Effect of dietary fats with odd or even numbers of carbon atoms on metabolic response and muscle damage with exercise in Quarter Horse–type horses with type 1 polysaccharide storage myopathy

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Objective—To evaluate effects of fats with odd and even numbers of carbon atoms on muscle metabolism in exercising horses with polysaccharide storage myopathy (PSSM).

Animals—8 horses with PSSM (6 females and 2 males; mean ± SD age, 6.3 ± 3.9 years).

Procedures—Isocaloric diets (grain, triheptanoin, corn oil, and high-fat, low-starch [HFLS] feed) were fed for 3 weeks each; horses performed daily treadmill exercise. Grain was fed to establish an exercise target, and HFLS feed was fed as a negative control diet. Daily plasma samples were obtained. For each diet, a 15-minute exercise test was performed, and gluteus medius muscle specimens and blood samples were obtained before and after exercise.

Results—Feeding triheptanoin, compared with the corn oil diet, resulted in exercise intolerance; higher plasma creatine kinase (CK) activity and concentrations of C3:0- and C7:0-acylcarnitine and insulin; and lower concentrations of nonesterified fatty acids (NEFA) and C16:0-, C18:1-, and C18:2-acylcarnitine, without changes in concentrations of plasma glucose or resting muscle substrates and metabolites. Feeding grain induced higher CK activity and insulin concentrations and lower NEFA concentrations than did corn oil or HFLS feed. Feeding grain induced higher glucose concentrations than did triheptanoin and corn oil. In muscle, feeding grain resulted in lower glucose-6-phosphate, higher citrate, and higher postexercise lactate concentrations than did the other diets.

Conclusions and Clinical Relevance—Triheptanoin had detrimental effects, reflecting decreased availability of NEFA, increased insulin stimulation of glycogen synthesis, and potential inhibition of lipid oxidation. Long-chain fats are the best dietetic for PSSM. (*Am J Vet Res* 2010;71:326–336)

Polysaccharide storage myopathy is a form of ER that affects 12% of Quarter Horses.[1] It is characterized by high muscle glycogen concentrations (1.8 times as high as that for clinically normal horses) and accumulation of abnormal polysaccharide in muscle fibers.[2,3] Clinical signs develop after 10 to 30 minutes of submaximal exercise and include signs of muscle pain and stiffness, which are accompanied by high CK activity; these results are indicative of muscle damage.[4–6]

Most glycogen storage disorders are caused by deficiencies in enzymes involving glycogenolysis or glycolysis.[7] Excessive glycogen accumulation in the skeletal muscle of horses with PSSM has been investigated[8,9] and is not associated with a deficiency in glycolysis or glycogenolysis. Affected horses actually used

Abbreviations

BW	Body weight
CAC	Citric acid cycle
CK	Creatine kinase
ER	Exertional rhabdomyolysis
G6P	Glucose-6-phosphate
GYS1	Glycogen synthase gene
HFLS	High-fat, low-starch
NEFA	Nonesterified free fatty acids
PSSM	Polysaccharide storage myopathy
SET	Standardized exercise test

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more glycogen during near-maximal exercise than did clinically normal horses.[8] Results of other studies[2,10,11] suggest that horses with PSSM may have heightened sensitivity to insulin and enhanced glucose uptake by skeletal muscle, which may further increase glycogen synthesis. Recently, PSSM in Quarter Horses was found to be attributable to a novel gain-of-function mutation in *GYS1*, which resulted in an increase in glycogen synthase activity.[12]

Despite the apparent availability of glycogen for energy metabolism, horses with PSSM have an energy deficit during submaximal exercise, as indicated by an abnormally high concentration of inosine monophosphate in muscle fibers after exercise.[4] The precise mechanism for the development of ER in horses with PSSM is not known; however, we postulate that disrupting the flux of glycogen to pyruvate and acetyl CoA may impair generation of CAC intermediates and energy, which results in muscle membrane damage and potential leakage of CAC intermediates from PSSM-affected muscle cells. This mechanism has been proposed in animals and in humans with chronic rhabdomyolysis and metabolic myopathies.[13–15]

Feeding a low-starch, fat-supplemented diet that includes corn oil or an HFLS commercially prepared feed can decrease the number of episodes of ER in horses with PSSM.[6,16] The recommended amount of dietary fat for horses with PSSM varies from 12% of digestible energy (determined on the basis of a dietary trial[16]) to 25% of digestible energy (determined on the basis of an anecdotal report[17]); however, there are few data that quantify the amount and type of fat that is best utilized by horses with PSSM. Potential mechanisms for improvement when horses were fed fat diets include decreased glucose uptake and decreased insulin stimulation of glycogen synthesis. Additionally, provision of dietary long-chain fatty acids with an even number of carbon atoms may supply the CAC with an alternate energy source in the form of the 2-carbon acetyl-CoA moiety.

Triheptanoin, a 7-carbon fat, generates propionyl-CoA (in addition to acetyl CoA) and has been used in humans to replenish the CAC by providing succinyl-CoA.[14,18] Dietary supplementation with triheptanoin has been used to treat patients with disorders, such as carnitine palmitoyltransferase II deficiency,[19] pyruvate decarboxlyase deficiency, and type II glycogen storage disease (ie, Pompe's disease), by supplying 5-carbon ketone bodies.[14,20]

Measuring plasma acylcarnitine concentrations is an accepted method of monitoring oxidation of fats of specific chain lengths. Excessive accumulation of acylcarnitine of a specific chain length indicates impaired metabolism disorder of the corresponding short-, medium-, or long-chain fatty acid.[14,20,21] Analysis of results of a recent study[22] indicated that triheptanoin was absorbed and metabolized by healthy Thoroughbreds on the basis of evaluation of corresponding plasma concentrations of acylcarnitines.

We hypothesized that CAC intermediates could become depleted in PSSM horses with chronic ER and that provision of a fat with an odd number of carbon atoms (such as triheptanoin) would increase these intermediates in skeletal muscle, thus potentially attenuating ER during submaximal exercise. The objectives of the study reported here were to determine whether dietary triheptanoin (compared with dietary corn oil) would reduce muscle damage in horses with PSSM as indicated by plasma CK activity after submaximal exercise and whether dietary triheptanoin or corn oil would affect substrate availability during submaximal exercise. Another objective was to compare the metabolic responses for dietary triheptanoin and corn oil with those for grain (which is known to induce ER) and to a commercially available HFLS feed that has been reported[16] to decrease episodes of ER in horses with PSSM.

