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Stephanie J. Valberg
Michigan State University

Zoë J. Williams
Michigan State University

Carrie J. Finno
University of California-Davis

Abigail Schultz
Michigan State University

Deborah Velez-Irizarry
Michigan State University

See next page for additional authors

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Type 2 polysaccharide storage myopathy in Quarter Horses is a novel glycogen storage disease causing exertional rhabdomyolysis

Stephanie J. Valberg[1] | Zoë J. Williams[1] | Carrie J. Finno[2] | Abigail Schultz[1] | Deborah Velez-Irizarry[1] | Marisa L. Henry[1] | Keri Gardner[1] | Jessica L. Petersen[3]

[1]Large Animal Clinical Sciences, College of Veterinary Medicine, Michigan State University, East Lansing, Michigan, USA

[2]Department of Population Health and Reproduction, School of Veterinary Medicine, University of California-Davis, Davis, California, USA

[3]Department of Animal Science, University of Nebraska-Lincoln, Lincoln, Nebraska, USA

Correspondence
Stephanie J. Valberg, Large Animal Clinical Sciences, College of Veterinary Medicine, Michigan State University, East Lansing, MI 48824, USA.
Email: valbergs@msu.edu

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Abstract

Background: Both type 1 (PSSM1) and type 2 polysaccharide storage myopathy (PSSM2) are characterised by aggregates of abnormal polysaccharide in skeletal muscle. Whereas the genetic basis for PSSM1 is known (R309H *GYS1*), the cause of PSSM2 in Quarter Horses (PSSM2-QH) is unknown and glycogen concentrations not defined.

Objectives: To characterise the histopathological and biochemical features of PSSM2-QH and determine if an associated monogenic variant exists in genes known to cause glycogenosis.

Study design: Retrospective case control.

Methods: Sixty-four PSSM2-QH, 30 PSSM1-QH and 185 control-QH were identified from a biopsy repository and clinical data, histopathology scores (0–3), glycogen concentrations and selected glycolytic enzyme activities compared. Coding sequences of 12 genes associated with muscle glycogenoses were identified from whole genome sequences and compared between seven PSSM2-QH and five control-QH.

Results: Exertional rhabdomyolysis in PSSM2-QH occurred predominantly in barrel racing and working cow/roping performance types and improved with regular exercise and a low starch/fat-supplemented diet. Histopathological scores, including the amount of amylase-resistant polysaccharide (PSSM2-QH 1.4 ± 0.6, PSSM1-QH 2.1 ± 0.3, control-QH 0 ± 0, $p < 0.001$), and glycogen concentrations (PSSM2-QH 129 ± 62, PSSM1-QH 175 ± 9, control-QH 80 ± 27 mmol/kg, $p < 0.0001$) were intermediate in PSSM2-QH with significant differences among groups. In PSSM2-QH, abnormal polysaccharide had a less filamentous ultrastructure than PSSM1-QH and phosphorylase and phosphofructokinase activities were normal. Seventeen of 30 PSSM2-QH with available pedigrees descended from one of three stallions within four generations. Of the 29 predicted high or moderate impact genetic variants identified in candidate genes, none were present in only PSSM2-QH and absent in control-QH.

Main limitations: Analyses of PSSM2-QH and PSSM1-QH were performed on shipped samples, controls on frozen samples.

Conclusions: PSSM2-QH is a novel glycogen storage disorder that is not the result of a mutation in genes currently known to cause muscle glycogenoses in other species.

KEYWORDS

glycogen, glycogenolysis, glycolysis, horse, muscle disease, polyglucosan

1 | INTRODUCTION

Equine polysaccharide storage myopathy (PSSM) is a specific form of exertional rhabdomyolysis in Quarter Horses (QH) and related breeds. The hallmark feature that led to the discovery of PSSM was aggregates of amylase-resistant polysaccharide (Ar-PS) in periodic acid–Schiff (PAS) stains of skeletal muscle.[1] Amylase-resistance is defined by the presence of PAS positive polysaccharide in muscle fibres following incubation in amylase. Normal glycogen is amylase-sensitive, meaning that all PAS staining in muscle fibres is removed following amylase pre-incubation. Aggregates of amylase-sensitive polysaccharide (As-PS) were subsequently added as a diagnostic feature of PSSM.[2] These PAS positive inclusions ranged from small aggregates of glycogen to course granular inclusions.[2] In 2008, a dominant nonsynonymous gain-of-function mutation in the glycogen synthase 1 (*GYS1*) gene was identified in PSSM-QH that were phenotyped by increased muscle glycogen concentrations and Ar-PS.[3] Of the 99 PSSM-QH that were used to identify the *GYS1* mutation, 72% possessed the *GYS1* mutation and 28% did not. This led to the term type 1 PSSM (PSSM1) being applied to horses with the *GYS1* mutation[4] and type 2 PSSM (PSSM2) applied to those horses that had abnormal aggregates of Ar- or As-PS that did not possess the *GYS1* mutation.[4,5] Thus, the term PSSM2 represents a histopathological description of PAS-stained polysaccharide and not a specific aetiology. To date, muscle glycogen concentrations have not been reported in PSSM2-QH.

In Arabian and Warmblood horses diagnosed with PSSM2, glycogen is predominantly As-PS and concentrations are not abnormally increased compared with breed matched controls.[6–8] A subset of Arabian and Warmblood horses diagnosed with PSSM2 had aggregates of desmin in scattered myofibres and myofibrillar disarray in ultrastructural studies, leading to adoption of the term 'myofibrillar myopathy' for this subset of Warmblood and Arabian PSSM2 horses.[7,9] PSSM2 in these two breeds likely represents a potential early phase of myofibrillar myopathy.[9,10] In comparison, desmin aggregates typical of myofibrillar myopathy were not identified in 163 PSSM2-QH diagnosed with PSSM2.[11] Thus, PSSM2-QH appears to have a different aetiopathology from myofibrillar myopathy.

The first objective of our study was to characterise the clinical features and response to diet and exercise recommendations of adult PSSM2-QH. The second objective was to compare muscle glycogen concentrations, phosphofructokinase (PFK) enzyme activity, histopathology and ultrastructure in PSSM2-QH compared with PSSM1-QH and control-QH. The third objective was to evaluate PSSM2-QH familial relationships and determine if a monogenic variant in any of 12 genes known to cause glycogenosis in other species is associated with PSSM2-QH.

2 | MATERIALS AND METHODS

PSSM2-QH cases were retrospectively selected from the Michigan State University Neuromuscular Diagnostic Laboratory database using submissions between December 1993 and November 2021. Selection criteria included QH (defined as QH, American Paint or Appaloosa horses), ≥2 years of age, a history of rhabdomyolysis, negative for the *GYS1* mutation and a diagnosis of moderate PSSM2 in semimembranosus, semitendinosus or gluteal muscle biopsy specimens. Moderate PSSM2 was defined by the presence of either Ar-PS or As-PS aggregates in muscle fibres or PAS staining that was darker than a control standard as determined by visual inspection. Genotyping for the *GYS1* mutation was originally performed as previously described.[3] To confirm the PSSM2-QH diagnosis, DNA was subsequently submitted to the Veterinary Diagnostic Laboratory at the University of California, Davis for *GYS1* genotyping in all PSSM2-QH. Additionally, the diagnostic laboratory also genotyped the PSSM2-QH for the ryanodine receptor (*RYR1*) mutation causing malignant hyperthermia,[12] the myosin heavy chain 1 (*MYH1*) mutation causing myosin heavy chain myopathy[13,14] and the mutation causing glycogen branching enzyme (*GBE1*) deficiency.[15]

Criteria for retrospectively selecting control-QH from the biopsy repository included no history of a myopathy, ≥2 years of age, negative for the *GYS1* mutation and no evidence of histopathology in gluteal muscle biopsies. Positive controls consisted of 30 PSSM1-QH with gluteal or semimembranosus muscle biopsies submitted to the diagnostic laboratory between 2004 and 2021.

