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From Mutation Discovery to Population Screening: Equine Juvenile Spinocerebellar Ataxia and Inherited Disease Burden in American Quarter Horses

By

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THESIS

Submitted in partial satisfaction of the requirements for the degree of

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DAVIS

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2025

Dedication

To my mom, Tracy, whose belief in my potential set me on the path to pursue my dreams. Thank you for being a constant source of encouragement, wisdom, and love throughout my education and life.

To my fiancé, Timothy, whose confidence in me supported my journey these past ten years. I am grateful for your partnership, patience, and the countless ways you made this achievement possible.

And to my sister, Victoria, who keeps me grounded and reminds me of the importance of authenticity. Your perspective enriches both my work and life.

Abstract

Equine veterinary genetic research has contributed significantly to the veterinary community, horse owners and breeders, animal welfare, and breed longevity. The discovery of causative mutations for inherited equine diseases allows veterinarians to recognize the clinical signs of disease, achieve definitive diagnoses via genetic testing, and make recommendations based on a patient's breed or lineage. Owners and breeders benefit from these genetic discoveries, as they are not only offered more comprehensive veterinary care, but are also given the tools to test their horses for disease status in order to avoid breeding affected animals. Some inherited diseases in equines are fatal, so preventing transmission is highly critical for animal welfare as well as the cost of breeding and pregnancy. In Chapter 1 of this thesis, we report the causative genetic mutation for Equine Juvenile Spinocerebellar Ataxia (EJSCA), a novel, fatal autosomal recessive disease in young American Quarter Horse (AQH) foals. This study includes the functional validation of this variant and EJSCA allele and carrier frequencies. This new genetic test adds to the six genetic tests already available in this breed.

Allele frequency studies allow researchers to estimate the genetic disease alleles within populations, which is important to monitor over time and especially when novel mutations are discovered. These studies give the veterinary community, breeders, and breed associations a clear picture of the risk an inherited disease poses because they highlight the temporal response to genetic test availability, any potential gaps in community education, and the prevalence of often devastating novel diseases. This allows breeders to make informed decisions about potential pairings and helps prevent unnecessary suffering. The persistence of disease alleles is harmful to breed longevity, since the greater the number of individuals with a

disease variant, the more likely that allele will be passed onto offspring. In Chapter 2 of this thesis, we report the allele, carrier, and affected frequencies of seven critical diseases within the AQH breed, including EJSCA, in a randomly selected cohort and seven top performing cohorts of AQHs registered with the American Quarter Horse Association.

Chapter 1: An intronic variant in *Ferredoxin Reductase (FDXR)* creates a cryptic exon in Quarter Horses with Equine Juvenile Spinocerebellar Ataxia

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Abstract

Equine Juvenile Spinocerebellar Ataxia (EJSCA) is a novel autosomal recessive neurologic disease in Quarter Horses. Affected foals display a progressive proprioceptive ataxia by 1-5 weeks of age, leading to recumbency and necessitating humane euthanasia. Whole genome sequencing was performed on 7 EJSCA cases, 4 obligate carriers, 4 unaffected half or full-siblings, and 28 unrelated, unaffected Quarter Horses. An 82 kb region of association was identified (EquCab3.0, chr11: 6963986-7045999), containing 9 candidate SNPs across three genes (*FDXR, GRIN2C* and *TMEM104*). Decreased *FDXR* mRNA expression and a cryptic exon 2 was identified in spinal cord tissue from EJSCA cases via RNA-sequencing. One of the 9 associated SNPs (*FDXR-203* c.177+1778G>C) was the eighth base pair of this cryptic exon. Affected foals were homozygous for the variant. Protein concentrations of FDXR were lower in EJSCA cases in spinal cord and liver. The *FDXR-203* c.177+1778G>C mutation represents the first non-coding disease-causing genetic variant in Quarter Horses. Additionally, this is the first genetic cause of a degenerative axonopathy in the horse and a spontaneous disease model to study FDXR pathology in humans.

Author Summary

The American Quarter Horse is the most popular horse breed in North America. A rapidly progressive spinocerebellar ataxia, termed equine juvenile spinocerebellar ataxia (EJSCA), was first identified in Quarter Horse foals in 2020. EJSCA was inherited as an autosomal recessive trait. Whole-genome sequencing on 7 ESJCA cases and RNA-sequencing of spinal cord identified an intronic variant in *FDXR* creating a cryptic third exon in affected foals. Four additional affected foals were subsequently genotyped and were all homozygous for the intronic variant. Decreased transcript and protein expression of FDXR was observed in spinal cord and liver. The *FDXR-203* c.177+1778G>C mutation represents the first disease-causing genetic variant for an inherited degenerative axonopathy in the horse and a spontaneous disease model to study FDXR pathology in humans.

Introduction

In 2020, the first identified case of equine juvenile spinocerebellar ataxia (EJSCA) in the American Quarter Horse (QH) was recognized [1]. Over the next 4 years, an additional eleven cases were identified. All foals were presented between 1-5 weeks of age with an acute history of neurological deficits, with the pelvic limbs more severely affected. The spinal ataxia rapidly progressed to include paraparesis, resulting in recumbency within 0-18 days and necessitating humane euthanasia [1]. Postmortem lesions were confined to histologic evidence of dilated myelin sheaths and digestion chambers throughout the dorsal spinocerebellar tract of entire spinal cord, but most severe in the cervicothoracic region. Some foals had additional lesions

within the fasciculus cuneatus and ventromedial tracts [1]. The pedigree analysis of cases supported an autosomal recessive mode of inheritance, with relatedness tracing aback within 4-6 generations on both sides of the pedigree [1].

Inherited defects that result in a spinocerebellar ataxia have been reported in dogs [2-9], cattle [10-15] and sheep [16-18]. In horses, a distinct spinocerebellar ataxia, termed equine neuroaxonal dystrophy / degenerative myelopathy, has been described but horses develop less severe clinical signs later in life (typically 1-3 years of age) [19]. There is currently no genetic mutation identified for eNAD/EDM and EJSCA is a clinically and histologically distinct disorder [1].

We performed whole-genome sequencing in seven affected QH foals with EJSCA to detect genetic variants associated with the phenotype. We identified an intronic variant, resulting in a cryptic exon in *FDXR* c.177+1778G>C, that significantly associated with the EJSCA phenotype. Decreased FDXR transcript and protein expression supported loss-of-function in the spinal cord and liver of affected foals, which aligned with clinical findings.

Results

Animals

Clinicopathologic findings of foals affected with EJSCA were previously described [1]. Briefly, the study included n=11 confirmed EJSCA foals (n=9 fillies and n=2 colts) that appeared healthy at

birth, but developed an acute onset of neurologic deficits within the first months of life (**S1 Table**). Deficits consisted of a spinocerebellar ataxia of all four limbs, with mild asymmetric paraparesis in some cases. Clinicopathologic findings included elevated gamma-glutamyl transferase (GGT) and hyperglycemia on biochemical profiles, with normal muscle enzymes. All foals rapidly progressed to recumbency and were euthanized. Postmortem evaluation identified dilated myelin sheaths with digestion chambers throughout the entire spinal cord but most severe in the dorsal spinocerebellar tracts of the cervicothoracic region [1]. Control horses were considered healthy if they had lived past six months of age with no evidence of ataxia.

Whole-Genome Association Study

A whole-genome association study was performed to investigate the genetic cause of EJSCA. Significantly associated variants were identified, and their effects predicted using the whole-genome sequence of n=7 EJSCA foals, n=3 carrier dams, n=1 carrier sire, n=3 unaffected half-siblings, n=1 unaffected full-sibling and n=28 unrelated control Quarter Horses (**S1 Table**). Within the filtered .vcf file, 77 variants were associated with EJSCA at $p<1 \times 10^{-11}$, and all were homozygous in EJSCA foals and heterozygous in the carriers. The 77 associated variants were located in an 82 kb region on chr11 from 6963986-7045999Mb (**S2 Table**). To prioritize variants for further investigation, the variants within this region were further filtered by effect ('MODERATE' and 'HIGH') and screened in public databases (https://ncbi.nlm.nih.gov/subs/sra/ and [20]). No 'HIGH' effect nonsense variants were identified. All 'MODERATE' effect variants were found in other breeds with no reported cases of this neurological condition (**S2 Table**). Of the remaining single nucleotide variants (SNVs), only 9 were not identified in any other breed

(**Table 1**). These variants spanned three genes: *Ferredoxin reductase (FDXR*, 1 synonymous and 4 intronic SNVs), *Glutamate ionotropic receptor NMDA type subunit 2C (GRIN2C*, intronic SNV) and *Transmembrane protein 104 (TMEM104* (intronic SNVs) (**Fig. 1**). All genes are expressed in human spinal cord RNA (https://www.proteinatlas.org/). *FDXR* and *GRIN2C* are expressed at the RNA level in equine spinal cord and cerebellum, but *TMEM104* is not [21]. Scores were determined for each orthologous human variant using the 100-vertebrate score by phastCons (https://genome.ucsc.edu/). All conservation scores were 0, except for chr11:6973334, which was 0.05 (**Table 1**).

mRNA-sequencing

To quantify gene expression across the three genes of interest in the candidate region (*FDXR, GRIN2C* and *TMEM104*), mRNA-sequencing on spinal cord tissue was performed on n=5 EJSCA cases and n=6 unaffected age-matched controls (**S1 Table**). There were no genes that were significantly different at a $P_{FDR}<0.05$. However, of the three positional candidate genes (*FDXR*, *GRIN2C* and *TMEM104*), only *FDXR* was significantly decreased in EJSCA foals at $P_{unaadjusted}<0.05$ (p=0.0008) (**Fig. 2A**). Raw .bam files were then visualized for FDXR and a cryptic exon was identified in intron 2, corresponding to the intronic variant identified at chr11:6973334 (G/C) (**Fig. 2B**).