Materials and Methods

Horses—Eight Quarter Horse–type horses in which PSSM had been diagnosed were used in the study. Three of the horses were registered Quarter Horses, 1 was a registered Appaloosa, 2 were Quarter Horse–Arabian crossbred horses, and 2 were derived from mating a Quarter Horse mare with a Quarter Horse–Thoroughbred crossbred stallion. The diagnosis of PSSM was based on detection of amylase-resistant abnormal polysaccharide in gluteal muscle biopsy specimens.[3] Subsequent to the study, all 8 horses were found to be heterozygous for a dominant mutation in *GYS1*, which is known to cause PSSM.[12]

The horses (6 mares, 1 stallion, and 1 gelding) ranged from 2 to 14 years of age (mean ± SD, 6.3 ± 3.9 years). Body weight ranged from 277 to 567 kg (mean, 466 ± 104 kg). The horses were housed on a drylot and fed grass hay for at least 1 year prior to the study. During the study, all horses were housed in an accredited facility and were cared for in accordance with principles outlined by the Animal Use and Care Committee at the University of Minnesota.

Diets—Four isocaloric diets were formulated to provide a total digestible energy of 50 kcal/kg of BW/d and to meet minimum daily nutrient requirements (**Appendix**).

Grain

A high-starch grain diet that in other studies[10,16] induced subclinical increases in plasma CK activity in horses with PSSM was fed to determine an exercise target for each horse. The diet consisted of molasses-supplemented grain (3.4 g/kg of BW), calcium-balanced rice bran (1.8 g/kg of BW), and ration balancer (1 g/kg of BW) and was divided into 2 feedings/d (7:30 AM and 4:30 PM). The grain consisted of 37% corn, 32% wheat middlings, 15% oats, 5.4% soy hulls, 4% soybean meal, 3.5% molasses, 1% limestone, and 0.5% vitamin-mineral premix (dry-weight basis). The rice bran was a commercially available rice bran pellet[a] and contained a minimum of 12.5% crude protein, a minimum of 20.0% crude fat, a maximum of 4.0% free fatty acids, a maximum of 13.0% crude fiber, 2.2% to 3.2% calcium, and a minimum of 1.8% phosphorus (dry-weight basis). The ration balancer was a soybean meal base formulated for grass hay and contained 30% protein, 1.0% fat, 5.0% crude fiber, 4.5% to 5.5% calcium, and 2.0% phosphorus (dry-weight basis) and additional vitamins and minerals to meet minimal daily requirements.

Triheptanoin and corn oil

Both oils were in liquid form, and diets provided corn oil[b] or triheptanoin[c] (1.5 mL/kg of BW/d). Oil rations were allowed to soak into 1.5-kg cubes of timothy hay, which were split into 3 feedings/d (7:30 AM, 12:30 PM, and 4:30 PM). Horses were fed ration balancer (1 g/kg of BW) and grass hay (15 g/kg of BW/d) in addition to the oil-soaked hay cubes. The same individual prepared oil diets daily throughout the study, and all other personnel were not aware of the oil that the horses were consuming. All horses consumed the daily oil ration, although for 2 horses, the triheptanoin was palatable only when placed on dry (rather than moistened) hay cubes.

HFLS diet

Horses were fed a commercially available HFLS diet[d] (5.4 g/kg of BW/d), which was split into 2 daily feedings (7:30 AM and 12:30 PM). Grass hay was fed at 17 g/kg of BW/d. The HFLS diet consisted of 12.5% protein, 12.5% fat, 22% fiber, and 10% starch (dry-weight basis). This diet was used for comparison with the other diets because it had been reported[16] to lower plasma CK activity in horses with PSSM, compared with results for the grain diet.

Exercise target—Horses were acclimated to a treadmill over a period of 6 to 11 days prior to the study, initially performing 4 minutes of walking (1.9 m/s) and then gradually adding alternating intervals of 2 minutes of walking and 2 minutes of trotting (3.0 to 3.8 m/s) on a day-by-day basis. Each horse had the same handler on the treadmill throughout the entire study, and horses were exercised in the same order at approximately the same time each day (between 7 AM and 3 PM). Because of the length of the study and variations in rate of consumption of feed among horses, it was not possible to exercise horses at a specific interval after feeding each day.

The handlers closely monitored each horse's adaptation to exercise to determine exercise tolerance on the treadmill. If horses moved with ease at an even gait, another exercise interval was added. If horses had an uneven gait, shortened hind limb stride, reluctance to continue, sweating, or a tucked-up abdomen, the exercise session was terminated. All horses completed the acclimation period for treadmill exercise.

Grain diet evaluation—The grain diet was gradually introduced during the initial treadmill acclimation over a period of 5 days. When feeding of the complete grain diet was attained, the grain diet period began.

Exercise

An initial exercise period was performed by each horse, as established from the treadmill acclimation. This initial exercise period differed among horses and was 20 minutes (alternating intervals of 2 minutes of walking and 2 minutes of trotting) for horses that readily adapted to the treadmill; horses that had exercise intolerance for the treadmill exercise completed a shorter initial exercise period with fewer intervals of walking and trotting. The exercise target for each horse for the remainder of the study was established as the mean number of 2-minute walking-trotting intervals a horse was able to comfortably perform (up to a 20-minute maximum, including the warm-up interval [4 minutes of walking]), while being fed the grain diet. Horses were exercised daily Monday through Friday for 3 weeks and allowed to rest in box stalls on Saturday and Sunday.

Plasma samples

Venous blood samples were collected into heparin-coated vacuum tubes Monday through Friday at 4 hours after exercise. Plasma for analysis of CK activity was separated immediately by use of centrifugation and analyzed within 24 hours. Aliquots of plasma samples were stored at –20°C for subsequent analysis of glucose, insulin, and NEFA concentrations.

Performance of an SET

At the end of each diet period (ie, on the 15th day of exercise), horses performed a 15-minute SET that consisted of 4 minutes of walking followed by 2-minute intervals of walking and trotting. Because of variations in the rate of consumption of feed, it was not possible to perform the SET for each horse at a specific interval after feeding. A catheter was placed in a jugular vein prior to the SET, and venous blood samples were obtained before and immediately after the SET. Blood samples were stored on ice and centrifuged within 30 minutes after collection; plasma was harvested, and aliquots were stored at –20°C for analysis of glucose and NEFA concentrations and at –80°C for analysis of insulin and acylcarnitine concentrations. Venous blood samples were collected 4 hours after the SET and analyzed to determine plasma CK activity.