All PSSM2-QH and PSSM1-QH samples had been shipped on icepacks to the Neuromuscular Diagnostic Laboratory and processed within 48 h of when the muscle biopsy was taken. Samples that were large enough were divided into two sections prior to freezing. One piece was mounted on cork in OCT media and frozen in isopentane suspended in liquid nitrogen for histopathological analysis. The second portion was frozen directly in liquid nitrogen for biochemical assays. Control gluteus medius muscle samples had been obtained from healthy horses for prior research studies, with the samples divided into two portions. One portion for histopathology was rolled in talc, frozen on-site in liquid nitrogen, and later mounted on cork in OCT

medium. A second portion for biochemical analyses was immediately frozen in liquid nitrogen after sampling.

2.1 | Clinical data

Registered name, performance type, sex, age at presentation for a muscle biopsy, recorded clinical signs and serum creatine kinase (CK) and aspartate transaminase (AST) activities were retrieved from the biopsy submission forms (where provided). Owners of PSSM2-QH horses with biopsy submissions between 2012 and 2021 ($N = 31$) or their referring veterinarians were contacted by email or telephone in November 2021. At the time of diagnosis of PSSM2, horse owners had received recommendations for management that included a low starch fat supplemented diet (hay with <12% nonstructural carbohydrate, concentrate with <15% nonstructural carbohydrate and 10%–12% fat by weight) and regular daily exercise.[16] Respondents within two contact attempts were asked whether the horse was fed the recommended diet and given regular daily exercise. Additionally, they were asked if their horse's clinical signs improved with recommendations and if horses returned to their expected performance level.

2.2 | Muscle glycogen concentrations and PFK enzyme activity

Glycogen concentrations were assayed fluorometrically as glucose residues after boiling 2–10 mg of gluteal or semimembranosus/tendinosus muscle tissue for 2 h in 1 M HCl.[17] PFK activity was assayed in seven PSSM2-QH, five PSSM1-QH and six control-QH using a colorimetric assay (BioVision, Milpitas, CA). For controls, semimembranosus muscle samples were selected that had been shipped on icepacks to the laboratory, had no evident histopathology and had been stored at −80°C for an equivalent amount of time to PSSM2-QH samples. Briefly, 20 mg of muscle tissue was homogenised in 400 μl of ice-cold assay buffer, the supernatant was removed and diluted 1:40 in phosphate buffered saline. Fifty microlitres of sample was aliquoted to 96-well plates in duplicates and samples were read every 2 min for 30 min (OD_{450}). Two time points in the linear range and an NADH standard curve were used to calculate PFK activity expressed as μmol/g/min.

2.3 | Muscle histopathology

All muscle biopsy samples were stained with a minimum of haematoxylin and eosin (H&E), PAS and amylase PAS. Briefly, for PAS staining 7 μm thick sections were fixed in 10% neutral buffered formalin, prior to application of 0.5% periodic acid which was followed by Schiff reagent and haematoxylin counterstaining. Sections for amylase PAS were fixed in Carnoy's and then incubated at 37°C in 1.0% diastase of malt combined with human saliva prior to PAS staining. The incubation times in the diastase solution had varied over time between 30 and 45 min based on the ability of the diastase solution to completely remove glycogen from a standard sample. Other stains applied to the majority of PSSM1-QH and PSSM2-QH cases included modified Gomori Trichrome, oil red O and nicotinamide adenine dinucleotide tetrazolium reductase and desmin.[9,18] Myophosphorylase staining was retrospectively performed on muscle samples from 12 PSSM2-QH, 5 PSSM1-QH and 5 control-QH submitted between 2014 and 2021.[18]

2.3.1 | Histopathological scores

H&E sections from all PSSM2-QH, PSSM1-QH and 25 control-QH were reviewed by one observer (SJV) during a 2-week period, and scored (0 absent, 1 mild, 2 moderate, 3 marked) for histopathological features: muscle fibre size variation, anguloid atrophy, angular atrophy, macrophage infiltration and subsarcolemmal vacuoles.[7] PAS and amylase PAS stains were reviewed and scored 0–3 for overall intensity of the PAS stain. The abnormal polysaccharide was recorded as As-PS or Ar-PS and then scored (0–3) for the overall number of fibres with polysaccharide aggregates in the sample, the amount under the sarcolemma and the amount in the cytoplasm.

2.3.2 | Electron microscopy

A region from paraffin-embedded tissue blocks of one control-QH and two PSSM2-QH (both negative for the *GBE1* mutation) were excised. The samples were sequentially deparaffinated in decreasing concentrations of xylol/ethanol. Muscle fragments (1 mm^3) were fixed in 2.5% glutaraldehyde in 0.166 M sodium cacodylate buffer (pH 7.4) at 4°C and post-fixed in 1% osmium tetroxide in 0.166 M sodium cacodylate buffer (pH 7.4). En bloc staining was performed using 2% uranyl acetate in water. Muscles were sequentially dehydrated in increasing concentrations of acetone and infiltrated in Spurr resin. Samples were embedded in Spurr and polymerised at 60°C. Selected blocks were sectioned at 70–80 nm and stained with uranyl acetate and lead citrate before imaging. Thin sections were also stained with PAS to ensure abnormal polysaccharide was present.

Electron micrographs processed from fresh muscle were also included from two additional PSSM cases because they had superior image resolution to the formalin-fixed specimens. One case was related to one of the PSSM2-QH and had a similar limited amount of Ar-PS as PSSM2-QH (Figure S1), however samples were obtained 15 years prior to the discovery of the *GYS1* mutation and a genotype was unavailable. The second case was a PSSM1-QH included for comparison.

2.4 | Data analysis

Shapiro–Wilk testing was used to determine normality. Data that were not normally distributed (age, CK, AST) were $\log_{10}$ transformed. Serum CK and AST activities were compared between PSSM2-QH

and PSSM1-QH using an unpaired *t*-test. Ages, muscle glycogen concentrations and PFK activity were compared using a one-way ANOVA among PSSM2-QH, PSSM1-QH and control-QH. Histopathological scores were compared using Mann–Whitney tests between control and PSSM2-QH and PSSM2-QH and PSSM1-QH. An alpha level of 0.05 was used to determine significance. Data were analysed using Graph Pad Prism software (version 9.1.2).

2.5 | Genetic analyses

Pedigrees of PSSM2-QH with registered names available were retrieved from online databases and evaluated for common ancestors. Seven of the PSSM2-QH that had either the highest number of fibres with Ar-PS ($N = 4$) or glycogen concentrations between 157 and 346 mmol/kg ($N = 3$) and five control-QH were selected for whole genome sequencing (WGS). DNA was isolated from hair bulbs, buffy coat, whole blood or muscle tissue using Gentra Puregene (Germantown, MD).

2.5.1 | Whole genome sequencing

Seven DNA PSSM2-QH samples were submitted to Michigan State University Research Technology Support Facility Genomics Core. Illumina TruSeq Nano DNA Library Preparation Kit (Illumina, San Diego, CA) with IDT for Illumina Unique Dual Index adapters were used following manufacturer's recommendations. Libraries were quality controlled and quantified using a combination of Qubit dsDNA HS (ThermoFisher, Waltham, MA) and Agilent 4200 TapeStation HS DNA1000 (Agilent, Wood Dale, IL). Completed libraries were pooled in equimolar quantities and the pool quantified using the Invitrogen Collibri Quantification qPCR kit (Thermofisher). This pool was loaded onto one lane of an Illumina NovaSeq 6000 SP flow cell and sequencing was performed in a 2× 150 bp paired end read format using a NovaSeq 6000 v1.5 300 cycle reagent kit (Illumina). Base calling was done by Illumina Real Time Analysis (RTA) v3.4.4 and output of RTA was demultiplexed and converted to FastQ format with Illumina Bcl2fastq v2.20.0. Following quality trimming, reads were mapped to the EquCab3.0 reference genome. Genotypes were called using GATK Haplotype Caller after mapping was completed. Five control-QH DNA samples were sequenced by the University of California, Davis Pioneer project (CJF). Library preparation was completed using a Kapa Hyper library for Illumina and quality control performed using Qubit. Samples were sequenced to 20× coverage on an Illumina 4500 using paired-end 150 bp reads. Data are available at NCBI using the BioProject ID PRJNA841859.