Variant Validation via Sanger Sequencing and Genotyping

DNA from n=11 EJSCA foals, n=12 dams, n=4 sires, n=11 siblings, and n=8 unrelated healthy control horses underwent Sanger sequencing performed to confirm the putative *FDXR* variant

detected from whole genome sequencing data (c.177+1778G>C). All EJSCA foals were homozygous for the variant while all dams and sires were heterozygous. Unrelated control samples were homozygous wildtype (**Fig. 2C**). An additional 1,060 other available presumed unaffected Quarter Horses were randomly selected from the biorepository at the UC Davis Veterinary Genetics Laboratory and genotyped for this variant. From the 1,060 horses, 25 were heterozygous and the remainder of the horses were homozygous wildtype. The allele frequency for the *FDXR* variant was calculated from this sample set for the Quarter Horse population (q=0.012). An additional 181 horses across 26 breeds were screened and no other carriers were identified in other breeds (**S3 Table**).

***FDXR* c.177+1778G>C Functional Validation**

Since the putative variant for EJSCA was deep within an intron, 1778 bp away from exon 2, and the RNA-sequencing data did not clearly define the boundaries of the cryptic exon, we pursued reverse-transcriptase PCR (RT-PCR) in spinal cord, liver and gluteal muscle from EJSCA affected foals and age-matched controls. Primers flanking exons 2 and 3 of *FDXR*-203 (https://useast.ensembl.org/Equus_caballus/Info/Index) amplified two PCR products, one prominent band and one band with less intensity in control horses (~350 bp and ~480 bp, respectively, **Fig. 2D**) and three products were observed in EJSCA foals (~350, 480 and 650 bp, **Fig. 2D**).

Gel extraction of these bands from controls and cases was performed and subsequent Sanger sequencing was repeatedly unsuccessful, even with multiple attempts of nested PCR. Long-read

PromethION Nanopore sequencing of the resulting RT-PCR products from spinal cord tissue was therefore performed in n=2 EJSCA foals and n=4 controls to define these fragments. Reads that were 98% similar to each other, based on global sequence identity, were clustered, and four significant clusters were identified with >10% of at least one sample's total reads in them. These four identified clusters contained 82.5% of all analyzed reads. A consensus sequence was then identified and two clusters (Cluster 24 and 26, both 306 bp on average) demonstrated allele sharing across all six samples (EJSCA and controls) while the two other clusters (Cluster 0, 398 bp and Cluster 144, 541 bp) were unique to EJSCA foals only (**Fig. 3A**, **S4 Table**). Clusters 0 and 144 each contained sequence that aligned over the cryptic exon 2 that had been identified with the RNA-sequencing data (**Fig. 3B**). Cluster 144 also contained a longer 5' end of canonical exon 3 (**Fig. 3B**). Within both Clusters 0 and 144, the G>C substitution was evident at 8 bp (Cluster 0) or 29 bp (Cluster 144) from the start of the reads.

Western blotting of spinal cord and liver demonstrated decreased protein expression in EJSCA foals (**Fig. 4**). Additionally, decreased expression was observed in myocardium and gluteal muscle from EJSCA foals; however, the overall expression in these tissues was lower (**Fig. 4**). Biologic replicates of western blotting from spinal cord is included in **S1 Figure**.

Discussion

There are currently six well-characterized disease variants in the American Quarter horse that are recommended for testing as part of the American Quarter Horse Association Health Panel. These include glycogen branching enzyme deficiency (GBED), hereditary equine regional dermal

asthenia (HERDA), hyperkalemic periodic paralysis (HYPP), malignant hyperthermia (MH), polysaccharide storage myopathy Type 1 (PSSM1) and myosin-heavy chain myopathy (MYHM). Testing for this panel is required by the breed association for stallions before resulting foals are registered. The variants for these six diseases are all coding in nature, including missense (HERDA, HYPP, MH, PSSM1, MYHM) and nonsense (GBED) mutations. The *FDXR* c.177+1778G>C mutation identified in this study represents the first intronic variant that results in a severe fatal neurologic disease in Quarter Horses. Additionally, this *FDXR* variant represents the first identified cause for an inherited degenerative axonopathy in the horse and a spontaneous disease model to study FDXR pathology in humans.

In humans, FDXR mutations are associated with mitochondriopathies defined by decreased electron transport chain function, increased reactive oxygen species and iron overload [22]. The first link between FDXR and mitochondrial disease in humans was published in 2017, where recessive mutations in *FDXR* resulted in optic atrophy, ataxia and hypotonia in a cohort of individuals [23]. Functional studies in cells from these patients showed reduced FDXR activity and mitochondrial overload. That same year, eight children with auditory neuropathy and bilateral defect of the optic nerves were identified with recessive *FDXR* mutations [24]. Since then, the disease has been renamed FDXR-related mitochondriopathy (FRM) and symptoms were expanded to include movement disorders and global developmental delay [25]. Over 80% of the variants described to date in humans are missense. Foals with EJSCA did not appear to have visual or auditory deficits, but electroretinography and brainstem auditory-evoked

potential testing was not performed. Optic nerves are not routinely collected during veterinary necropsies, but should be evaluated in any future cases of this disorder

Deep intronic mutations can disrupt normal splicing patterns by creating new splice sites, altering splicing enhancers or silencers or interfering with canonical splice sites. Deleterious DNA variants that are more than 100 bp away from exon-intron junctions commonly lead to pseudo-exon inclusion [26]. With EJSCA, the deep intronic *FDXR* c.177+1778G>C mutation creates a cryptic splice site, which likely activates nonsense-mediated decay, as evidenced by reduced mRNA expression and our fainter RT-PCR bands. The advantage of performing whole-genome sequencing, versus whole exome sequencing, was clearly highlighted in this study, as an exome only approach would have failed to detect this variant . Pairing our whole-genome results with reads obtained from RNA-sequencing was critical in identifying a putative functional intronic variant in *FDXR*.

While EquCab3.0 has undoubtedly improved the reference assembly for the horse, annotation of specific isoforms still relies primarily on in silico predictions through Ensembl. There are six transcripts of *FDXR* annotated in Ensembl (FDXR-201,202,203,204,205 and 206), with FDXR-203 labeled as the “canonical” isoform. Using the NCBI annotation, which relies more on RNA-seq data to identify coding regions, there are five transcripts (XM_023652066.1 or variant X4, XM_023652067.1 or variant X5, XM_023652064.1 or variant X1, XM_023652065.1 or variant X2 and XM_001492615.6 or variant X2). Using the Ensembl annotation, the main variation

between isoforms occurs in the 5' UTR region and 3' to exon 3, with alternative splicing annotated between exons 3 and 4, 4 and 5 and after exon 10. In NCBI, the variation is 3' to exon 10, with alternative splicing denoted between exons 11, 12 and 13. Notably, none of these isoforms have coding sequence predicted between canonical exons 2 and 3 and none of these isoforms support a longer 5' end to exon 3 that was noted in our EJSCA foals (as shown by PromethION long-read sequencing results). Thus, this disease is associated with a novel splice variant of *FDXR*.