Muscle biopsy specimens

Gluteal muscle biopsy specimens were obtained before and immediately after completion of the SET through the same incision by use of SC administration of lidocaine and a percutaneous needle biopsy technique.[23] Specimens were obtained within a 2-cm square on an 18-cm line from the highest point of the tuber coxae to the head of the tail at a depth of 6 cm. Biopsy specimens were immediately frozen in liquid nitrogen and stored at –80°C until biochemical analysis was performed.

Triheptanoin and corn oil diets evaluation—After the 3-week grain diet period, horses were allowed a 10-day washout period. Then, a randomized crossover design was used, with half of the horses fed the triheptanoin diet and the other half fed the corn oil diet for 3 weeks. A 10-day washout period was then provided, and the horses were then fed the other oil-based diet. For the first 7 days of the washout period, the horses were fed grass hay only, and then the alternate diet was gradually introduced over a period of 3 days.

The exercise target for each horse determined during feeding of the grain diet was used as the daily exercise format for each diet period. Each horse was closely monitored during exercise, and exercise was terminated if the horse had excessive sweating, stiffness, or an inability to maintain pace with the treadmill prior to reaching the exercise target. Daily blood samples were

collected and processed as described previously. If overt signs of muscle stiffness were observed or plasma CK activity was > 50,000 U/L, the number of intervals a horse performed was decreased the following day to 4 minutes or less. Exercise was then gradually resumed over the subsequent days with the goal of returning to the exercise target within 4 days. The number of minutes exercised daily was recorded for each horse. The percentage of the exercise target achieved each day for each diet was calculated for each horse by use of the following equation: (min completed/target min) × 100. A mean percentage of the exercise target for each day was calculated for the triheptanoin, corn oil, and HFLS diets.

For subsequent diets, collection of daily blood samples, performance of an SET and collection of blood samples before and after the SET, and collection of gluteal muscle biopsy specimens were performed as described for the grain diet. Gluteal muscle specimens were obtained from the opposite side (ie, left vs right) from the side used for the preceding SET.

HFLS diet evaluation—After both oil-based diets were fed, horses were allowed a 10-day washout period. The horses were fed grass hay only during the first 7 days of the washout period, and then the HFLS diet was gradually introduced over a period of 3 days. Horses were then fed the HFLS diet for 3 weeks. Horses were exercised daily and monitored (including daily blood samples) as described previously. An SET, collection of blood samples before and after the SET, and collection of gluteal muscle biopsy specimens were performed as described previously. The number of minutes for each exercise session and percentage of exercise target achieved were calculated as described for the triheptanoin and corn oil diets.

Analysis of plasma samples—Plasma CK activity was analyzed by use of an automated chemistry analyzer. Plasma glucose concentration was measured spectrophotometrically by use of the hexokinase method,[e] and NEFA concentration was assayed by use of the enzymatic colorimetric method.[f] Insulin concentration was measured by use of a radioimmunoassay validated for use in samples obtained from horses.[24] Concentrations of free, C2:0-, C3:0-, C5:0-, C7:0-, C16:0- (ie, hexadecanoic acid), C18:1- (ie, oleic acid), and C18:2- (ie, linoleic acid) acylcarnitines were assayed by use of mass spectrometry[24] on plasma samples obtained before and after exercise from horses only for the triheptanoin and corn oil diet periods.

Biochemical analysis of muscle biopsy specimens—Frozen gluteal muscle specimens were lyophilized; dissected free of blood, fat, and connective tissue; and then weighed. Glycogen concentration was determined fluorometrically in muscle biopsy specimens as glucose residues remaining after portions (1 to 2 mg) of muscle tissue were boiled for 2 hours in 1M HCl.[25] A comparison of glycogen concentrations before and after exercise was not made because little difference was anticipated on the basis of analysis of concentrations determined after 20 minutes of submaximal exercise[26] and because variability of detected glycogen concentrations can be high when fibers with abnormal polysaccharide are included in the sample analyzed. A separate portion (4 to 6 mg) of muscle was homogenized by crushing with a glass rod in 1.5M perchloric acid and then cold centrifuged for 10 minutes at 9,300 × *g*. The supernatant was neutralized with 1M $KHCO_3$ and centrifuged again, and the remaining supernatant was used for analysis of lactate, G6P, citrate, pyruvate, and ATP concentrations via fluorometric techniques.[22,25]

Statistical analysis—Data were analyzed by use of commercially available software packages.[g,h] A repeated-measures ANOVA was used to analyze effects of diet on logarithmically transformed plasma CK activity, percentage of daily exercise target achieved among diets, and daily plasma glucose, insulin, and NEFA concentrations. A 2-way repeated-measures ANOVA was used to analyze effects of diet and exercise on CK activity; plasma concentrations of glucose, insulin, NEFA, and acylcarnitines; and muscle concentrations of ATP, lactate, G6P, pyruvate, and citrate in samples obtained before and after exercise. A 1-way ANOVA was used to determine the effect of diet on resting muscle glycogen concentrations. The post hoc Tukey-Kramer multiple comparison test was used. Significance was set at values of $P < 0.05$. Results were expressed as mean ± SD.

Results

Animals—All horses completed the daily exercise period during feeding of each of the diets; however, not all horses achieved the exercise target every day for all diets. Daily blood samples could not be obtained by jugular venipuncture from 1 horse because of excitability, which necessitated placement of a long-term indwelling catheter during feeding of the grain, triheptanoin, and corn oil diets. Because of an inability to maintain a patent catheter in this horse during feeding of the HFLS diet, blood was only obtained before and after the SET. Values for daily blood samples for this horse were not included in the statistical analyses.

Weight gain—Mean ± SD weight gain during feeding of the grain diet was 8.0 ± 11.3 kg. Mean weight gain for horses during feeding of the triheptanoin and corn oil diets was 6.3 ± 9.4 kg and 5.9 ± 6.3 kg, respectively. Mean weight gain during feeding of the HFLS diet was 9.5 ± 3.2 kg. There was no significant difference in mean weight gain among the diets.