2.5.2 | Comparisons of selected genes

Twelve genes known to cause skeletal muscle glycogen storage disorders in other species were identified via literature review (November 2021). The chromosomal location of genes associated with Ar-PS: phosphofructokinase (*PFKM*), glycogen synthase (*GYS1*), glycogen branching enzyme (*GBE1*), glycogenin (*GYG1*), adenosine monophosphate kinase (*PRKAG2*) and RANBP2-type and C3HC4-type zinc finger containing 1 (*RBCK1*); as well as glycogenoses (myophosphorylase [*PGYM*], phosphoglucomutase [*PGAM2*], aldolase [*ALDOA*], lactate dehydrogenase [*LDHA*], beta enolase [*ENO3*], α1-3-glucosides [*GAA*]) were identified using Ensembl (EquCab 3.0; Table S1). SnpSift was used to filter variants based on region and both SnpE and Ensembl's Variant Effect Predictor were used to predict the functional effects of detected variants within the 12 selected genes.[19,20] The genotypes of variants with moderate or high predicted impact and deleterious SIFT scores, as determined by Ensembl, were compared in PSSM2-QH and control-QH using a Fisher's exact test, with significance set at $p < 0.05$.

3 | RESULTS

3.1 | Clinical data

There were 64 PSSM2-QH, 30 PSSM1-QH and 185 control-QH included in our study (Table 1). Mean ages ranged from 8.6 to 8.9 years across groups (Table 1). For PSSM2-QH performance types, 26 were racing (24 barrel racers, 2 racing), 11 roping/cow horse, 6 Western pleasure, 4 reining, 2 cutting, 2 trail, 1 jumping, 1 driving and 11 had unspecified use. Of the 64 PSSM2-QH, 55 were reported to have exertional rhabdomyolysis, 2 horses developed rhabdomyolysis on pasture and 7 had both exertional and nonexertional rhabdomyolysis. Additional reported clinical signs in PSSM2-QH included a low-grade lameness ($n = 11$), stiffness (8), fasciculations (7) and atrophy (2). Mean normalised CK ($p = 0.28$) and AST ($p = 0.08$) activities were above the normal ranges and elevated to a similar extent in PSSM2-QH and PSSM1-QH (Figure 1). Median values for PSSM2-QH were CK 1981 U/L ($N = 35$ with reported values), AST 998 U/L ($N = 29$) and for

TABLE 1 The number of horses, age, sex and breeds, glycogen concentrations and PFK activity in the type 2 PSSM (PSSM2-QH), type 1 PSSM (PSSM2-QH) and control (control-QH) groups

	N	Age (years)	Sex	Breeds	Glycogen (μmol/g)	PFK activity (μmol/g/min)
Control-QH	185	8.7 ± 5.2	145 F, 33 G, 3 S, 4 Unk	175 QH, 10 PA	80 ± 27[a]	404 ± 112[a]
PSSM2-QH	64	8.9 ± 4.7	38 F, 25 G, 1 Unk	57 QH, 6 PA, 1Ap	129 ± 62[b]	385 ± 37[a]
PSSM1-QH	30	8.6 ± 4.2	16 F, 11 G, 2 S, 1 Unk	26 QH, 3 PA, 1 Ap	175 ± 91[c]	572 ± 103[b]

Note: Different letters indicate significant differences within columns.
Abbreviations: Ap, Appaloosa; F, female; G, gelding; PA, American Paint; PFK, phosphofructokinase; PSSM, polysaccharide storage myopathy; QH, Quarter Horse; S, stallion; Unk, unknown sex.

PSSM1-QH, CK 3716 U/L ($N = 19$), AST 2307 U/L ($N = 14$). Reference range CK 194–346 U/L, AST 127–412 U/L.

None of the 64 PSSM2-QH possessed the *RYR1* mutation. Four (6.3%) PSSM2-QH possessed the *MYH1* mutation. Two *MYH1* homozygotes had Ar-PS, one presented with nonexertional and one exertional rhabdomyolysis; however, neither reported atrophy. The two *MYH1* heterozygotes had As-PS and presented with exertional rhabdomyolysis. Ten (15.6%) PSSM2-QH were heterozygous for the *GBE1* mutation (3 Ar-PS, 7 As-PS).

3.1.1 | Response to diet and exercise recommendations

Of the 31 veterinarians/horse owners contacted, 19 responded with follow-up information. Of the 19 horses, 15 (79%) followed the dietary recommendations (one of these fed low starch without adding fat). Of the 19 horses, 14 (74%) were given regular daily exercise or provided turn out on days not exercised. Of the 13 horses that followed both recommendations, 13 (100%) had no further episodes of rhabdomyolysis, 12 (92%) returned to their previous performance type and 1 (8%) switched from barrel racing to pleasure riding to prevent rhabdomyolysis. Owners emphasised that regular daily exercise was critically important for management, and two owners indicated that additional amino acid supplements were also beneficial. Of the six horses that did not follow the diet and exercise regimes, one was regularly exercised and successfully used dantrolene prior to exercise, one had navicular disease and could not exercise, one had not yet started back to work due to injury, one was subjected to euthanasia due to soreness and uncontrollable behaviour under saddle, one was donated to a rescue and one was subjected to euthanasia due to constant poor performance and other health issues.

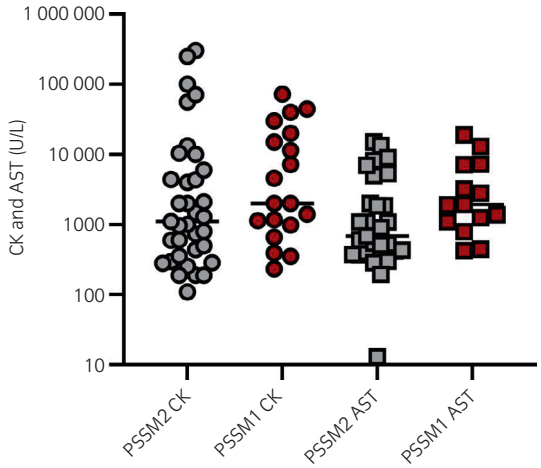

FIGURE 1 Increased serum CK and aspartate transaminase activities in PSSM2-QH and PSSM1-QH. There was no significant difference between PSSM2-QH and PSSM1-QH. Reference range CK 194–346 U/L, AST 127–412 U/L. CK, creatine kinase; PSSM-QH, polysaccharide storage myopathy in Quarter Horses

3.2 | Muscle glycogen and PFK enzyme activity

Muscle glycogen concentrations in PSSM2-QH ($p < 0.0001$) were 1.6-fold higher and, in PSSM1-QH, 2.2-fold higher than control-QH ($p < 0.0001$) (Table 1 and Figure 2A). PSSM1-QH glycogen concentrations were also significantly higher than PSSM2-QH ($p < 0.001$). Forty-six of 64 PSSM2-QH and 29 of 30 PSSM1-QH had glycogen concentrations above the 95% confidence limit for control-QH (confidence interval [CI] for controls 76–84 mmol/kg). The activity of PFK in PSSM2-QH was not different from shipped control samples ($p = 0.92$) and 1.5-fold lower than PSSM1-QH ($p = 0.006$) (Table 1).

3.3 | Histopathology

Myophosphorylase enzyme activity was present in histochemical stains of PSSM2-QH, control-QH and PSSM1-QH (Figure 2A).