Since we were unable to amplify cDNA from the region between canonical exons 2 and 3 of *FDXR* using standard nested PCR and Sanger sequencing techniques, we elected to leverage cDNA Nanopore long-read sequencing technology to sequence each particular fragment. Major isoforms of genes have been readily identified in human cell lines using Nanopore long-read sequencing protocols [27]. Gene expression estimates from Nanopore long-read RNA-seq data showed the lowest estimation error overall when compared to various RNA-seq protocols [27]. In our study, this long-read sequencing technology provided detailed insight into the alternative splicing that resulted from the *FDXR-203* c.177+1778G>C variant. This study represents the first use of Nanopore long-read sequencing technologies in the horse to identify novel splice events resulting from intronic variants.

Clinically, foals with EJSCA are presented with a spinocerebellar ataxia and clinicopathologic evidence of liver injury (high GGT concentrations). Muscle enzymes (creatine kinase [CK] and

aspartate aminotransferase [AST]) are within normal limits on these foals. This clinical presentation is reflected in the tissue-specific expression that we identified for FDXR. Expression was highest, at both the RNA and protein level, in spinal cord and liver and lower in gluteal muscle and myocardium. In the human protein atlas (https://www.proteinatlas.org), FDXR expression is noted to be highest in adrenal gland (RNA and protein levels), testis and epididymis (protein only) and fallopian tube and ovary (protein only). Within the nervous system, expression is quantifiable, but much lower, in human cerebellum and spinal cord at the RNA level only. We were able to leverage the functional atlas of animal genomes (FAANG) dataset that was created in the horse to evaluate equine-specific tissue patterns of gene expression and regulation (https://www.ebi.ac.uk/ena/data/view/ERA1487553) [21]. *FDXR* was expressed at the RNA level in equine spinal cord and cerebellum, with no functional elements (promoters, enhancers, silencers) predicted at the site of the EJSCA variant (*FDXR-203* c.177+1778G>C). Since *FDXR* expression was identified in equine spinal cord and cerebellum, the FAANG dataset provided species-specific gene expression information that was advantageous to this investigation.

It is worthwhile to note that, of the 9 putative variants (**Table 1**), the *FDXR-203* c.177+1778G>C variant was the only one that did not have a conservation score of 0. While variants that disrupt canonical splice site elements often adhered to a highly conserved consensus sequence, de novo mutations that create cryptic exons do not [28]. Thus, while using conservation scores to prioritize putative variants remains an important tool, variants should not be excluded as candidates simply due to low conservation scores.

In summary, this study identified a putative variant associated with EJSCA in the American Quarter Horse (*FDXR-203* c.177+1778G>C). This *FDXR* variant represents the first to explain an inherited degenerative axonopathy in the horse and a spontaneous disease model to study FDXR pathology in humans.

Materials and Methods

Animals

All procedures were approved by the University of California-Davis Institutional Animal Care and Use Committee (#23015) and carried out in accordance with guidelines and regulations. Written consent from the owners was obtained for all sample collections. Clinicopathologic findings of affected foals were previously described [1]. The study included n=11 confirmed EJSCA foals (n=9 fillies and n=2 colts) that appeared healthy at birth, but developed severe neurologic deficits within the first months of life (**S1 Table**). A total of n=7 EJSCA affected foals underwent whole-genome sequencing (**S1 Table**). For RNA-sequencing, spinal cord tissue from n=5 affected EJSCA foals was profiled (all fillies, median age 30 days, range 14-30 days, **S1 Table**). For RT-PCR of spinal cord, liver and gluteal muscle, a subset of n=3 affected EJSCA foals were used (**S1 Table**). For Western blot analysis, spinal cord, liver, and gluteal muscle from n=3 affected foals, and cardiac muscle from n=1 affected foal, were used (**S1 Table**).

Control horses. For the genetic investigation, blood samples were provided on all of the affected foals' dams, of which n=3 were used for whole-genome sequencing, along with n=1 sire, n=3 unaffected half-siblings and n=1 unaffected full-sibling (**S1 Table**). Siblings had lived past six months of age with no evidence of ataxia. Publicly available whole-genome sequences from unaffected unrelated Quarter Horses (https://www.ebi.ac.uk/ena/data/view, SUB11328561, SUB13501748 and SUB15353402) was leveraged as an additional set of control samples. For RNA-sequencing, spinal cord tissue from n=5 unaffected age-matched foals was profiled (2 fillies, 3 colts, median age 180 days, range 21-2190 days, **S1 Table**). For RT-PCR of spinal cord and gluteal muscle, a subset of n=3 control foals were used. For RT-PCR of liver, a subset of n=2 control foals were used. For Western blot analysis, spinal cord and gluteal muscle from n=3 control foals, liver from n=2 control foals and myocardium from n=1 control foal were used (**S1 Table**).

Blood DNA Extraction

Genomic DNA was isolated from whole blood samples according to the WIZARD Blood DNA Extraction Kit protocol (Promega, Madison, WI). Sample DNA concentrations were measured using QIAxpert (QIAGEN, Hilden, Germany) and diluted to 20 ng/µl.

Whole-Genome Sequencing

Genomic DNA from the 15 horses were sequenced on the Illumina HiSeq4500 at approximately 30X coverage. Whole genome sequences were deposited in the NCBI Sequence Read Archive (https://ncbi.nlm.nih.gov/subs/sra/). For publicly available files, Fastq files were obtained from

the SRA archive (https://www.ebi.ac.uk/ena/data/view, SUB11328561, SUB13501748 and SUB15353402) and trimmed for quality. Reads were mapped to the EquCab3.0 equine reference sequence[29] using BWA for Illumina mapping program [30]. Mapping quality was assessed using Samtools Flagstat [31]. SNP, INDEL discovery, and genotyping across all samples was performed for the discovery of a variant using Genome Analysis Toolkit (GATK, v4.2) [32] Variant calling was performed using GATK's HaplotypeCaller to generate genomic variant call format (gVCF) files for each sample. Joint genotyping was conducted with GenotypeGVCFs to create a multi-sample VCF. The resulting VCF was filtered using SNPSift [33] to filter the resulting .vcf file by quality, using a variant Phred threshold of 30 ($Q \geq 30$).

A whole-genome association study was performed using n=7 EJSCA foals, n=4 parents (coded as unknown) and n=32 control Quarter Horses. Case/control status was assigned using SNPSift caseControl [34], followed by filtering based on an allelic *P* value of 1×10^{-11}. In addition to assessing significantly associated variants called with FreeBayes, raw bam files were visually inspected using Integrative Genome Viewer [35] in the candidate regions for any structural variants, including duplications, inversions, and large deletions or insertions. SNPEff was then used to predict the effect on protein function of the alternate variants, classifying variants as having 'HIGH', 'MODERATE', 'LOW', or 'MODIFIER' putative effects [34]. Chromosomal distribution of the resulting significantly associated variants ($P<1 \times 10^{-11}$) was assessed. Variants with predicted 'HIGH' or 'MODERATE' effects were then screened in the publicly available database of equine whole-genome sequences using NCBI's Sequence Read Archive (SRA)

database (https://www.ncbi.nlm.nih.gov/sra) and excluded if found in breeds other than the Quarter Horse.

mRNA-sequencing

To quantify gene expression across the three genes of interest in the candidate region (*FDXR, GRIN2C* and *TMEM104*), mRNA-sequencing on spinal cord tissue was performed on n=5 EJSCA cases and n=6 unaffected age-matched controls (**S1 Table**). RNA was isolated from spinal cord tissue as previously described [21] and RNA integrity scores (RIN) assessed using the BioAnalyzer High Sensitivity RNA Assay. RIN scores below 7.0 were considered to have failed quality control. Libraries were prepared using PolyA selection with the NEBNext Ultra II Directional Prep Kit. The resulting libraries were sequenced on Illumina, with 150 base pair paired end reads at a depth of 40 million reads per sample. Adapter trimming, poly A trimming, quality trimming, length filtering, and removal of PCR duplicates were conducted using HTStream, version 1.3.3 [36]. The resulting reads were aligned to EquCab.3.0 using STAR, version 2.7.10b [37]. Differential expression analyses were conducted using the limma-voom Bioconductor pipeline (limma version, edgeR version, in R version 4.2.2 (2022-10-31) [38]), using a model with effects for disease status, sex, and age. Low expressed genes were filtered prior to analysis using the function filterByExpr in edgeR, leaving 16,449 genes. Raw bam files of *FDXR* were visually inspected using Integrative Genome Viewer [35]