Daily exercise target and plasma CK activity—The exercise target established during the grain diet ranged from 12 to 18 minutes. There was a significant ($P < 0.001$) effect of diet on exercise intolerance. Horses completed less of their exercise target when fed the triheptanoin diet (achieved 83% of the exercise target) than when fed the corn oil diet (achieved 99% of the exercise target; **Figure 1**). There was no difference in the exercise target achieved between horses when fed the corn oil diet and the HFLS diet (97%).

Mean daily plasma CK activities were higher than the reference range (96 to 620 U/L) during feeding of the triheptanoin and corn oil diets (**Table 1**). There was a significant ($P < 0.001$) effect of diet on logarithmically transformed plasma CK activity. Logarithmically transformed plasma CK activities were higher during

feeding of the triheptanoin diet than during feeding of the corn oil diet (Figure 1). Horses had higher logarithmically transformed plasma CK activities when fed the grain diet than when they were fed the corn oil or HFLS diets; however, the highest CK activities were detected when horses were fed the triheptanoin diet. Horses had similar CK activities when fed the corn oil and HFLS diets. Day of exercise did not have a significant effect on logarithmically transformed CK activity.

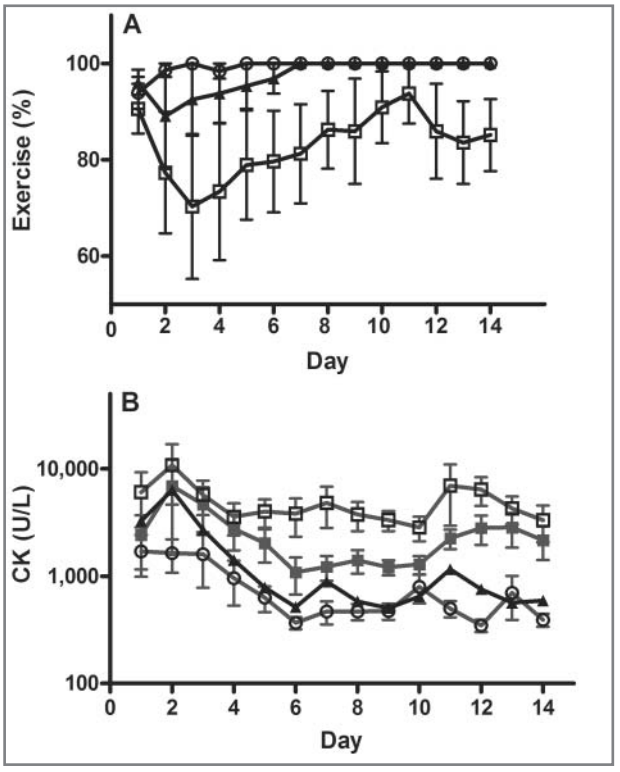

Figure 1—Mean ± SD percentage of individual exercise target completed by horses each day while fed the triheptanoin (white squares), corn oil (white circles), and HFLS (black triangles) diets (A) and mean plasma CK activity in samples obtained 4 hours after exercise in horses while they consumed the grain (black squares), triheptanoin, corn oil, and HFLS diets (B). In panel A, the individual exercise target for each of 8 horses was calculated as the mean amount of time each horse could exercise while being fed the grain diet. Mean percentage of exercise target completed was calculated for each of the 3 other diets as follows: (minutes of daily exercise completed each day/individual exercise target) X 100. Horses completed significantly ($P < 0.05$) less of their exercise target while being fed the triheptanoin diet than while being fed the corn oil or HFLS diets; no other significant differences were detected among the other diets. In panel B, logarithmically transformed plasma CK activity was significantly ($P < 0.05$) higher for the triheptanoin diet than for the corn oil or HFLS diets; no other significant differences were detected among the other diets. Plasma CK activity for the HFLS diet represents results for only 7 horses. Day 1 = First day on which the complete amount of the specified diet was fed; horses were exercized for 14 days with a 10-day washout period between subsequent diets.

Daily plasma glucose, insulin, and NEFA concentrations—Diet had a significant ($P < 0.001$) effect on daily plasma glucose concentrations; however, concentrations were not significantly different between the triheptanoin and CO diets (Table 1). Feeding the grain diet resulted in significantly higher concentrations than when the HFLS diet was fed.

Diet had a significant ($P < 0.001$) effect on insulin concentrations. Daily plasma insulin concentrations were significantly higher for the triheptanoin diet than for the corn oil diet. Feeding the grain diet resulted in higher plasma insulin concentrations than for the corn oil and HFLS diets, but insulin concentrations did not differ between the corn oil and HLFS diets.

Diet had a significant ($P < 0.001$) effect on NEFA concentrations. Feeding corn oil resulted in higher NEFA concentrations than for all other diets (Table 1). Daily plasma NEFA concentrations were similar when grain and triheptanoin diets were fed.

Effect of SET on plasma CK activity—Mean ± SD logarithmically transformed plasma CK activity in samples obtained 4 hours after SET was higher when horses were fed the triheptanoin diet ($\log_{10}$ 3.3 ± 0.63) than when fed the corn oil ($\log_{10}$ 2.6 ± 0.18) or HFLS ($\log_{10}$ 2.7 ± 0.22) diets, and CK activity was similar to that for the grain diet ($\log_{10}$ 3.1 ± 0.53; **Table 2**).

Effect of SET on plasma glucose, insulin, and NEFA concentrations—Diet had a significant ($P = 0.02$) effect on glucose concentrations. Plasma glucose and insulin concentrations obtained before and after the SET did not differ between the triheptanoin and corn oil diets. Feeding the triheptanoin diet resulted in lower blood glucose concentrations before the SET, compared with concentrations before the SET when the HFLS diet was fed. Glucose concentrations were significantly ($P < 0.001$) decreased after the SET; however, differences among horses between glucose concentrations obtained before and after the SET within the same diet were not identified in post hoc tests (Table 2). There was an overall significant ($P < 0.001$) decrease in insulin concentrations after the SET; however, only the HFLS diet had a significant change in concentrations determined in samples obtained before and after the

Table 1—Mean (range) plasma CK activity and mean ± SD glucose, insulin, and NEFA concentrations in samples obtained after exercise daily for 3 weeks in 8 horses while they were fed a grain, triheptanoin, corn oil, or HFLS diet.