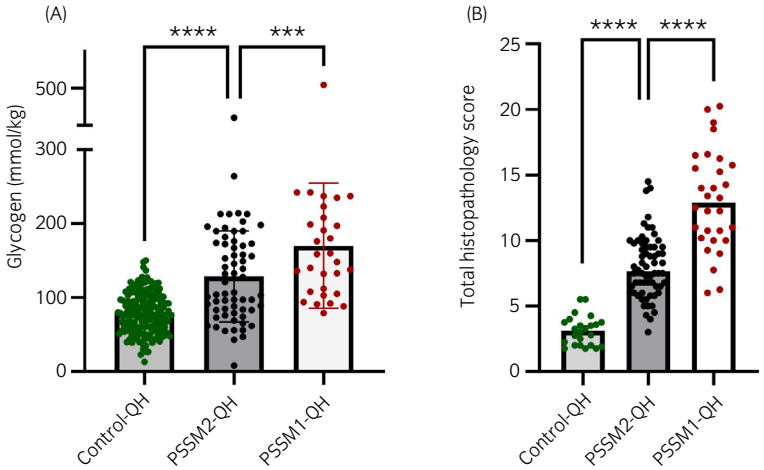

FIGURE 2 (A) Skeletal muscle glycogen concentrations in control-QH, PSSM2-QH and PSSM1-QH (points represent one horse). PSSM2-QH glycogen concentrations were significantly higher than control horses ($p < 0.0001$) and lower than PSSM1-QH ($p < 0.001$). (B) Mean and individual total histopathological scores for fibre size variation, anguloid atrophy, angular atrophy, macrophage infiltration, subsarcolemmal vacuoles, intensity of the PAS stain, total amount of polysaccharide, amount of polysaccharide under the sarcolemma and in the cytoplasm, each graded as absent (0), mild (1), moderate (2), severe (3). PSSM2-QH had total scores that were significantly higher than control-QH and lower than PSSM1-QH ($p < 0.0001$). *** $p < 0.001$, **** $p < 0.0001$. PSSM-QH, polysaccharide storage myopathy in Quarter Horses

TABLE 2 The mean ± SD of histopathological scores for fibre size variation (Size Var), anguloid atrophy (Angd), internalised myonuclei (IN), macrophages, subsarcolemmal vacuoles (Subsarc Vac), PAS staining intensity, abnormal polysaccharide (PS), granular cytoplasmic glycogen (Cyto PS), subsarcolemmal glycogen (subsarc PS), total histopathological score and number of horses with amylase resistant (Ar) and amylase sensitive (As) polysaccharide in muscle biopsies from control-QH, type 2 polysaccharide storage myopathy (PSSM2-QH) and type 1 PSSM (PSSM2-QH)

	N	Size Var	Angd atrophy	IN	Macrophages	Subsarc Vac	PAS intensity	Abnormal PS	Granular Cyto PS	Subsarc PS	Total score	Ar:As (N)
Control-QH	25	0.8 ± 0.7	0.3 ± 0.4	0 ± 0	0 ± 0	0 ± 0	1.9 ± 0.2	0 ± 0	0 ± 0	0.08 ± 0.3	3.1 ± 1.1	0:0
PSSM2-QH	64	1.2 ± 0.8*	0.6 ± 0.6*	0.4 ± 0.6***	0.1 ± 0.3	0.1 ± 0.4***	1.9 ± 0.5	1.4 ± 0.6***	1.0 ± 0.8***	0.8 ± 0.7***	8.0 ± 2.4***	18:46***
PSSM1-QH	30	1.4 ± 0.6	1.2 ± 0.6^^^	0.5 ± 0.6	0.4 ± 0.6^^	1.3 ± 1.1^^^	2.1 ± 0.6	2.0 ± 0.3^^^	2.0 ± 0.4***	1.2 ± 0.8^	13.0 ± 3.8^^^	27:3***

Abbreviation: PAS, periodic acid–Schiff.
Significant differences between PSSM2-QH and control-QH ($p < 0.05$; ***$p < 0.001$). ^Significant differences between PSSM2-QH and PSSM1-QH (^$p < 0.05$; ^^$p < 0.01$; ^^^$p < 0.001$).

Granular polysaccharide inclusions in PSSM2-QH and PSSM1-QH stained darkly for myophosphorylase (Figure 2A). Total scores for histopathology were significantly higher in PSSM2-QH (128.7 ± 61.6) than control-QH (79.9 ± 27.2) ($p < 0.001$) (Figure 2B). PSSM2-QH had significantly more variation in muscle fibre sizes, anguloid atrophied fibres, fibres with internalised myonuclei, subsarcolemmal vacuoles, total abnormal polysaccharide and more abnormal polysaccharide in both cytoplasmic and subsarcolemmal locations than control-QH (Table 2, Figures 3A–I and S2). Compared with PSSM1-QH, PSSM2-QH had significantly less anguloid atrophy, macrophage infiltration, subsarcolemmal vacuoles, abnormal polysaccharide and polysaccharide inclusions in cytoplasmic locations (Table 2, Figures 3D–L and S2). Total scores for PSSM1 horses were 170.2 ± 84.7.

3.3.1 | Characteristic of abnormal polysaccharide

In all control-QH, polysaccharide was evenly distributed throughout the cell, was sensitive to amylase-digestion and not associated with aggregation of phosphorylase (Figures 3A–C and S2). Polysaccharide in 61% (39/64) PSSM2-QH had either a dark, homogeneous As-PS staining pattern with increased subsarcolemmal glycogen near blood vessels or formed small As-PS inclusions (Figures 3D–F and S2). In seven PSSM2-QH (7/64; 11%), course granular inclusions appeared identical to those of PSSM1-QH in PAS stains, however, they completely digested with 45 min of pre-incubation in amylase unlike PSSM1-QH (Figures 3G–I and S2). In 18 (28%) PSSM2-QH, the course granular inclusions were Ar-PS. The amount of Ar-PS was significantly less in PSSM2-QH (18/64; 28%) than PSSM1-QH (27/30 PSSM1-QH, 90%, $p < 0.0001$). Fibres with Ar-PS or course granular As-PS were often found along the periphery of muscle fascicles in PSSM2-QH (Figure 3G,H). Glycogen concentrations in PSSM2-QH with Ar-PS (131 ± 52 mmol/kg) were not significantly different from concentrations in PSSM2-QH with As-PS (128 ± 67 mmol/kg, $p = 0.80$).

3.3.2 | Ultrastructure

In an area of PSSM2-QH muscle that had abnormal granular polysaccharide, myofibrils were displaced by beta glycogen particles and an amorphous material with a frosted-glass appearance and variable electron density (Figure 4A–D). This material was also present in subsarcolemmal regions (Figure 4C). It was not seen in the control QH. In the fresh PSSM sample of a related horse with a similar amount of abnormal PS in light microscopy, material with a ground glass appearance and variable electron density was identified (Figure 4E,F) that contained a small amount of filamentous material resembling polyglucosan (Figure 4G). In contrast, the polyglucosan in electron microscopic (EM) images of a PSSM1-QH had abundant filamentous material (Figure 4H).

