body weight) was 4·81 (SD 0·40) and 6·07 (SD 0·42) for the test group and control group respectively and the values for fat intake (g/kg body weight) were 1·30 (SD 0·11) and 0·20 (SD 0·01) for the test group and the control group respectively. The ponies maintained their body weight throughout the study: initial and final body weights were respectively 158 (SD 18) kg and 160 (SD 19) kg for the control group and 165 (SD 30) kg and 162 (SD 30) kg for the test group.

Table 2. Activities (nmol/min per mg protein) 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†

(Mean values and standard deviations for six ponies, except CPT-1 control values where values are for five ponies)

	Control diet		High-fat diet	
Enzyme	Mean	SD	Mean	SD
ACC	0·015	0·003	0·007	0·002**
FAS	0·192	0·024	0·084	0·019***
DGAT	0·163	0·021	0·158	0·068
CPT-I	8·01	1·77	6·26	1·56

Mean values were significantly different from those for the control diet **$P < 0·01$, ***$P < 0·001$.

† For details of diets and procedures see Table 1 and p. 32.

Enzyme activities in liver

The influence of 45 d fat feeding on the activity of a number of key enzymes of lipid metabolism in the liver is presented in Table 2. The specific activity of DGAT was unaffected, but the activities of ACC and FAS were diminished significantly in fat-fed animals ($P < 0·01$ and

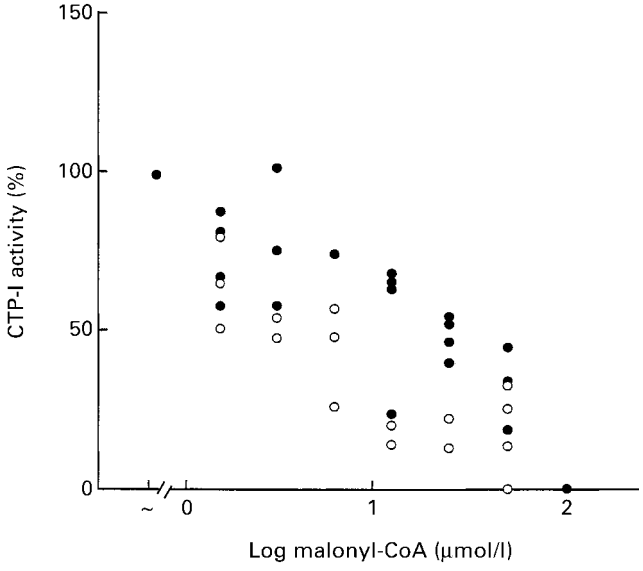

Fig. 1. Effect of dietary fat on the sensitivity of hepatic carnitine palmitoyltransferase-I (CPT-I) to inhibition by malonyl-CoA. Enzyme activity was determined in mitochondria isolated from ponies fed a high-fat (●) or control diet (○) in the presence of increasing concentrations of malonyl-CoA. The 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. For details of diets and procedures, see Table 1 and p. 32.

Table 3. Activities (nmol/min per mg protein) 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†

(Mean values and standard deviations for six ponies, except CTP-1 control values in semitendinosus where values are for five ponies)

	Control diet		High-fat diet	
	Mean	SD	Mean	SD
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*
CPT-I	4·60	0·67	6·73	0·63**
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

Mean values were significantly different from those for the control diet *$P < 0·05$, **$P < 0·001$.
† For details of diets and procedures, see Table 1 and p. 32.

$P < 0·001$ respectively). The measurement of ACC activity is subject to modification by several factors (Geelen *et al.* 1997) and so mass measurement of ACC was performed by ELISA. The results showed that the livers of fat-fed ponies contained substantially less (2·37 (SD 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 shown in Fig. 1, fat feeding resulted in a loss of sensitivity of hepatic CPT-I to malonyl-CoA.

Enzyme activities in muscle

In homogenates of the different muscle samples, the activity of PFK as an 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 effects of diet on the activities of PFK and 3-HAD (Table 3). The activity of CS was enhanced significantly in the masseter muscle of ponies fed the high-fat diet ($P < 0·05$). The activity of CPT-I, a regulatory enzyme of fatty acid oxidation, was significantly higher in mitochondria of the masseter muscle from fat-fed ponies ($P < 0·001$).

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 ($P < 0·05$). The glycogen concentration was lower in all four muscles of the fat-fed ponies and this effect reached statistical significance in the masseter ($P < 0·05$), gluteus ($P < 0·01$) and semitendinosus muscle ($P < 0·01$; Table 4).

Discussion

Consistent with previous observations (Duren *et al.* 1987; Orme *et al.* 1997; Geelen *et al.* 1999), ponies fed the high-fat diet had lower plasma concentrations of TAG than did their counterparts fed the control diet. On day 45 of the experiment, the values were 123 (SD 70) and 274 (SD 107) μmol/l respectively ($P < 0·016$). The present 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 shown by the decreased activities of ACC and FAS in the liver. Rates of hepatic *de novo* fatty acid synthesis are associated with rates of secretion of TAG-rich VLDL (Beynen *et al.* 1981). Thus, we conclude that the decreased plasma levels of TAG reported in fat-supplemented equines is due not only to increased removal of lipoprotein particles (Geelen *et al.* 1999) but also to lower production rates.

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 catalysed by ACC. The hepatic concentration of malonyl-CoA is related directly to the rate

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

(Mean values and standard deviation for six ponies)

	Control diet				High-fat diet			
	TAG		Glycogen		TAG		Glycogen	
Muscle	Mean	SD	Mean	SD	Mean	SD	Mean	SD
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*
Gluteus	156·3	62·3	583·8	218·6	165·0	59·9	285·8	65·1**
Semitendinosus	74·2	26·3	978·4	376·0	134·4	52·6*	390·8	140·7**

Mean values were significantly different from those for the control diet. *$P < 0·05$, **$P < 0·01$.
† For details of diets and procedures, see Table 1 and p. 32.

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 decrease 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 human subjects and Boyadjiev, (1996) for rats fed a high-fat diet, and indicates 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 activities of 3-HAD and CS are in large excess and that an increase in the activity of the enzyme that shuttles fatty acids into mitochondria, CPT-I, will be most effective in upgrading the capacity for fatty acid oxidation and thus of energy production. In agreement with previous results (Orme *et al.* 1997; Geelen *et al.* 2000) dietary fat induced an increase in masseter CS activity, where as 3-HAD activity was not significantly affected. This finding 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 muscles of low aerobic capacity (semitendinosus). In all four muscle types dietary fat reduced the glycogen content markedly; the reduction was statistically significant in masseter, gluteus and semitendinosus ($P < 0{\cdot}05$, $P < 0{\cdot}01$ and $P < 0{\cdot}01$ respectively). This result is in agreement with data reported for horses by Greiwe *et al.* (1989) and Pagan *et al.* (1987). However, others researchers have shown an increased muscle glycogen concentration in response to a comparable level of fat supplementation (Oldham *et al.* 1990; Harkins *et al.* 1992; Jones *et al.* 1992). The variability in results between the different studies could be due to many factors, including 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 with results reported by Snow (1983) and Essen-Gustavson *et al.* (1984), the TAG content of highly-aerobic muscle was less than that of low-aerobic muscle. The semitendinosus muscle accumulated significantly more TAG in the ponies fed the fat-supplemented diet ($P < 0{\cdot}05$). Accordingly, in human subjects 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 (Orme *et al.* 1997;Geelen *et al.* 1999). The results of the present 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, which in turn could 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 and may explain why Webb *et al.* (1987) observed that extra dietary fat provided more energy for work in the cutting horse.

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