Diet	CK* (U/L)	Glucose (mg/dL)	Insulin (μU/mL)	NEFA (mEq/L)
Grain	2,758[a] (232–44,343)	103.8 ± 18.1[a]	17.0 ± 14.0[a]	0.084 ± 0.025[a,b]
Triheptanoin	5,623[b] (223–50,040)	89.1 ± 17.7[b]	15.0 ± 14.0[a,b]	0.077 ± 0.039[a,b]
Corn oil	856[c] (195–7,257)	88.9 ± 17.7[b]	8.4 ± 4.9[c]	0.139 ± 0.046[c]
HFLS†	1,491[c] (157–39,942)	96.6 ± 11.5[a,b]	11.0 ± 7.2[b,c]	0.095 ± 0.036[b]

*Statistical comparisons for CK activity were performed on logarithmically transformed values to normalize the data. †Represents results for only 7 horses because the catheter for collection of blood samples did not remain patent in 1 horse.

[a–c]Within a column, values with different superscript letters differ significantly ($P < 0.05$).

SET. No differences were apparent among diets for insulin concentrations before and after the SET. Concentrations of NEFA were significantly ($P < 0.001$) affected by diet, and NEFA concentrations before the SET were lower for the triheptanoin diet than for the corn oil diet. No differences were found when comparing NEFA

Table 2—Mean ± SD values for plasma CK activity in samples obtained 4 hours after exercise and glucose, insulin, and NEFA concentrations in samples obtained before and after an SET for 8 horses while they were fed a grain, triheptanoin, corn oil, or HFLS diet.

		Glucose (mg/L)		Insulin (μU/mL)		NEFA (mEq/L)	
Diet	**CK (U/L)**	**Before**	**After**	**Before**	**After**	**Before**	**After**
Grain	2,215 ± 2,153[a]	99.4 ± 4.1[a,b]	83.5 ± 13.3	21.7 ± 9.9	10.6 ± 4.2	0.030 ± 0.021[a,b]	0.046 ± 1.043[a,b]
Triheptanoin	4,114 ± 4,876[a,b]	82.4 ± 16.8[a]	82.1 ± 11.0	18.5 ± 8.4	9.2 ± 3.0	0.021 ± 0.006[a]	0.028 ± 0.010[a]
Corn oil	411 ± 151[a]	93.3 ± 8.6[a,b]	74.6 ± 13.9	12.3 ± 6.7	4.5 ± 1.3	0.080 ± 0.028[b]	0.091 ± 0.060[b]
HFLS	538 ± 308[a]	105.6 ± 18.3[b]	88.8 ± 21.1	23.0 ± 19.5	8.7 ± 4.9*	0.040 ± 0.019[a,b]	0.056 ± 0.035[a,b]

*Value differs significantly ($P < 0.05$) from value before exercise.
See Table 1 for remainder of key.

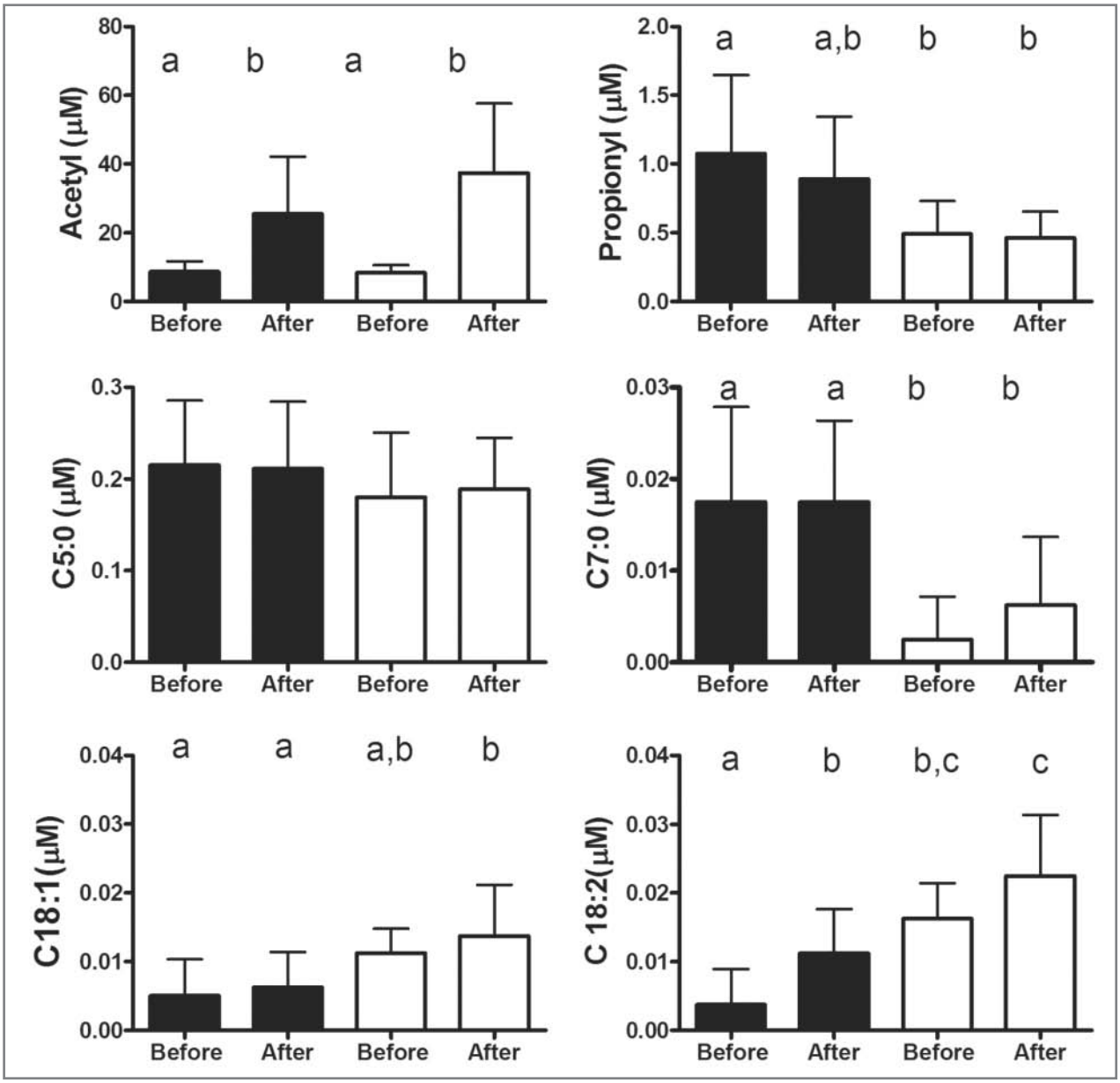

Figure 2—Mean ± SD concentrations of plasma acylcarnitines with various numbers of carbon atoms obtained before and after a 15-minute SET in horses when they were fed the triheptanoin (black bars) and corn oil (white bars) diets. Notice that scales on the y-axis differ among the graphs. [a–c]Values with different letters differ significantly ($P < 0.05$). When horses were fed the triheptanoin diet, they had higher concentrations of propionyl and C7:0 acylcarnitines, whereas when horses were fed the corn oil diet, they had higher concentrations of long-chain fats (C18:2 acylcarnitine).