Sanger Sequencing Validation and Genotyping

To confirm the putative variants identified on whole-genome sequencing, primers were designed using Primer3Plus software [39] and DNA oligonucleotides synthesized by Integrated DNA Technologies (F 5'TGCATCCTCAAGACGACCTG3', R 5'TTCCACCCTCATGCATTCCC3'). Amplification of products was performed using end-point PCR. Products were loaded with Invitrogen™ TrackIt™ Cyan/Orange Loading Buffer and electrophoresis was performed using a 1.5% agarose gel stained with SYBR™ Safe DNA Gel Stain (Invitrogen™; s33102). Fragments were visualized using the Azure™ c150 Gel Imaging Workstation. PCR was performed using the Invitrogen™ Platinum™ *Taq* DNA Polymerase kit (10966). Each 25-µl PCR reaction comprised 0.1 ul of Platinum™ *Taq* DNA Polymerase, 2.5 µl of 10x PCR Buffer -Mg, 0.75 ul of 5 mM $MgCl_2$, 0.5 ul of 10 mM dNTPs, 0.25 ul of 20 uM forward and reverse primers (Integrated DNA Technologies), 1 ul KB Extender, and 1 µl of 50 ng/ul genomic DNA. Standard PCR conditions were performed as follows: 94 °C for 2 min and 35 cycles of 94 °C denaturation for 30 s, 57 °C annealing for 30 s, and 72 °C extension for 1 min. Sanger sequencing was performed using the commercially available services provided by the UC Berkeley DNA Sequencing Facility (https://ucberkeleydnasequencing.com/home). Resulting sequences were aligned to EquCab3.0 (http://www.ncbi.nlm.nih.gov/genome/145) and analyzed with SEQUENCER® software (Gene Codes Corp.). The n=11 affected foals, n=12 dams, n=4 sires, n=11 siblings, and n=8 unrelated control horses were genotyped (**S1 Table)**. To estimate the Quarter Horse population allele frequency and test additional animals for the presence of this variant, 1,060 presumed unaffected Quarter Horses with samples banked at the UC Davis Veterinary Genetics Laboratory (VGL) were genotyped for the *FDXR* intronic variant. Samples were randomly

selected from those with consent to use for research purposes and individuals with known first degree relationships were removed. Genotyping was performed using the assay developed and now commercially available at the VGL (https://vgl.ucdavis.edu/test/equine-juvenile-spinocerebellar-ataxia-ejsca). The allele frequency for the *FDXR* variant was calculated as:

$$f_D = (H_{D/N} + 2H_{D/D}) / 2n$$

$$SE_{fD} = \sqrt{((f_D * (1 - f_D)) / 2n)}$$

Where:

f_D represents the frequency of the disease allele

SE_{fD} represents the standard error of f_D

$H_{D/N}$ represents the number of horses with one disease allele (heterozygous)

$H_{D/D}$ represents the number of horses with two disease alleles (homozygous)

n represents tested population number

An additional 181 horses across 26 breeds were screened and no other carriers were identified in other breeds (**S3 Table**).

FDXR conservation and expression

Scores were determined for each orthologous human variant using the 100-vertebrate score by phastCons (https://genome.ucsc.edu/) since conservation scores are not available in the EquCab3.0 genome browser within UCSC (https://genome.ucsc.edu/). *FDXR* expression was evaluated in tissues available from the equine FAANG initiative (https://www.ebi.ac.uk/ena/data/view/ERA1487553) [21].

FDXR c.177+1778G>C Functional Validation

RT-PCR

To define the boundaries of the cryptic exon identified in *FDXR*, RT-PCR was performed on spinal cord, liver and gluteal muscle of n=3 EJSCA foals and n=3 controls (**S1 Table**). Primers were designed to anneal to exon 2 and exon 5 of *FDXR*-203, as annotated in the Ensembl database for EquCab3.0 (https://useast.ensembl.org/Equus_caballus/Info/Index). Primer sequences were F'CTTCTACACGGCCCAACACC 3', R 5' GAGCCCATTGTACCAGCCC 3'. Gel extraction and Sanger sequencing of the resulting bands was unsuccessful. Therefore, long-read sequencing was performed on the resulting bands from EJSCA and control horses.

PromethION Nanopore Long-read Sequencing

Long-read PromethION Nanopore sequencing of the resulting RT-PCR products from spinal cord tissue was performed in n=2 EJSCA foals and n=4 controls (**S1 Table**). Barcoding PCR was performed using the PBC001 kit, barcoded samples were pooled and the sequencing library was prepared using LSK114 kit. The libraries were sequenced on R10.4.1 PromethION flow cell. Data was base called in the super accurate mode using dorado v4.3.0, 400 bps. The yield was 12M reads with more than 1M reads per barcode. Only data >Q10 was used for downstream analysis. For analysis, reads were randomly subsampled at 100,000 reads per sample and primer sequences trimmed. Samples were then merged into a single file and the clustering algorithm CD-HIT [40] was used to cluster reads that were 98% similar to each other based on global sequence identify. Significant clusters were defined as those that had >10% of at least

one sample's total reads in them. With these four identified clusters, that contained 82.5% of all analyzed reads, 25 sequences from each cluster were again randomly subsampled and ClustalW [41] was used to identify a consensus sequence using default parameters. Sequences were aligned to EquCab3.0 using BLAST.

Western Blotting

For total protein extraction, 10-20 mg of gluteal muscle, liver, and heart and 30-40 mg of spinal cord was used. Tissues were transferred to 1.5 mL microcentrifuge tubes on ice, 600 uL RIPA lysis buffer was added, and tissues were homogenized with plastic pestles. Contents were agitated on a plate shaker for 2 h at 4°C, centrifuged at 13,000 x g for 20 minutes at 4°C, and supernatant was collected. Isolated protein sample concentrations were quantified using the Pierce™ BCA Protein Assay Kit (Thermo Scientific™; Cat no. 23225) and samples were stored at -80°C. All protein concentrations were diluted to 1 ug/1 ul with 1x Tris Glycine SDS and 15 ul lysate samples mixed with 7.13 ul 4x Laemmli sample buffer (Bio-Rad; Cat no. 1610747) and 0.75 ul 2-Mercaptoethanol (Bio-Rad; Cat no. 1610710). Samples were heated for 5 minutes at 90°C. SDS-PAGE and blotting was performed using the Bio-Rad Mini-PROTEAN Tetra cell, 10% Mini-PROTEAN® TGX™ Precast Protein Gels, 10-well, 30 µl (Cat no. 4561033), and Bio-Rad Nitrocellulose Membranes, Precut, 0.2 µm, 7 x 8.4 cm (Cat no.16201460). 15 ug total protein was loaded for each sample.

To detect FDXR protein, a polyclonal rabbit IgG primary antibody (Novus Biologicals NBP2-38706) was used at a dilution ratio of 1/10000 and a goat anti-rabbit HRP-linked secondary

antibody (Cell Signaling Technology 7074P2) was used at a dilution ratio of 1/10000. To detect actin, a mouse monoclonal IgG1 κ beta Actin (C4) primary antibody (Santa Cruz Biotechnology sc-47778) was used at a dilution ratio of 1/10000 and a goat anti-mouse IgG (H+L) cross-adsorbed HRP-linked secondary antibody (Invitrogen A16072) was used at a dilution ratio of 1/5000. Imaging was performed using the Bio-Techne ProteinSimple FluorChem E System according to the manufacturer's instructions.

Data Access

The whole-genome sequence data generated in this study have been submitted to the NCBI BioProject database (https://www.ncbi.nlm.nih.gov/bioproject/).

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Author Contributions

BNB was responsible for methodology, investigation, formal analysis, and writing of the manuscript. CJF was responsible for the conceptualization, funding acquisition, methodology, investigation, formal analysis, resources and writing of the manuscript. RG and RRB contributed

to methodology, genotyping analysis, and funding related to testing the identified variant in QH and other breeds. All authors have contributed to and/or reviewed the final manuscript.

Competing Interests

A portion of the revenue generated by UC Davis Veterinary Genetics Lab (UCD-VGL) for tests discovered in the Finno Laboratory (MYHM and EJSCA) provides funding for additional research conducted in the Finno Laboratory.

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W.S.S.G. owns shares in Oxford Nanopore Technologies. The other authors declare no competing interests.