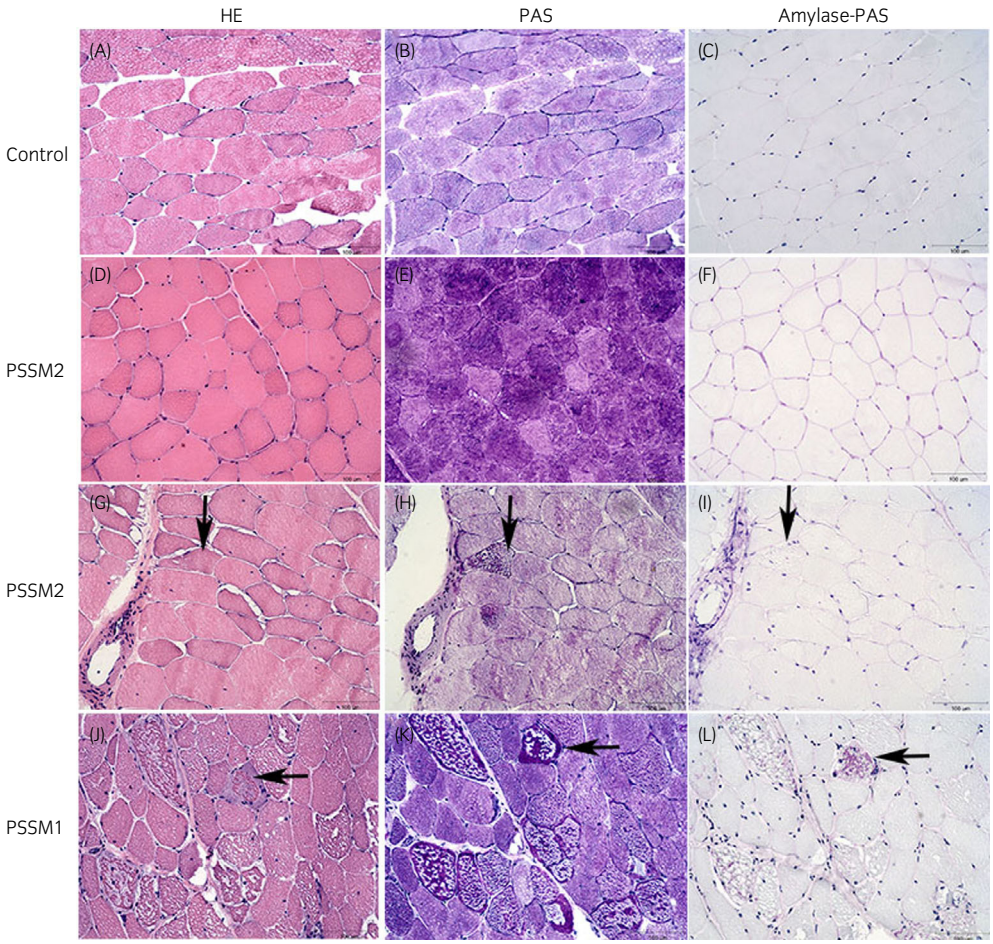

FIGURE 3 Cross section of gluteal (A–C) or semimembranosus (D–L) muscle stained with H&E (A, D, G, J), PAS (B, E, H, K) or amylase PAS (C, F, I, L) 20×. (A–C) Control horse with amylase sensitive glycogen. (D–F) Normal myofibre morphology and fine granular dark PAS positive polysaccharide that is amylase-sensitive in a PSSM2-QH. (G–I) Increased anguloid atrophy, internalised myonuclei and course granular amylase-sensitive polysaccharide in a few fibres of a PSSM2-QH. (J–L) Increased fibre size variation, internalised myonuclei, subsarcolemmal vacuoles and numerous PAS positive amylase resistant subsarcolemmal and cytoplasmic inclusions in a PSSM1-QH. Arrow indicates same fiber in serial sections. H&E, haematoxylin and eosin; PAS, periodic acid–Schiff; PSSM-QH, polysaccharide storage myopathy in Quarter Horses

3.4 | Genetic analyses

3.4.1 | Pedigree analysis

Thirty pedigrees from PSSM2-QH were available for evaluation. A relationship among horses within 4–6 generations was found for three subsets of PSSM2-QH. Seven PSSM2-QH were related to one stallion and three PSSM2-QH related to an additional stallion on one side within four generations (Figure 5A). Within four more generations, these two pedigrees could be connected and included the PSSM-QH with a fresh sample used for electron microscopy (Figure 5A). In the third family, seven horses were related to one stallion within four generations. The dam and two half siblings of one of these PSSM2-QH also had a history of exertional rhabdomyolysis (Figure 5B).

3.4.2 | Genome sequences

Sequencing depth across the candidate genes averaged 8.9× while that of the controls, sequenced for another study, was 27.2×. There were 13 million variants total with over 52 000 having a predicted impact on gene function (Table S1). From these data across the 12 candidate genes, 75 variants were identified in our analysis, with 3 predicted to be of high impact, 26 moderate and 46 low impact (Tables 3 and S2). At least one variant with high or moderate impact was identified in each of the 12 candidate glycogenoses genes in at least one PSSM2-QH or control-QH. There were no variants present in the majority of PSSM2-QH and absent in a minority of control-QH and none of the variants were significantly associated with PSSM2-QH (Table 3). Two variants with low impact scores were present in 100% of PSSM2-QH but were also present in 67%–83% of control-QH (Table 3). There were three variants annotated as deleterious according to SIFT scores, two in *PFKM* and one in *PRKA2* (Table 3). The deleterious *PFKM* variants were only present in one PSSM2-QH and this horse had normal PFK activity. The third deleterious variant in *PRKAG2* was found in only one PSSM2-QH. Four variants had deleterious SIFT scores, with low confidence, and were present in 0–4 PSSM2-QH and 1–4 control-QH.

4 | DISCUSSION

The results of our study clearly show that PSSM2 in Quarter Horse-related breeds is a glycogenosis characterised by 1.6-fold higher muscle glycogen concentrations and small numbers of fibres with Ar- or granular As-PS inclusions that resemble polyglucosan. In several species, muscle glycogenoses are caused by monogenic