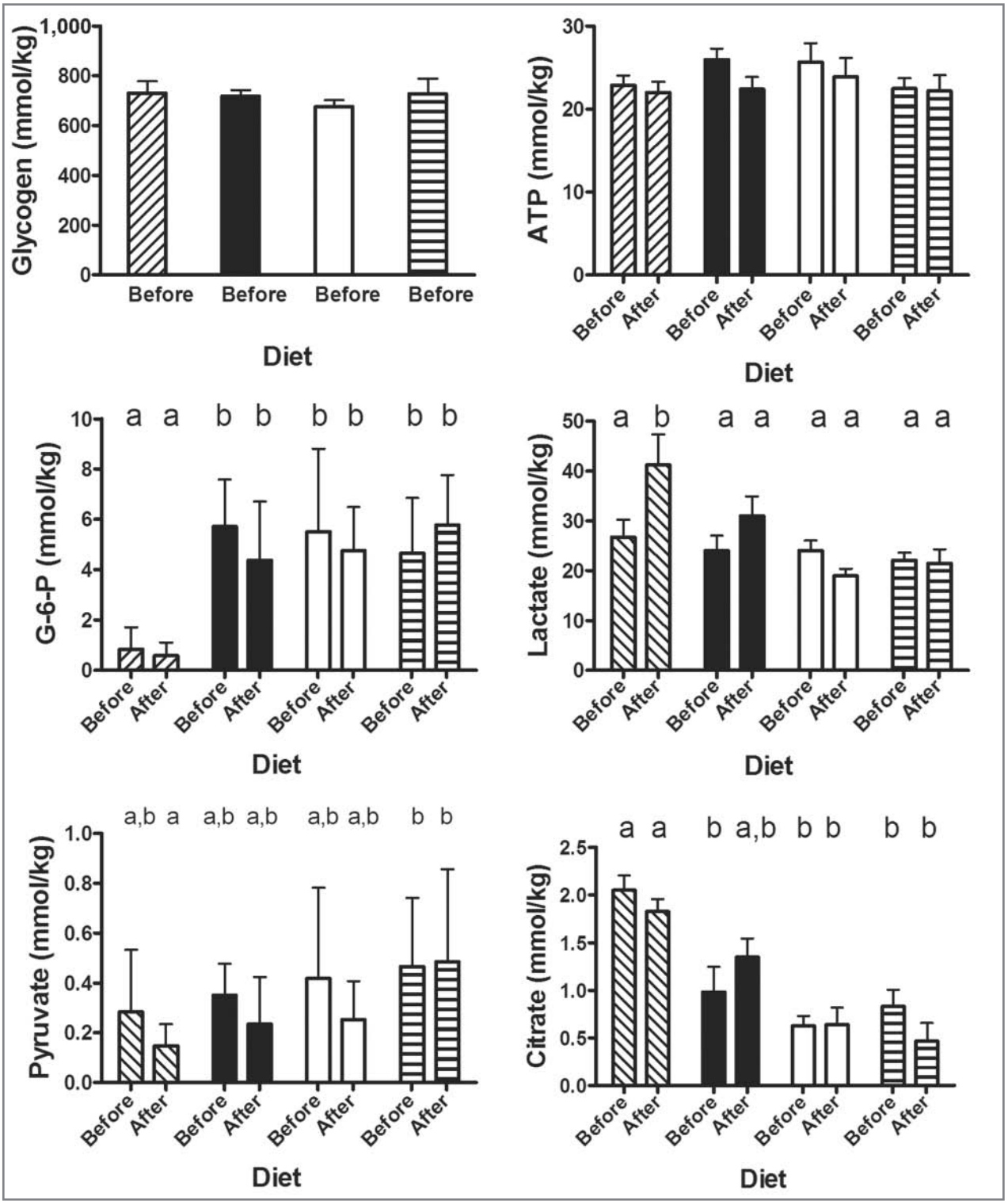

Figure 3—Mean ± SD concentrations of substrates and metabolites in gluteal muscle biopsy specimens obtained before and after a 15-minute SET in horses when fed the grain (diagonal-striped bars), triheptanoin (black bars), corn oil (white bars), and HFLS (horizontal-striped bars) diets. Values are reported on a dry-weight basis. *See* Figure 2 for remainder of key.

concentrations between the grain, triheptanoin, and HFLS diets for samples obtained before or after the SET. Concentrations of NEFA after the SET for the triheptanoin diet were lower than for the corn oil diet. Overall, the SET did not have a significant effect on NEFA concentrations.

Effect of SET on plasma acylcarnitine concentrations—Concentrations of C2:0-acylcarnitines increased significantly ($P < 0.001$) after the SET, compared with concentrations before the SET (**Figure 2**). Concentrations of C3:0- and C7:0-acylcarnitines were not significantly affected by the SET but were significantly ($P <$

0.001) higher for the triheptanoin diet, compared with concentrations for the corn oil diet. Mean ± SD concentrations of C16:0-acylcarnitine were not significantly affected by the SET but were significantly (P = 0.03) lower for the triheptanoin diet (0.0113 ± 0.004µM and 0.0138 ± 0.005µM before and after the SET, respectively) than for the corn oil diet (0.0188 ± 0.004µM and 0.0150 ± 0.008µM before and after the SET, respectively). Concentrations of C18:1-acylcarnitines were not significantly affected by the SET but were significantly (P = 0.002) lower after the SET for the triheptanoin diet than for the corn oil diet. Concentrations of C18:2-acylcarnitine were significantly (P < 0.001) lower for the triheptanoin diet than for the corn oil diet and increased significantly (P = 0.006) after the SET for the triheptanoin diet. Free and C5:0-acylcarnitine concentrations were not significantly affected by the SET and did not differ significantly between the triheptanoin and corn oil diets. Free acylcarnitine concentrations for the triheptanoin diet were 18.19 ± 6.95µM and 21.89 ± 8.42µM before and after the SET, respectively; free acylcarnitine concentrations for the corn oil diet were 21.04 ± 7.12µM and 23.22 ± 5.89µM before and after the SET, respectively.