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Figures

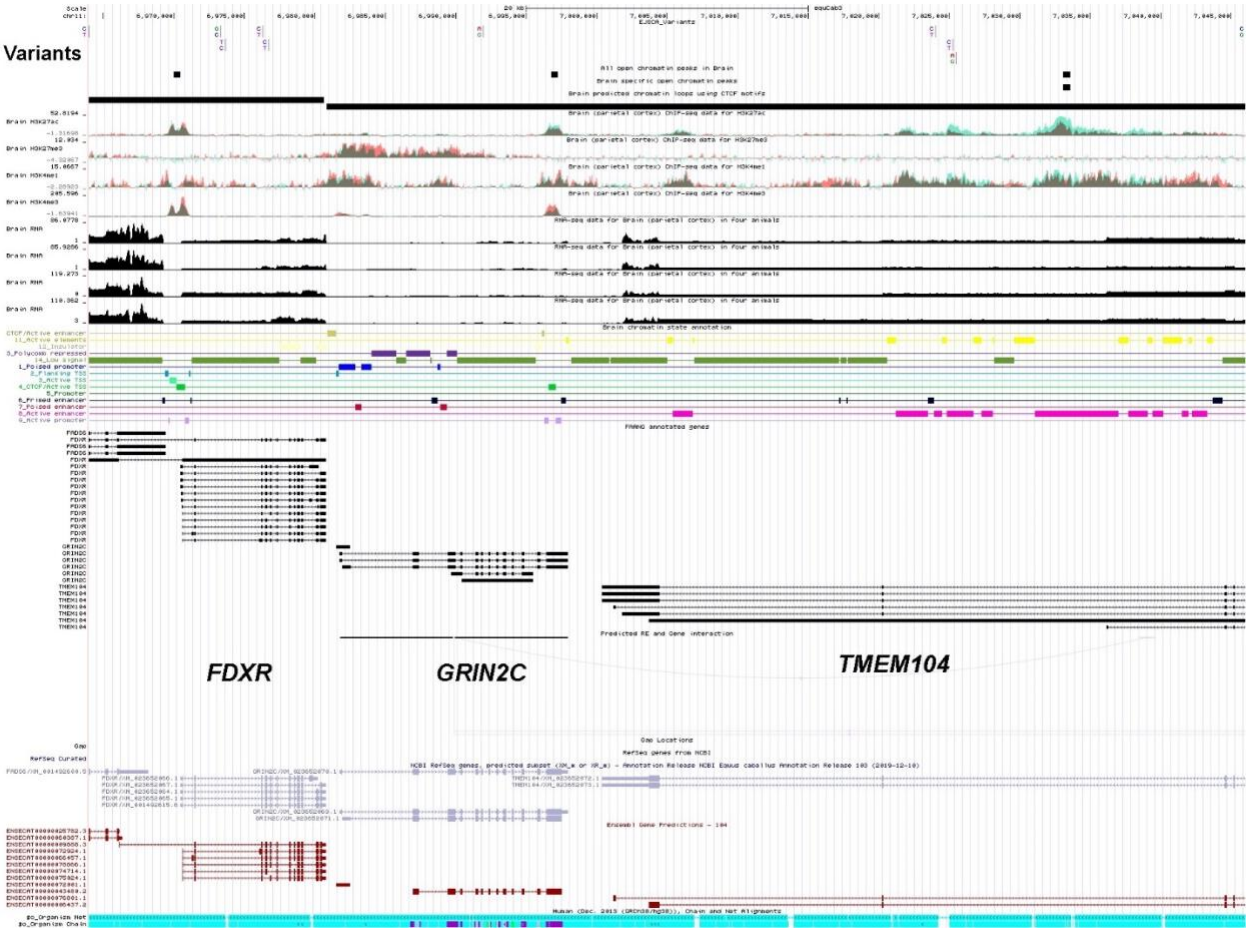


Figure 1—Whole genome association study identified nine homozygous SNPs across the genes *FDXR*, *GRIN2C*, and *TMEM104* that were only present in EJSCA foals. Whole genome association testing of 7 EJSCA cases, 4 obligate carriers, 3 unaffected half-siblings, 1 unaffected full-sibling, 28 unrelated QHs identified an 82 kb candidate region of interest ($p=2.9 \times 10^{-12}$) that was homozygous in all affected foals. After filtering by effect ('MODERATE' and 'HIGH') and screening in public databases (https://ncbi.nlm.nih.gov/subs/sra/ and [20]), nine homozygous SNPs across the genes *Ferredoxin reductase (FDXR*, 1 synonymous and 4 intronic SNVs), *Glutamate ionotropic receptor NMDA type subunit 2C (GRIN2C*, intronic SNV) and *Transmembrane protein 104 (TMEM104* (intronic SNVs) were only present in EJSCA foals.

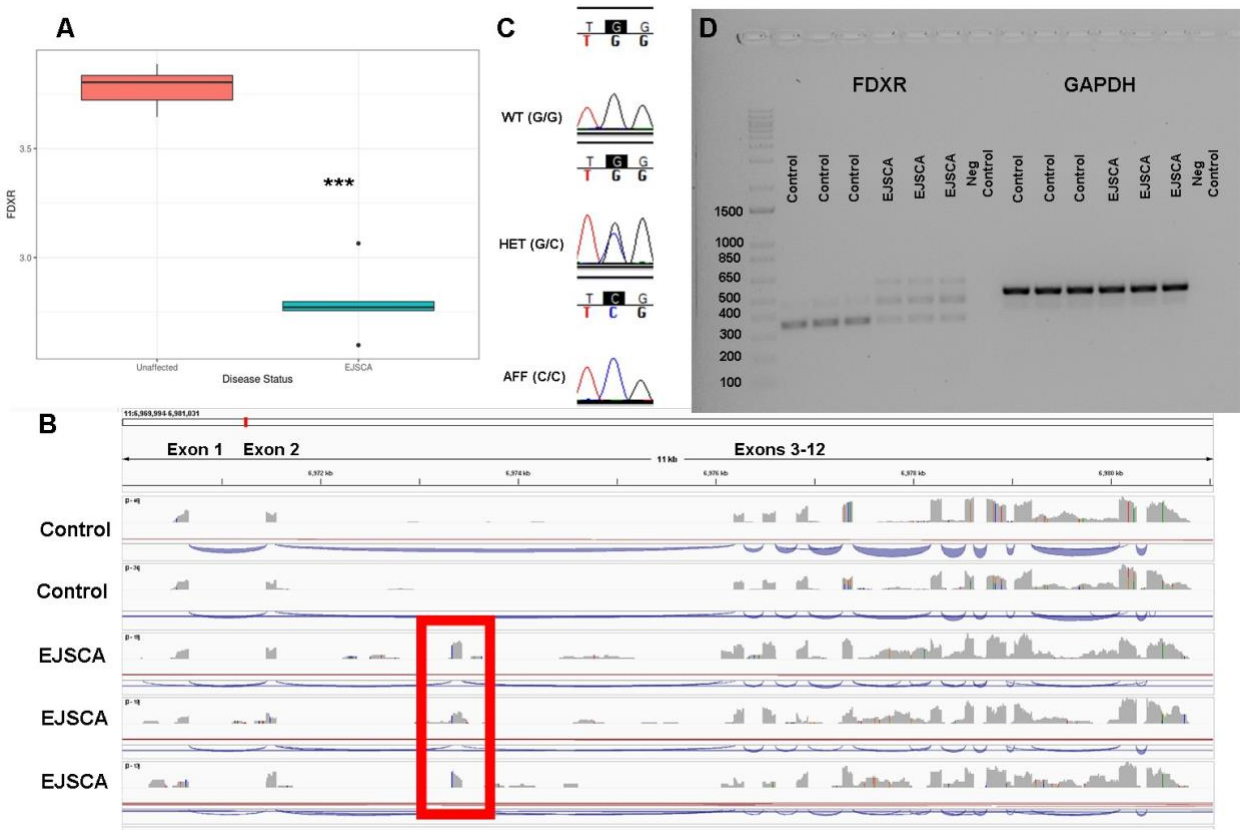


Figure 2—**FDXR investigation and functional validation**. **(A)** Bulk mRNA sequencing of spinal cord tissue performed on n=5 EJSCA cases and n=6 unaffected age-matched controls identified significantly decreased *FDXR* expression in EJSCA foals at $P_{unaadjusted}$<0.05 (p=0.0008). **(B)** Raw mRNA .bam files over *FDXR* show a cryptic exon (indicated in red box) in intron 2 in EJSCA foals, corresponding to one of the *FDXR* deep intronic variants identified in whole genome association study (chr11:6973334, G/C). **(C)** Sanger sequencing of n=11 EJSCA foals, n=12 dams, n=4 sires, n=11 siblings, and n=8 unrelated healthy control horses confirmed perfect segregation of the c.177+1778G>C variant. All EJSCA foals were homozygous C/C, all dams and sires were heterozygous G/C, and all unrelated horses were wildtype G/G. **(D)** RT-PCR of *FDXR* exons 2-5 shows three faint amplicons in EJSCA foals and two amplicons (one bold, one faint) in unrelated control horses.