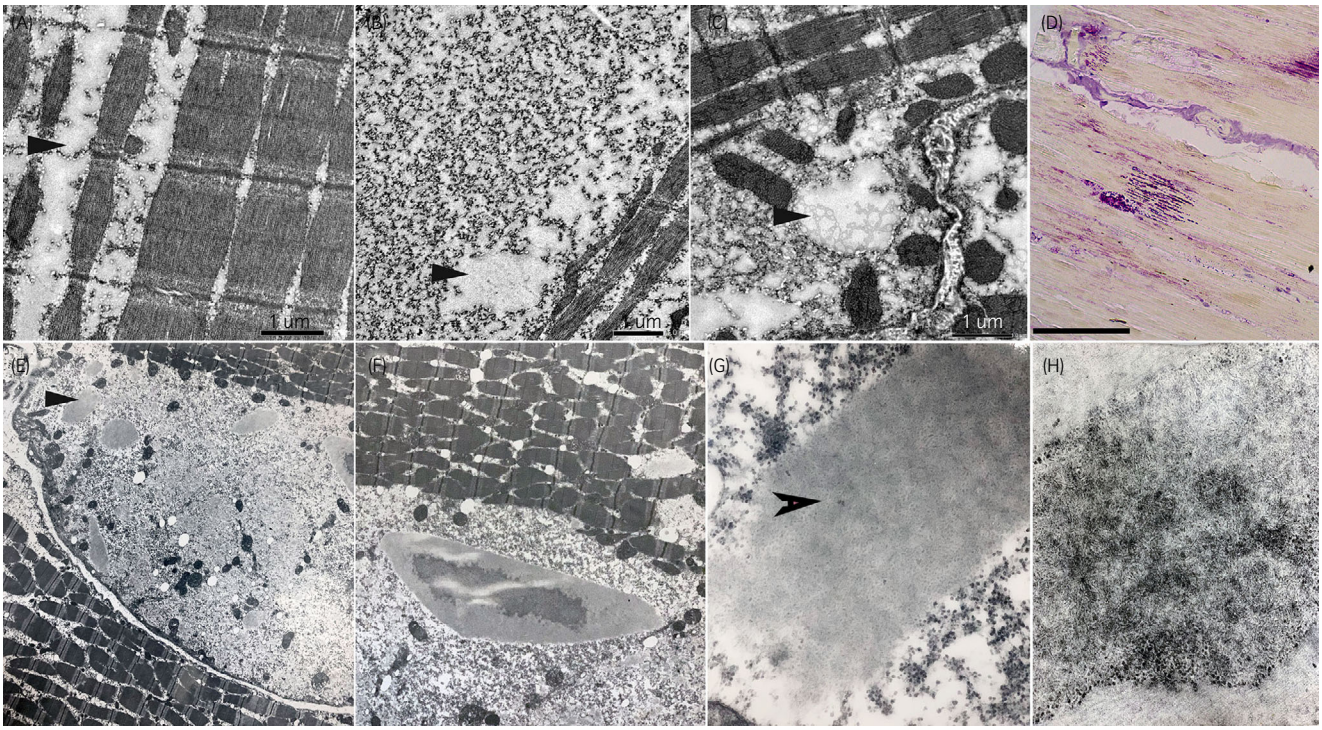

FIGURE 4 Electron microscopic images of semimembranosus muscle from a PSSM2-QH prepared from paraffin embedded formalin-fixed blocks (A–D), gluteal muscle prepared from fresh tissue from a related PSSM-QH (E–G) and from a PSSM1-QH (H). (A) A large amount of amorphous material with a frosted-glass appearance (arrowhead) together with mitochondria and beta glycogen particles separate myofibrils 6000×. (B) Accumulation of beta glycogen and amorphous material (arrowhead) under the sarcolemma 5000×. (C) Presumed polyglucosan body (arrowhead) positioned under the sarcolemma that has variable electron density and is surrounded by beta glycogen particles and mitochondria 6000×. (D) PAS stain of thin sections of the region used for EM showing a fibre with abnormal granular polysaccharide. Bar 100 μm. (E) Subsarcolemmal accumulation of beta glycogen, polyglucosan bodies (arrowhead) and mitochondria 5800×. (F) A polyglucosan body with an electron dense centre surrounded by beta glycogen particles and a few mitochondria 9600×. (G) Higher magnification of the polyglucosan body (arrow in E and G) showing a small amount of filamentous material 58 000×. (H) Abundance of filamentous material surrounded by beta glycogen particles in a polyglucosan body from a PSSM1-QH 74000×. EM, electron microscopic; PSSM-QH, polysaccharide storage myopathy in Quarter Horses

mutations in 12 genes encoding enzymes involved in glycogen synthesis, metabolism or lysosomal/proteasomal degradation.[21,22] We identified 75 variants in coding sequences of the 12 glyco(geno)lytic genes in both PSSM2-QH and control-QH, including three variants with high and 26 variants with moderate predicted impact scores. None of the variants, however, were significantly associated with PSSM2-QH. Therefore, PSSM2-QH appears to be a novel glycogenosis potentially caused by variants in genes not currently associated with skeletal muscle glycogen storage disease or in genes that either alter the regulation of glycogen-related genes or cause post-translational modification of their product. Alternatively, there could be multiple genes that together contribute to PSSM2-QH.

Nonspecific myopathic features of PSSM2-QH included mild anguloid atrophy, rare internalised myonuclei and rare macrophage infiltration. The hallmark of PSSM2-QH was intense PAS staining, subsarcolemmal glycogen accumulation and/or granular polysaccharide inclusions in a few fibres along the perimeter of muscle fascicles. The course granular polysaccharide that was apparent in PSSM2-QH resembled to that seen in PSSM1-QH, except that in PSSM2-QH the granular polysaccharide had variable resistance to amylase-digestion. In order to determine if the polysaccharide was polyglucosan, we evaluated EM images derived from formalin-fixed tissue from two PSSM2-QH. Polyglucosan bodies consist of beta glycogen particles and filamentous material that represents insoluble[23] long straight (α1–4) chains of glucose with few branch points making them resistant to amylase digestion.[24] Images from a related horse with exertional rhabdomyolysis that had a similar amount of Ar-PS to PSSM2-QH were included because they had better image resolution having been processed from fresh muscle. This horse lacked a *GYS1* genotype because the electron microscopy was performed before genetic testing was available. Abnormal material with variable electron density that resembled that seen in the two PSSM2-QH was apparent in this horse and the material contained a small amount of filamentous material that resembled polyglucosan. The amount of filamentous material in the polyglucosan bodies of the PSSM-QH was less than that seen in PSSM1-QH, which could potentially explain why PSSM2-QH polysaccharide inclusions were more sensitive to amylase-digestion than PSSM1-QH. Ultrastructural studies of fresh

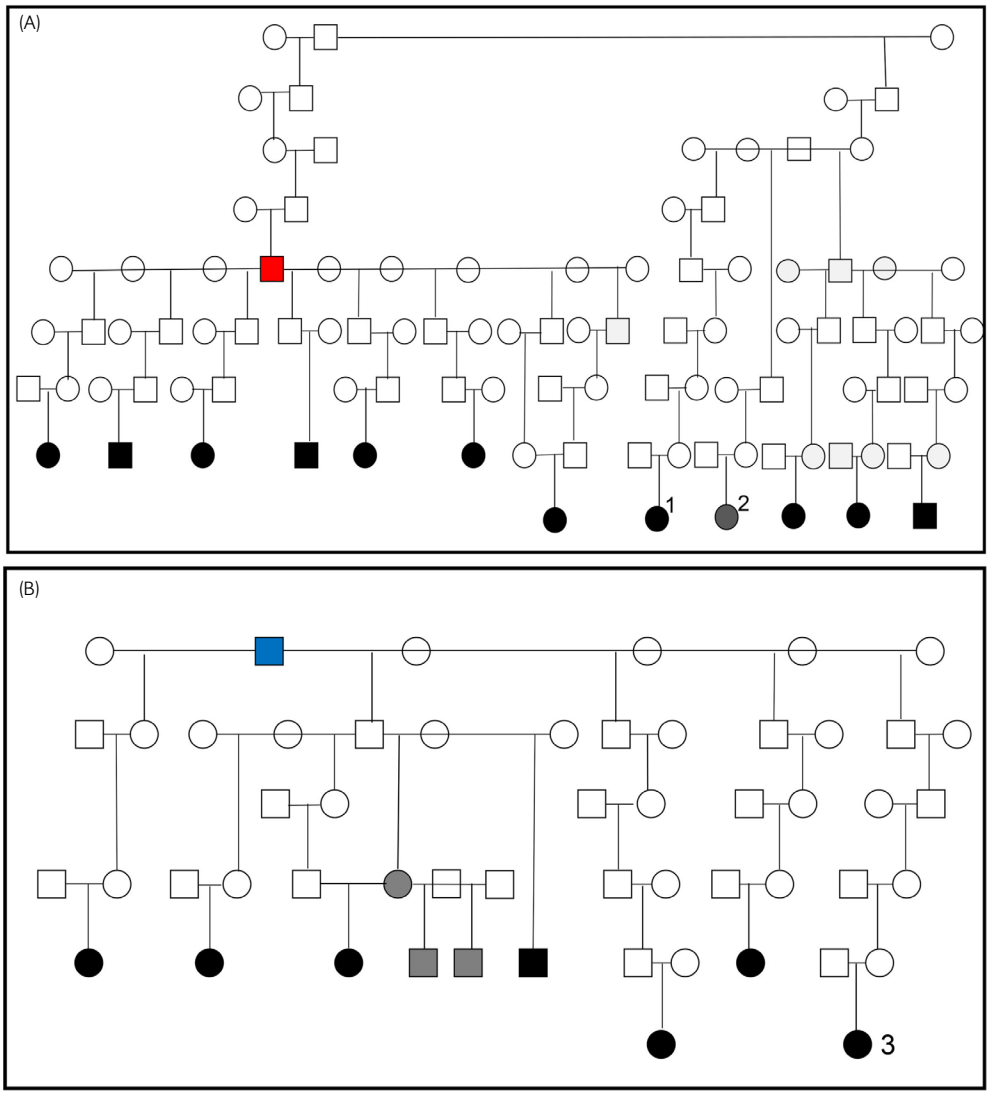

FIGURE 5 Pedigrees for PSSM2-QH (black). (A) Seven PSSM2-QH were related to one stallion (red) and three PSSM2-QH related to an additional stallion (blue) within four generations. These two pedigrees could be connected within four additional generations and included a PSSM2-QH used for WGS (1) and a PSSM-QH used for EM (2). (B) Seven horses were related to one stallion (blue) within five generations. The dam (grey) and two half siblings (grey) for one PSSM2-QH also had a history of exertional rhabdomyolysis. Horse 3 was used for WGS. Male squares and female circles. PSSM-QH, polysaccharide storage myopathy in Quarter Horses; WGS, whole genome sequencing

muscle samples fixed immediately in glutaraldehyde from more horses with PSSM2 are needed to confirm that the abnormal material in PSSM2-QH muscle is polyglucosan.