Muscle biochemical analysis—Resting muscle glycogen concentrations were not significantly affected by diet **(Figure 3)**. Muscle ATP concentrations were not significantly affected by diet or by the SET. Concentrations of G6P were significantly lower for the grain diet than for any other diet, but G6P concentrations were not significantly different among the triheptanoin, corn oil, and HFLS diets. Muscle lactate concentrations were significantly (P < 0.001) affected by diet but not by the SET. Lactate concentrations were not significantly different between the triheptanoin and corn oil diets. When horses were fed the grain diet, lactate concentrations after the SET were significantly higher than concentrations after the SET when horses were fed the other 3 diets. Muscle pyruvate concentrations were significantly (P = 0.006) affected by diet but not by the SET. Muscle pyruvate concentrations after the SET were significantly lower for the grain diet than for the HFLS diet. Muscle citrate concentrations were significantly (P < 0.001) affected by diet but not by the SET. Concentrations of citrate before the SET were significantly higher for the grain diet than for the triheptanoin, corn oil, and HFLS diets. Muscle citrate concentrations were not significantly different between the triheptanoin and corn oil diets. Muscle citrate concentrations before and after the SET were significantly higher for the grain diet than for the corn oil or HFLS diets.

Discussion

The study reported here clearly indicated that the type of fat ingested by horses with PSSM affects both exercise intolerance and muscle damage. Triheptanoin was selected for inclusion in the study because as a fat with an odd number of carbon atoms, it supplied acetyl-CoA (similar to the effect for long-chain fats) and also could potentially replenish the CAC through the provision of succinyl-CoA via methyl malonyl-CoA.[18] Triheptanoin appeared to be absorbed when fed, as indicated by higher concentrations of plasma C3:0- and C7:0-acylcarnitines, compared with results when horses were fed corn oil. Concentrations of C5:0-acylcarnitine, which is produced from C7:0-acylcarnitine metabolism in the liver, were not different between corn oil and triheptanoin diets in the present study, but were significantly higher for triheptanoin versus corn oil in another study[22] in which oils were administered by nasogastric tube 120 minutes before exercise. It is possible that in the study reported here, the variability in consumption of oils may have influenced the ability to detect differences in C5:0-acylcarnitines and that C5:0-acylcarnitine arising from C7:0-acylcarnitine consumption may be rapidly converted to acetyl CoA and propionyl CoA, which resulted in similar acylcarnitine concentrations between the oil diets. Rather than benefiting PSSM horses, provision of this alternate energy source exacerbated exercise intolerance and resulted in even greater muscle damage (as indicated by plasma CK activity) than for the grain diet. When fed to healthy Thoroughbreds in another study,[22] triheptanoin was tolerated well and resulted in postexercise CK activity higher than the reference range in only 1 of 8 horses.

Because triheptanoin appears to be of benefit for other metabolic myopathies, it is difficult to explain its unexpected detrimental effect in horses with PSSM. One potential explanation for its lack of a beneficial effect may be that the horses with PSSM in the present study did not have depletion of CAC intermediates. Muscle citrate concentrations in horses with PSSM in this study were higher than those measured in healthy Thoroughbreds in another study.[22] A further explanation for the potential detrimental effect of triheptanoin may relate to its ability to stimulate insulin secretion. Daily insulin concentrations were as high when horses with PSSM were fed the triheptanoin diet as when those same horses were fed the grain diet. In healthy horses, a dose of 217 mL of triheptanoin given via nasogastric tube resulted in a significant increase in the insulin concentration 2 hours later.[22] Thus, one of the detrimental properties of triheptanoin in horses with PSSM may be its ability to further increase glycogen synthase activity via insulin and glucose, which thereby disturbs energy flux in a manner similar to that for high-starch diets.[27] However, muscle metabolic responses when horses were fed triheptanoin differed from the responses when horses were fed the grain diet in that lactate was not elevated after exercise and G6P concentrations were higher for the triheptanoin diet than for the grain diet.

Triheptanoin could have had a further negative impact on the supply of long-chain fats by inhibiting lipolysis through elevated insulin concentrations and by increasing malonyl-CoA concentrations (via postexercise citrate concentrations, similar to the effect for the grain diet), which further inhibits transport of long-chain fats.[28] Less utilization of long-chain fat in horses for the triheptanoin diet was suggested by lower plasma NEFA concentrations and lower plasma concentrations of C16:0- and C18:2-acylcarnitine when horses were fed the triheptanoin diet, compared with the corresponding concentrations when horses were fed the corn oil diet. Triheptanoin, when fed to rats and human patients, is extremely gluconeogenic,[14,21] which provides additional

glucose, an increased insulin response, and a decrease in lipolysis. Although these various mechanisms may all have contributed to impaired energy generation during submaximal exercise in horses with PSSM when they were fed the triheptanoin diet, we were unable to detect significant differences in muscle substrates or metabolites between horses when they were fed the triheptanoin or corn oil diet.

Feeding the grain diet in the study reported here resulted in exercise intolerance and rhabdomyolysis within 18 minutes after initiation of light exercise in horses with PSSM. There is evidence from other studies that horses with PSSM have impaired energy generation in muscle[4] and lower oxygen consumption during exercise,[29] compared with results for healthy horses. When horses with PSSM were fed the grain diet in the present study, they had a modest increase in lactate concentrations and did not have a significant decrease in muscle pyruvate concentrations with exercise, which is in contrast to findings for humans with glycogen storage diseases (myophosphorylase or phosphofructokinases deficiencies).[15,30] Thus, if there were substrate limited–oxidative metabolism in horses with PSSM when fed the grain diet, it would appear that the limitation in flux lies downstream of the metabolism of glycogen to pyruvate. Detecting the precise site of substrate limitation may require study of individual muscle fibers. For example, the present study and another study[4,8] revealed that ATP concentrations in whole muscle homogenates did not decrease significantly after exercise. However, analysis of individual fibers dissected from the same whole muscle sample revealed accumulation of inosine monophosphate in some fibers of horses with PSSM.[4] It is likely that only a small number of muscle fibers are recruited after 15 to 20 minutes of exercise, and only those recruited fibers exhibited substrate limited–oxidative flux.