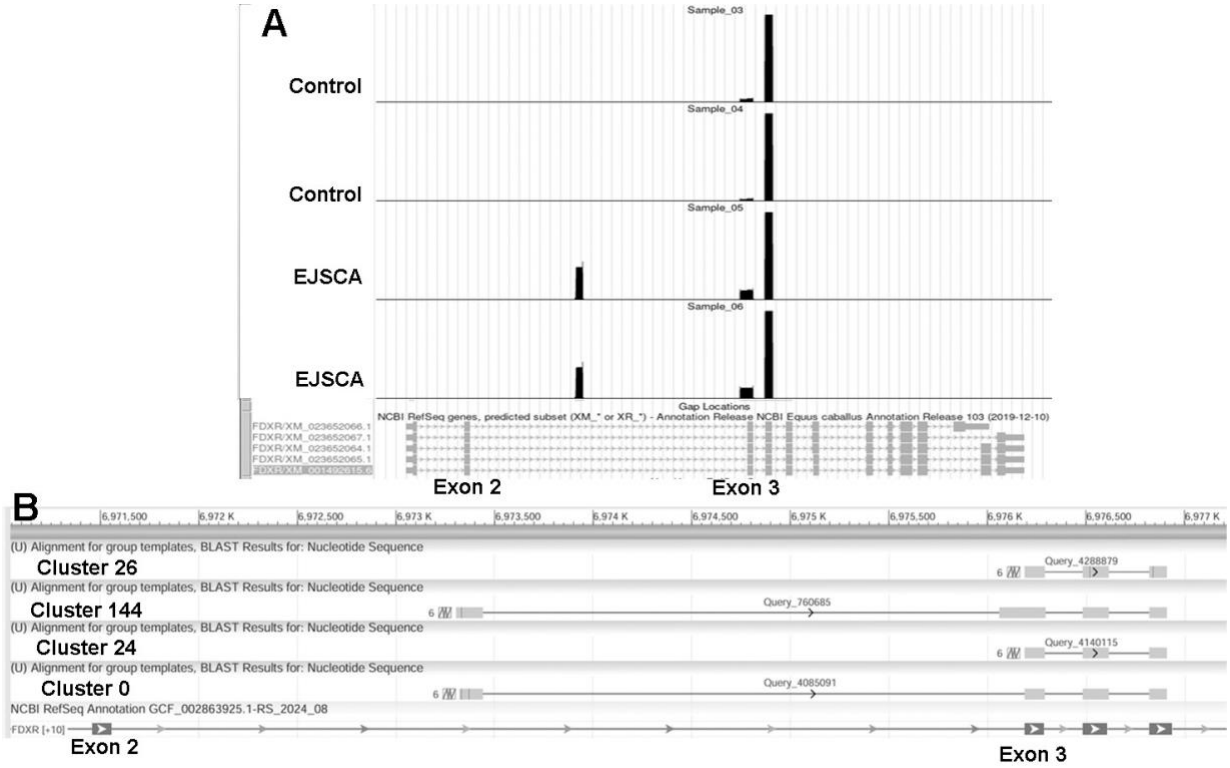


Figure 3—**Long-read PromethION Nanopore sequencing of *FDXR* cDNA amplicons from spinal cord tissue in n=2 EJSCA foals and n=4 control horses**. **(A)** Intronic *FDXR* reads were identified intron 2 only in EJSCA samples, consistent with cryptic exon localization. **(B)** Four significant read clusters were identified. Cluster 24 and 26, both ~306 bp, are present in both EJSCA and control samples. The two other clusters (Cluster 0, 398 bp and Cluster 144, 541 bp) were unique to EJSCA foals only. Clusters 0 and 144 each contained sequence that aligned over the cryptic exon 2.

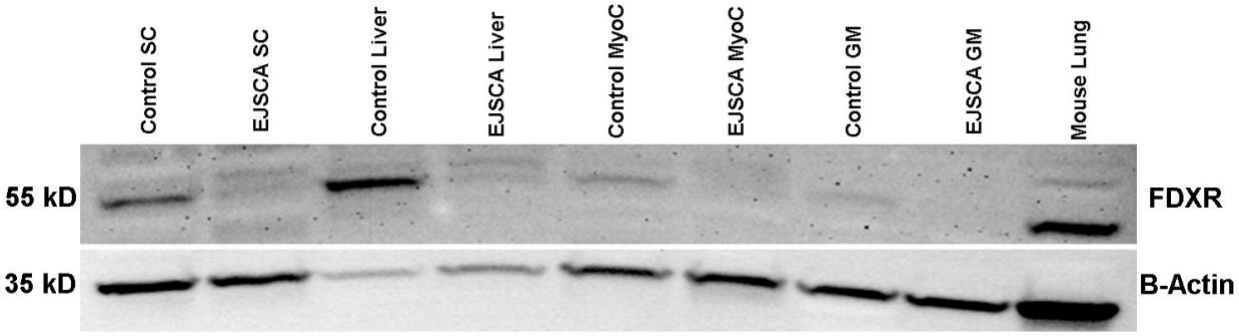


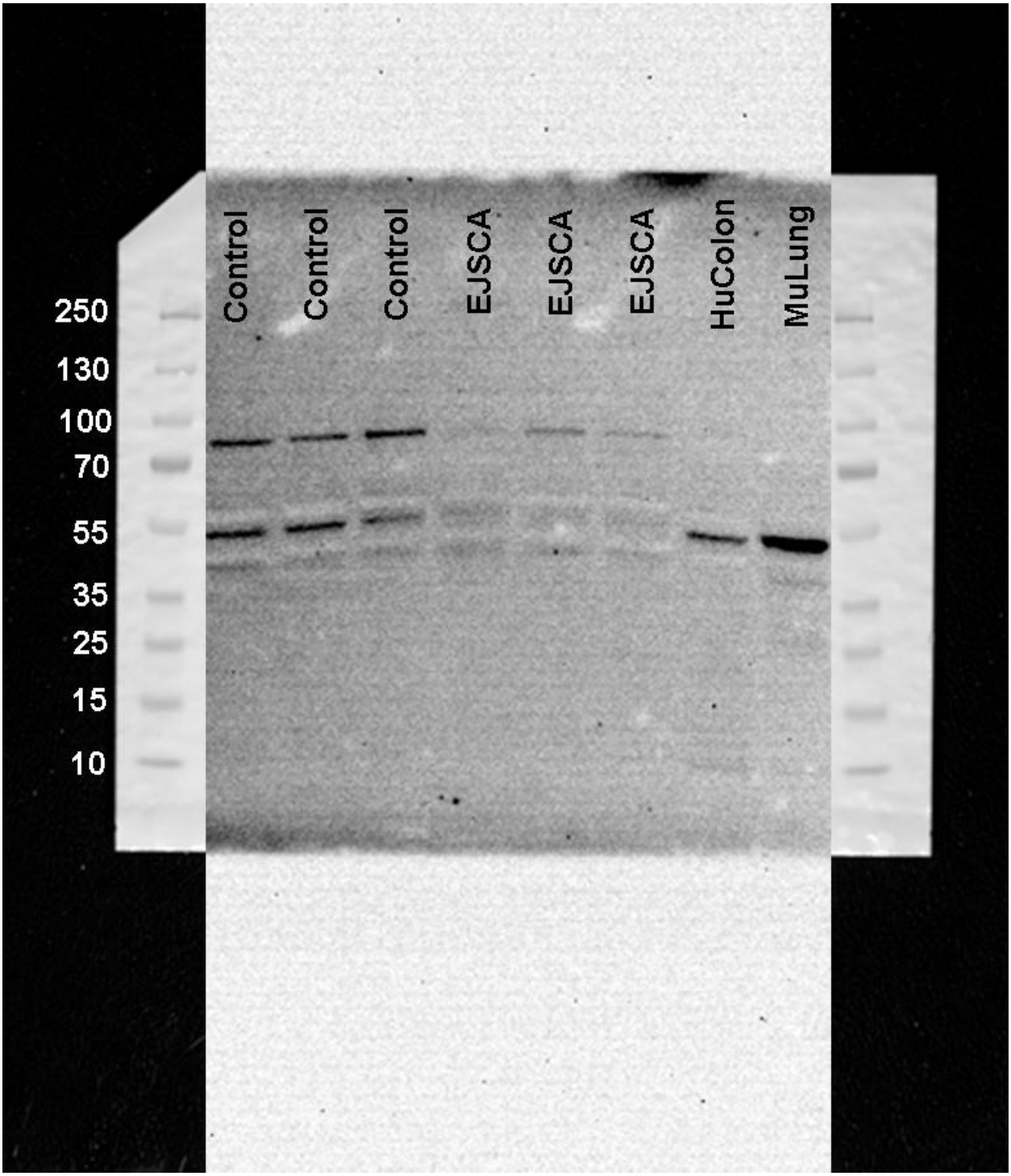
Figure 4—Western blot of protein from spinal cord, liver, myocardium, and gluteal muscle in an EJSCA foal and age-matched unrelated control. FDXR protein expression is decreased in all EJSCA foal tissues but is particularly evident in spinal cord and liver. Overall expression is decreased in myocardium and gluteal muscle.