A familial basis for PSSM2-QH was previously suggested by the fact that excessive glycogen storage occurred in three breeds that share a common genetic background, Quarter Horse, American Paint Horse and Appaloosa.[25] Although Warmblood and Arabian horses were formerly diagnosed with PSSM2 based on the appearance of As-PS, subsequent research identified normal muscle glycogen concentrations in these horses and desmin staining and ultrastructure was consistent with a form of myofibrillar myopathy.[7,9] Within our PSSM2-QH cohort, pedigree evaluation identified three 3–6 generation families that descended from three sires popular in barrel racing (two families) and working cow/roping horses (one family). One dam of a PSSM2-QH in these pedigrees was herself reported to have exertional rhabdomyolysis as did three of her offspring, one was a confirmed PSSM2-QH. The high prevalence of PSSM2-QH in barrel racing (45%) and working cow horses (21%) contrasts the low prevalence of these performance types in PSSM1 (highest in halter and pleasure), further supporting a distinct familial basis for PSSM2-QH.[26] The presence of PSSM2-QH in similar numbers of males and females did not support an X-linked pattern of inheritance. Consanguinity was rarely observed within six generations in the PSSM2-QH pedigrees. If enough generations are included in pedigree analysis, however, consanguinity can often be present because the Quarter Horse breed originated in 1940 from a small number of stallions.[27,28] Thus, a familial basis was suggested but not definitively identified by pedigree analysis.

One of the strongest candidate genes for PSSM2-QH was *PFKM* because a deficiency of PFK causes exertional rhabdomyolysis, high muscle glycogen concentrations, and, in some cases, Ar-PS in a few fibres, similar to PSSM2-QH.[21,24,29] There were five variants of high to moderate predicted effect identified in *PFKM*, however, they occurred in only one or two PSSM2-QH and in one to three control-QH. The 1 PSSM2-QH with 2 deleterious *PFKM* variants had normal PFK enzyme activity, as did 11 other PSSM2-QH. Thus, PFK deficiency is not the cause of PSSM2-QH.

TABLE 3 Gene, location of variants with high or moderate impact, reference and variant alleles, predicted SnpEFF impact, amino acid change, SIFT score, number of PSSM2-QH ($N = 7$) and control-QH ($N = 5$) that had the variant and Fisher's exact test p values

Gene	Location (Chr:bp; EquCab3.0)	Ref allele	Variant allele	Consequence	Predicted impact	Amino acid change	SIFT score	No. PSSM2-QH variant	No. control-QH variant	p value
ALDOA	13:20698094	G	C	Missense	Moderate	C/S	Tolerated low confidence (0.11)	3	5	0.3
ALDOA	13:20698181	G	A	Missense	Moderate	S/N	Tolerated low confidence (0.32)	3	4	0.6
ALDOA	13:20700810	A	G	Missense	Moderate	E/G	Deleterious low confidence (0)	3	4	0.6
ENO3	11:49909092	G	A	Missense	Moderate	S/F	Deleterious low confidence (0)	1	1	>0.9
GAA	11:2828748	T	G	Stop lost	High	*/Y	-	2	4	0.3
GAA	11:2829346	A	G	Missense	Moderate	V/A	Tolerated (0.38)	4	5	0.6
GAA	11:2829933	C	T	Missense	Moderate	G/S	Tolerated (0.25)	1	0	>0.9
GAA	11:2831262	C	T	Missense	Moderate	R/K	Tolerated (1)	4	5	0.6
GAA	11:2838024	C	A	Missense	Moderate	A/S	Tolerated (0.29)	3	4	0.6
GAA	11:2843207	C	A	Missense	Moderate	V/F	Tolerated (0.66)	5	6	0.5
GBE1	26:8845435	G	A	Missense	Moderate	A/T	Tolerated (0.06)	0	1	>0.9
GYS1	10:19209677	T	C	Missense	Moderate	S/G	-	1	0	>0.9
GYG1	16:83387969	A	G	Missense	Moderate	T/A	Tolerated (0.18)	2	0	0.5
LDHA	7:88827090		G	Missense	Moderate	T/A	Tolerated low confidence (0.35)	2	4	0.3
PFKM	6:66635283	AGGGGGGGG	GGGGG GGGG	Frameshift	High	GGG/GGGX	-	3	3	>0.9
PFKM	6:66635315	A	G	Missense	Moderate	E/G	-	1	1	>0.9
PFKM	6:66636449	T	A	Missense	Moderate	M/K	Deleterious low confidence (0)	2	1	>0.9
PFKM	6:66674053	A	G	Missense	Moderate	S/G	Deleterious (0)	1	0	>0.9
PFKM	6:66677818		A	Missense	Moderate	V/I	Deleterious (0.02)	1	0	>0.9
PGAM2	4:14823343	G	A	Missense	Moderate	T/I	Tolerated (0.46)	0	1	>0.9
PRKAG2	4:103265513	C	G	Missense	Moderate	E/Q	Deleterious (0.04)	1	0	>>0.9
PYGM	12:28606798	C	A	Missense		M/I	Tolerated (1)	1	0	>0.9
PYGM	12:28613145	C	T	Missense	Moderate	G/S	Tolerated (0.47)	1	0	>0.9
RBCK1	22:22579265	G	A	Missense	Moderate	P/L	Tolerated low confidence (1)	0	1	>0.9
RBCK1	22:22579366		GCGCGCGCGCG	In frame deletion	Moderate	PRARAR/ PRAR	-	1	2	>0.9

TABLE 3 (Continued)

Gene	Location (Chr:bp; EquCab3.0)	Ref allele	Variant allele	Consequence	Predicted impact	Amino acid change	SIFT score	No. PSSM2-QH variant	No. control-QH variant	*p* value
RBCK1	22:22579366		GCGCGCGCGCGCG	Frameshift	Moderate	PRARAR/PRARX	-	1	2	>0.9
RBCK1	22:22579433		C	Missense	Moderate	P/R	Tolerated low confidence (0.73)	0	1	>0.9
RBCK1	22:22579444		G	Missense	Moderate	A/P	Tolerated (0.3)	0	2	0.2
RBCK1	22:22579520	C	A	Missense	Moderate	R/L	Deleterious low confidence (0.04)	0	1	>0.9

Note: No variants were significantly associated with PSSM2 in QHs.
Abbreviations: PSSM, polysaccharide storage myopathy; QH, Quarter Horse.

Accumulation of Ar-PS also arises from mutations that disrupt glycogen synthesis or ubiquitin assembly.[24] Glycogen synthesis begins with the attachment of glucose to the protein glycogenin (encoded by *GYG1*) followed by the addition of (α1 → 4) glucose linkages catalysed by glycogen synthase (*GYS1*).[23] The glycogen branching enzyme (*GBE1*) cleaves the linear chain and creates numerous branches via an (α1 → 6) linkage. In horses, a gain of function mutation in *GYS1* and loss of function mutation in *GBE1* both result in the formation of polyglucosan bodies.[3,28] While the dominant *GYS1* mutation causes rhabdomyolysis, the recessive *GBE1* mutation primarily causes weakness and neonatal death.[3,30] A mutation in *PRKAG2* (adenosine monophosphate kinase) in pigs produces excessive glycogen storage and polyglucosan bodies, likely by enhancing glucose uptake in skeletal muscle.[31] Variants predicted to have a moderate impact on gene function were identified in the candidate genes *GYS1*, *GYG1*, *PRKAG2*, *RBCK1* and *GAA* however, they were typically found in a single PSSM2-QH or control-QH and therefore were not significantly associated with the PSSM2 phenotype in QH.