It is difficult to explain how a gain of function mutation in *GYS1*, which leads to enhanced glycogen synthase activity (even in the basal state),[12] would impair oxidative metabolism of substrates such as pyruvate or fatty acids. On the basis of results of the study reported here, we speculate that excessive stimulation of glycogen synthesis resulting from a gain of function in glycogen synthase (stimulated further by insulin) might be interpreted by nutrient sensors in cells as an indication that glycogenolysis and lipolysis need not be activated. A potential scenario in horses with PSSM fed the grain diet could be that nutrient sensors, such as AMP kinase, did not fully activate enzymes, such as pyruvate dehydrogenase, during exercise to produce adequate amounts of acetyl CoA for oxidative metabolism. Although acetyl CoA could also be supplied by fatty acid oxidation, horses with PSSM, when fed the grain diet, had low plasma NEFA concentrations, possibly as a result of suppression of lipolysis by high insulin concentrations.[31] An additional factor for reduction of fatty acid oxidation in horses with PSSM when fed the grain diet may have been the high muscle citrate concentrations identified. High muscle citrate concentrations have been reported[32] in muscle exposed to a high-glucose load. Citrate activates acetyl CoA carboxylase, which converts acetyl CoA to malonyl CoA, the committed step for fatty acid synthesis, thereby directing acetyl CoA away from the CAC cycle. Accumulation of malonyl CoA causes inhibition of carnitine palmytoyl transferase, which is the key enzyme necessary to transport long-chain fatty acids into the mitochondria for β-oxidation.[33,34] Thus, when horses with PSSM were fed the grain diet, they may have been unable to generate sufficient amounts of acetyl CoA from carbohydrate or fat metabolism to fuel muscle contraction during submaximal exercise.

The provision of long-chain fatty acids, such as those supplied by the corn oil diet, significantly decreased exercise intolerance and rhabdomyolysis. This is consistent with results of a randomized study[16] conducted to evaluate effects of the HFLS diet in exercising horses with PSSM. Feeding the corn oil diet increased plasma NEFA concentrations and plasma C18:2-acylcarnitine concentrations and thus the availability of fats for oxidation in skeletal muscle. Increased β-oxidation of long-chain fats with exercise when horses were fed the corn oil diet was suggested by increased C2:0-acylcarnitine concentrations after exercise. The long-chain fat diets also appeared to increase glycogenolytic-glycolytic and oxidative flux in muscle of horses with PSSM, as indicated by higher G6P concentrations, lower lactate concentrations, and higher pyruvate concentrations (HFLS diet only) in muscle, compared with results for the grain diet. Thus, long-chain fat diets appear to provide ample energy for aerobic exercise in horses with PSSM.

The study reported here was designed as a randomized comparison to compare effects for the corn oil and triheptanoin diets. The HFLS diet was fed last as a negative control diet, which could have biased the assessment of the HFLS diet because training is beneficial for horses with PSSM. Corn oil consists primarily of linoleic (54%), oleic (29%), and palmitic (13%) acids,[31] and the HFLS diet contained fats from rice bran (oleic [38%], linoleic [34%], and palmitic [22%] acids)[35] and soy oil (linoleic [50%], oleic [20%], and palmitic [14%] acids).[36] Thus, the forms of long-chain fats supplied by the corn oil and HFLS diets were relatively similar; however, the amount of digestible energy from fat supplied by the corn oil diet (30%) was twice that supplied by the HFLS diet (15%). Furthermore, plasma NEFA concentrations when horses were fed the corn oil diet were 50% higher than when they were fed the HFLS diet. The results of this study could indicate that in fit horses with PSSM, feeding half as much fat in the form of the HFLS diet has a similar beneficial effect to feeding 750 mL of corn oil. Although some veterinarians recommend 0.45 kg of fat/d for horses with PSSM,[17] results of the study reported here suggest that in fit horses, a reduced amount of dietary fat is similarly beneficial and may help avoid weight gain, thereby averting further disruption of an already disturbed metabolism. Low amounts of dietary starch and sugar are also equally important for horses with PSSM.

Daily exercise is another important means by which to decrease exercise intolerance and rhabdomyolysis in horses with PSSM.[6,16] The significant effect of time on plasma CK activity among diets, with higher CK activities during the first week of exercise in the present

study, supports the benefit of daily exercise. Exercise may lower plasma insulin concentrations and increase plasma NEFA concentrations, and training may, over time, enhance uptake of fatty acids into skeletal muscle and improve muscle oxidative capacity.[16,37,38] It was notable that the plasma NEFA concentrations at 4 hours after exercise were at least double those in samples obtained before exercise. In addition, it is possible that daily exercise may improve nutrient sensing and thereby affect the balance of energy supplied as carbohydrate and fat for oxidative metabolism.[39,40] Regular exercise and diet do not appear to decrease muscle glycogen concentrations in horses with PSSM because these were unchanged in the horses of the present study as well as in horses in other studies.[8,11,29]

The study reported here revealed that a key factor in feeding horses with PSSM is the provision of long-chain fats, as compared with short-chain fats, as well as providing low-starch diets that maintain low daily plasma insulin concentrations. Although triheptanoin appears to be metabolized by horses with PSSM and is of benefit to humans with disorders of lipid metabolism and some glycogenoses, it did not have a beneficial effect for horses with the *GYS1* mutation.

a. EquiJewel, Kentucky Equine Research, Versailles, Ky.
b. ACH Food Co Inc, Memphis, Tenn.
c. Sasol, Witten, Germany.
d. Re-Leve, Kentucky Equine Research, Versailles, Ky.
e. Glucose/hexokinase, Marshfield Clinic Laboratories, Marshfield, Wis.
f. Waco NEFA C test kit, Waco Diagnostics, Richmond, Va.
g. NCSS, Kaysville, Utah.
h. Prism software, GraphPad Software Inc, San Diego, Calif.

Appendix

Percentage of digestible energy supplied by starch, fat, protein, and fiber in the grain, triheptanoin, corn oil, and HFLS concentrates.

Total digestible energy	Grain	Triheptanoin	Corn oil	HFLS
Starch (%)	33	19	19	24
Fat (%)	9	30	30	17
Protein (%)	22	18	18	22
Fiber (%)	36	33	33	37

All diets were isocaloric and provided 25 Mcal/500 kg of diet/d.

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