Tables

Table 1: Nine putative genetic variants for EJSCA based on whole-genome association study. CHROM=chromosome, POS=position, REF=reference allele, ALT=alternate allele, QH=Quarter Horse. POS, REF, and ALT are according to the Equcab3.0 assembly[29]

CHROM	POS (	REF	ALT	SnpEff Putative Effect	$P_{allelic}$
11	6963986	C	T	*FDXR* synonymous (FDXR c.579C>T, p.Asn193Asn)	2.99×10^{-12}
11	6973334	G	C	*FDXR* intronic	2.99×10^{-12}
11	6973640	T	C	*FDXR* intronic	2.99×10^{-12}
11	6976294	C	T	*FDXR* intronic	2.99×10^{-12}
11	6976720	C	T	*FDXR* intronic	2.99×10^{-12}
11	6991960	A	G	*GRIN2C* intronic	2.99×10^{-12}
11	7024027	C	T	*TMEM104* intronic	2.99×10^{-12}
11	7025248	C	T	*TMEM104* intronic	2.99×10^{-12}
11	7045999	C	G	*TMEM104* intronic	2.99×10^{-12}

Supplemental Figures



Supplemental Figure 1— Western blotting of FDXR in liver from additional cases and controls.

Supplemental Tables

Supplemental Table 1— Overall demographics of horses used for the whole-genome sequencing and subsequent functional validation. SC=spinal cord, GM=gluteal muscle, LIV=liver, HRT=myocardium.

Horse ID	Age at Sampling	Sex	Breed	Diagnosis	Whole-genome sequencing	RNA-sequencing	Sanger sequencing	RT-PCR	PromethION Nanopore Sequencing	Western Blot
113	180 d	M	QH	Control; unrelated			x	x	x	
208	1 y	F	QH	Control; unrelated	x					
226	1 y	F	QH	Control; unrelated	x					
235	1 y	F	QH	Control; unrelated	x					
241	2 y	F	QH	Control; unrelated	x					
263	180 d	F	Percheron	Control; unrelated		x	x	x	x	x (SC, GM, LIV, HRT)
269	21 d	M	QH	Control; unrelated		x	x	x (SC and GM only)		x (SC, GM)
415	7 y	MC	TB/WB	Control; unrelated			x		x	
416	8 y	MC	QH	Control; unrelated	x					
417	8 y	MC	QH	Control; unrelated	x					
504	210 d	F	QH	Control; unrelated		x	x			
558	11 y	F	QH	Control; unrelated	x					
603	11 y	F	QH	Control; unrelated	x					
604	14 y	F	QH	Control; unrelated	x					
606	15 y	MC	QH	Control; unrelated	x					
607	14 y	F	QH	Control; unrelated	x					
608	15 y	F	QH	Control; unrelated	x					
609	8 y	MC	QH	Control; unrelated	x					
610	13 y	F	QH	Control; unrelated	x					

611	14 y	F	QH	Control; unrelated	x					
722	12 y	MC	QH	Control; unrelated	x					
723	8 y	MC	QH	Control; unrelated	x					
850	10 y	F	QH	Control; unrelated	x					
851	12 y	F	QH	Control; unrelated	x					
853	13 y	F	QH	Control; unrelated	x					
854	11 y	F	QH	Control; unrelated	x					
113	180 d	M	QH	Control; unrelated		x				
1170	13 y	FS	QH	Control; unrelated	x					
1196	10 y	F	QH	Control; unrelated	x					
1198	12 y	F	QH	Control; unrelated	x					
1200	15 y	F	QH	Control; unrelated	x					
1212	13 y	F	QH	Control; unrelated	x					
1219	180 d	M	QH	Control; unrelated	x					
1307	14 d	F	QH	EJSCA	x	x	x			
1325	13 y	F	QH	Control; dam	x		x			
1326	7 y	M	QH	Control; sire	x		x			
1327	16 y	F	QH	Control; dam			x			
1329	14 y	F	QH	Control; dam	x		x			
1330	14 d	F	QH	EJSCA	x		x			
1331	15 y	F	QH	Control; dam	x		x			
1332	21 d	F	QH	EJSCA	x	x	x		x	
1353	10 y	MC	TB	Control; unrelated			x			
1447	14 d	F	QH	EJSCA	x		x			
1448	5 y	F	QH	Control; dam			x			
1449	5 y	M	QH	Control; sire			x			
1450	15 y	F	QH	Control; grand-dam			x			
1451	16 y	M	QH	Control; grand-sire			x			
1454	49 d	M	QH	EJSCA	x		x			

1455	13 y	F	QH	Control; dam			x			
1456	8 y	M	QH	Control; sire			x			
1658	1 y	F	QH	Control; half-sibling	x		x			
1659	305 d	F	QH	Control; half-sibling			x			
1660	1 y	F	QH	Control; half-sibling			x			
1661	305 d	F	QH	Control; half-sibling			x			
1662	1 y	M	QH	Control; half-sibling	x		x			
1663	305 d	M	QH	Control; half-sibling			x			
1664	150 d	M	QH	Control; half-sibling	x		x			
1665	1 y	M	QH	Control; full sibling	x		x			
1673	3 y	F	QH	Control; sibling	x		x			
1684	7 d	F	QH	Control; full-sibling			x			
1705	13 d	M	QH	Control; full-sibling			x			
1706	3 d	M	QH	Control; full-sibling			x			
1747	14 d	F	QH	EJSCA			x			
1749	unknown	F	QH	Control; dam			x			
1750	unknown	F	QH	Control; dam			x			
1751	14 d	F	QH	EJSCA	x		x			
1752	24 y	M	QH	Control; grand-sire			x			
1753	30 d	F	QH	EJSCA	x		x			
1759	8 y	F	QH	Control; dam			x			
1760	5 y	M	QH	Control; sire			x			
1886	5 y	MC	Defense Travels	Control; unrelated				x		x (SC, GM, LIV)

1968	13 y	F	QH	Control; dam			x			
1969	30 d	F	QH	EJSCA		x	x	x	x	x (SC, GM, LIV, HRT)
1983	11 y	F	QH	Control; dam			x			
1984	30 d	F	QH	EJSCA		x	x	x		x (SC, GM, LIV)
1987	6 y	MC	QH/Paint	Control; unrelated		x	x		x	
1989	30 d	F	QH	EJSCA		x	x	x		x (SC, GM, LIV)
1991	7 y	F	QH	Control; dam			x			

Supplemental Table 2—Partial vcf file and public dataset searches of 77 variants associated with EJSCA at at $p<1x10^{-11}$. Uploaded as an attached excel spreadsheet.

Supplemental Table 3— Summary of breeds screened for the EJSCA mutation and corresponding genotype and estimated allele frequencies.

Breed	N/N	N/JSA	JSA/JSA	Total	Allele frequency
Appaloosa	10	0	0	10	0
Appendix	2	0	0	2	0
Belgian	2	0	0	2	0
Brabant	1	0	0	1	0
Carolina Marsh Tacky	1	0	0	1	0
Connemara Pony	2	0	0	2	0
Crossbred	7	0	0	7	0
Draft Cross	3	0	0	3	0
Friesian	1	0	0	1	0
Friesian Sporthorse	3	0	0	3	0
Gypsy Cob	4	0	0	4	0
Gypsy Vanner	3	0	0	3	0
Haflinger	2	0	0	2	0
Half Andalusian	1	0	0	1	0
Half Arabian	1	0	0	1	0
Hanoverian	1	0	0	1	0
Holsteiner	1	0	0	1	0

Imperial Heritage Horse	1	0	0	1	0
Lusitano	2	0	0	2	0
Miniature Horse	1	0	0	1	0
Morgan Horse	8	0	0	8	0
Mustang	1	0	0	1	0
Nokota	1	0	0	1	0
Oldenburg	2	0	0	2	0
Other	1	0	0	1	0
Paint Horse	91	0	0	91	0
Paint Quarter Horse	4	0	0	4	0
Percheron	2	0	0	2	0
Pinto	2	0	0	2	0
Quarter Horse	1035	25	0	1060	**0.012**
Shire	1	0	0	1	0
Tennessee Walking Horse	2	0	0	2	0
Thoroughbred	5	0	0	5	0
Unknown	6	0	0	6	0
Warmblood	6	0	0	6	0
Grand Total	**1216**	**25**	**0**	**1241**	

Supplemental Table 4—Clusters identified by cDNA Nanopore long-read sequencing in control and EJSCA horses.