Mutations in four other genes encoding glycolytic enzymes are known to cause exertional rhabdomyolysis and, in some cases, excessive glycogen storage without Ar-PS.[21] Myophosphorylase deficiency was evaluated both by assessing enzyme activity in muscle sections, which was normal, as well as by evaluating the coding sequence of *PGYM*. Two variants with predicted moderate impact were identified, one was present in all but two control-QH and the other only in one PSSM2-QH. In the remaining glycolytic genes, six additional variants were identified, none of which was significantly associated with the PSSM2 phenotype in QHs. Thus, analysis of the predicted high and moderate impact variants in genes associated with glycol(gen)lytic and glycogen synthetic enzymes did not identify a putative monogenic variant associated with PSSM2 in QH, indicating that PSSM2-QH may be a unique glycogenosis.

The genetic analysis performed in our study investigated the potential for a single genetic variant in a gene previously known to cause glycogen storage diseases in other species to cause PSSM2-QH. If PSSM2-QH was Mendelian but with reduced penetrance, we would still expect most or all of the cases to have at least one copy of the disease variant while it should be absent or at low frequency in the controls. None of the variants identified in our study fit this pattern of inheritance. It is possible that a combination of genetic variants contributes to PSSM2-QH, and our approach in this study was limited to a monogenic form of inheritance. Investigation of a complex genetic trait requires further investigation. One limitation of our genomic analyses is that the current annotation of the equine genome could be lacking some isoforms of the genes studied. Also, while the average coverage of the cases was 8.9×, rare variation could also have been missed that could be identified with deeper sequencing. Revisiting these loci as the annotation improves and generates deeper sequence coverage of PSSM2 cases could in the future help identify rare variants that might contribute to the manifestation of PSSM2.

Commercial genetic tests for PSSM2 are offered for variants in genes that are not associated with glycogenoses (equiseq.com). In a

recent study, about 60% of QH were shown to possess one or more of these commercial variants, regardless of whether they were healthy or had histopathological evidence of PSSM2.[32] Thus, the results of our study, studies of variants used in commercial tests for PSSM2[33] and other equine studies[32] show that genetic variants of predicted high and moderate impact are a common occurrence in the equine genome and are not necessarily associated with disease states. Much work must be done to ensure that there is a strong association between phenotype and genotype and that a variant results in a logical functional effect before concluding that a proposed commercial genetic variant is causative of a disease.

In the absence of a genetic test, the diagnosis of PSSM2-QH is still dependent on histopathological characterisation of muscle biopsies. A histopathological diagnosis of PSSM2-QH is more difficult than PSSM1-QH because readily identifiable Ar-PS is lacking in 72% of PSSM2-QH vs. it being present in 99% of PSSM1-QH. Furthermore As-PS can be degraded during shipping, resulting in a false negative diagnosis in PSSM2-QH cases. The accuracy of diagnosis of PSSM2-QH could be improved by obtaining specimens of sufficient size (2 cm square surface area) to identify fibres with abnormal polysaccharide and rapidly shipping well-chilled samples to the laboratory. Fixing an addition piece of muscle in formalin could also help to preserve glycogen during the shipping process.[34]

Study limitations included the lack of availability of snap-frozen samples from diagnostic submissions. This was because samples were obtained at various locations across the United States by referring veterinarians and shipped overnight. With variability in chilling and duration of time for shipment of PSSM2-QH samples, metabolism or degradation of glycogen could have occurred resulting in muscle glycogen concentrations measuring lower than they were at the time of biopsy and with potentially fewer As-PS inclusions than originally present. There was considerable variability in glycogen concentrations in the horses in our study. In 18 PSSM2-QH (28%), muscle glycogen concentrations were below the 95% CI for control-QH, which could have been due to the impact of shipping. However, we ensured that PSSM2-QH selected for whole-genome sequencing had Ar-PS or muscle glycogen concentrations well above the CIs for controls. The inclusion of PSSM1-QH in our study as a positive control allowed us to determine that shipped samples from horses with glycogenoses have abnormally high glycogen concentrations. The only samples available from healthy horses were snap-frozen research samples because diagnostic specimens are not received from healthy horses. The CIs established for glycogen concentrations from control samples were likely higher than those that would have been established by using shipped control samples. This, however, gave us a greater degree of certainty that glycogen concentrations measured in many of the shipped samples of PSSM2-QH were indeed abnormally elevated.

Dietary intervention for muscle glycogenoses in humans appears to have limited success in controlling weakness or rhabdomyolysis, particularly for defects distal to myophosphorylase.[35] Controlled exercise in humans, however, can be of benefit by reducing dependence on glycogen and increasing fatty acid oxidation.[36] Thirteen responding owners of PSSM2-QH who followed both diet and exercise recommendations found that regular exercise was particularly important in managing PSSM2-QH. Similar to PSSM1-QH, PSSM2-QH also responded to a low nonstructural carbohydrate/fat supplemented diet.[16,37] This diet decreases insulin secretion, which may be beneficial in PSSM1-QH because insulin increases glycogen synthase activity.[38] PSSM1-QH have both enhanced insulin sensitivity as well as continually enhanced glycogen synthase activity.[39,40] It is important to point out that the interpretation of horses' response to the diet is based on a small number of survey respondents and the survey did not collect quantitative unbiased information. A controlled trial that included a placebo would be needed to provide stronger evidence for a diet effect. It would be of interest to investigate insulin sensitivity in PSSM2-QH since the metabolic defect in these horses appears to impact glycogen synthesis based on the accumulation of Ar-PS.

In conclusion, PSSM2-QH is a glycogen storage disorder causing exertional rhabdomyolysis that has less severe histopathology than PSSM1-QH and glycogen concentrations that are intermediately elevated between PSSM1-QH and control-QH. The abnormal polysaccharide in PSSM2-QH is present in a small number of fibres per biopsy and has a similar course granular appearance to that in PSSM1-QH, but it is more sensitive to amylase-digestion and has less filamentous material in electron microscopy than PSSM1-QH. In PSSM2-QH, genetic variants were identified in candidate genes known to cause glycogenoses in other species, but none of these variants were significantly associated with PSSM2-QH. Thus, PSSM2-QH appears to be a novel glycogenosis that at present can only be diagnosed by muscle histopathology. Fortunately, based on 13 owners that followed both diet and exercise recommendations, a low nonstructural carbohydrate, fat supplemented diets and regular exercise appear to be effective in managing PSSM2-QH.

AUTHOR CONTRIBUTIONS

Stephanie J. Valberg and Carrie J. Finno contributed to data collection. Zoë J. Williams, Marisa L. Henry, Keri Gardner and Stephanie J. Valberg contributed to laboratory analyses. Deborah Velez-Irizarry, Stephanie J. Valberg, Jessica L. Petersen contributed to data analysis. Stephanie J. Valberg, Zoë J. Williams, Jessica L. Petersen and Carrie J. Finno contributed to manuscript preparation; all authors approved the final manuscript.

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CONFLICT OF INTEREST

Stephanie J. Valberg and other colleagues licence commercial laboratories to perform type 1 PSSM testing and personally receive royalties. Stephanie J. Valberg runs the Neuromuscular Diagnostic Laboratory at Michigan State University that processes muscle biopsies through the Michigan State Veterinary Diagnostic Laboratory. She has no personal financial interest in muscle biopsy submissions. Other authors report no competing interests.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1111/evj.13876.

DATA AVAILABILITY STATEMENT

The sequence data is available at NCBI using the BioProject ID PRJNA841859.

ETHICS STATEMENT

An Institutional Animal Care and Use Committee and Animal Use Form exemption for archived samples from Michigan State University was in place for this study.

INFORMED CONSENT

Explicit owner consent for animals' inclusion in the study was not stated.

ORCID

Stephanie J. Valberg https://orcid.org/0000-0001-5978-7010
Zoë J. Williams https://orcid.org/0000-0002-2852-2914
Carrie J. Finno https://orcid.org/0000-0001-5924-0234
Jessica L. Petersen https://orcid.org/0000-0001-5438-8555

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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