ClusterID	Avg Read Length	Control1	Control2	Control3	Control4	EJSCA1	EJSCA2	Total
Cluster_0	398	969	933	908	1,053	14,790	31,853	31,854
Cluster_24	306	55,164	54,783	54,868	54,281	33,858	32,487	285,441
Cluster_26	306	14,482	16,751	15,933	16,180	10,062	9,490	82,898
Cluster_144	541	11	3	15	4	4,339	4,270	8,642

Chapter 2: Allele Frequencies of Seven Inherited Disorders in Performance and Random Cohorts of American Quarter Horses (2020–2024)

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Abstract

Objective: To estimate allele frequencies for genetic diseases in the American Quarter Horse (AQH) including Hyperkalemic Periodic Paralysis (HYPP), Type 1 Polysaccharide Storage Myopathy (PSSM1), Malignant Hyperthermia (MH), Myosin-Heavy Chain Myopathy (MYHM), Glycogen Branching Enzyme Deficiency (GBED), Hereditary Equine Regional Dermal Asthenia (HERDA) and Equine Juvenile Spinocerebellar Ataxia (EJSCA) and compare these frequencies among elite performance subgroups and a random cohort of registered horses.

Methods: Prospective genetic survey. DNA was extracted from 300 elite performance AQHs in reining, cutting, barrel racing, halter, racing, western all-around, and English all-around disciplines and 300 randomly selected AQHs born between 2020-2024. Genotyping was performed with the commercially available assays for each disease. Allele, carrier, and affected frequencies were calculated.

Results: Genotyping the top-performing AQHs revealed a high HYPP and PSSM1 frequency in halter horses (0.318 ± 0.050 and 0.386 ± 0.052, respectively). While none of the top performing reining or cutting horses were observed to have the PSSM1 allele, there was a higher GBED frequency in reining horses (0.109 ± 0.032), and a higher HERDA frequency in cutting horses (0.135 ± 0.035), as compared to the other disciplines. Genotyping a population of randomly selected AQHs identified low PSSM1 frequency (0.012 ± 0.004), and that MYHM frequency (0.066 ± 0.010) was significantly higher than any other disease allele in this population.

Conclusions: Disease allele frequencies in AQHs can be highly variable between performance subgroups.

Clinical Relevance: The establishment of allele frequencies allows for longitudinal monitoring to determine how genetic testing results are being utilized.

Keywords: equine, breeding, DNA, genetic, neuromuscular

Introduction

The American Quarter Horse Association (AQHA) requires that stallions be tested for six inherited diseases within the American Quarter Horse (AQH) breed prior to the registration of any foals. The AQH genetic health panel has historically been referred to as the five panel but with recent additional tests now available, it is now known as the AQHA five panel plus. The genetic variants causing disease assayed by this panel are Hyperkalemic Periodic Paralysis (HYPP), Glycogen Branching Enzyme Deficiency (GBED), Hereditary Equine Regional Dermal Asthenia (HERDA), Type 1 Polysaccharide Storage Myopathy (PSSM1), Malignant Hyperthermia (MH), and Myosin-Heavy Chain Myopathy (MYHM). These six diseases directly affect individual animal health, overall population health, and breed sustainability. The dominant diseases (HYPP, PSSM1, MH and MYHM) can sometimes be managed with specialized environmental, dietary, and exercise protocols, but in other cases have been shown to be fatal.[1-22] The autosomal recessive diseases, including GBED and HERDA, cause severe disease.[23-27] The identification of inheritance patterns and causal variants for health traits in horses has made it possible to use routine diagnostic testing to guide veterinary care and breeding practices. However, updated information on discipline specific allele frequencies for these diseases can provide insight into tailored breeding and management practices. Establishing allele frequencies for all known genetic diseases from the same random cohort will allow for continued monitoring and to best educate on the utilization of genetic tools.

HYPP is a muscle disorder caused by a c.4248C>G missense mutation in *SCN4A*.[1–3] Horses with one or two copies of the mutation may experience episodes of muscle tremors, weakness, or

collapse, sometimes resulting in sudden death.[1,4–9] The severity of clinical signs varies, but homozygotes are noted to be more severely affected than heterozygotes.[3,9] PSSM1 is a muscle glycogen storage disorder caused by a mutation in *GYS1*.[10] Horses with one or two copies of the mutation accumulate abnormal glycogen in skeletal muscle, leading to muscle pain, stiffness, and exercise intolerance, however PSSM1 can be managed by implementing dietary changes and regular exercise.[10–15] Homozygotes are generally more severely affected than heterozygotes.[16] MH is caused by a mutation in the *RYR1* gene.[17] Affected horses are susceptible to life-threatening hypermetabolic reactions when exposed to certain anesthetics, succinylcholine, or stress. Clinical signs include muscle rigidity, high body temperature, and arrhythmias.[17,18] The presence of the MH mutation can worsen the clinical course of other muscle diseases like PSSM1.[19] Lastly, MYHM is an autosomal codominant muscle disorder caused by a missense mutation in *MYH1* and can present as immune-mediated myositis with rapid muscle atrophy or as non-exertional rhabdomyolysis with severe muscle pain.[20–22] Environmental triggers such as infection or vaccination can precipitate disease.[20–22] Homozygotes again tend to have more severe clinical presentations.[20,21]

GBED is an autosomal recessive disease that leads to stillbirth, abortion, or early neonatal death.[23] Homozygous foals lack the enzyme needed to properly store glycogen, resulting in severe muscle weakness and vital organ dysfunction.[23] The causal variant for GBED is a c.102C>A nonsense mutation in *GBE1*.[24] HERDA is also an autosomal recessive condition, but this condition impacts the skin and affects young AQHs and related breeds.[25–27] Horses with two copies of the c.115G>A missense mutation in *PPIB* develop hyperextensible, fragile skin

that tears easily and heals poorly, often leading to euthanasia.[25,27] Clinical signs usually appear by age two, including regional swelling, blisters, open wounds, loose or sloughing skin, and easily damaged skin upon trauma, especially when horses begin training under saddle.[25] Heterozygotes (carriers) for both the GBED and HERDA mutations are clinically unaffected and live otherwise normal lives, but can pass the mutation to offspring.[25,27]

While it is not currently included in the AQHA five panel plus test, another severe genetic disease affecting AQHs is Equine Juvenile Spinocerebellar Ataxia (EJSCA).[28] EJSCA is a recently described (2024) autosomal recessive neurologic disorder in young AQH foals.[28] The causal variant for EJSCA is a c.177+1778G>C mutation deep in intron 2 of *FDXR* (Chapter 1). Foals with two copies of the *FDXR* mutation develop progressive hind- and forelimb ataxia between one and five weeks of age, which rapidly progresses to recumbency and necessitates humane euthanasia within days of disease onset.[28] Laboratory findings include a mixed hepatopathy and histopathological analysis reveals characteristic lesions in the spinocerebellar tracts of the cervical spinal cord.[28] Carriers do not show clinical signs but can pass the mutation to offspring, creating a 25% risk of producing an affected foal when two carriers are bred.[28] Genetic testing is now available at the University of California, Davis Veterinary Genetics Laboratory to allow breeders to identify carriers and avoid at-risk matings.[29]

An effective method for estimating the prevalence of genetic diseases is by determining the allele and carrier or affected frequency status of a smaller, representative populations. Allele frequency is the proportion of a particular allele of a gene in a population, such as a disease

allele. Carrier frequency is the proportion of all individuals of a population that are heterozygous for, or carriers of, the disease allele. Carrier frequency is applicable to recessive diseases only, such as GBED, HERDA, and EJSCA, in which certain individuals may not be affected with the disease but are carriers for the disease allele, and therefore have the potential to pass that allele onto offspring. Affected frequency is the proportion of all individuals in a population who have the genotype that results in expression of a genetic disease. Affected frequency is applicable to both autosomal dominant and recessive diseases, but the genotype that qualifies as "affected" differs by inheritance pattern. For autosomal dominant diseases, affected individuals can be heterozygous or homozygous for the disease allele. For autosomal recessive diseases, affected individuals are strictly homozygotes, not carriers.

In 2009, Tryon et al. published a similar allele frequency study involving 851 AQHs born 2001-2004 that estimated the allele, carrier, and affected frequencies of prevalent AQH diseases within the population of samples submitted for parentage, as well as across popular AQH performance and show disciplines.[30] That study demonstrated that the prevalence of certain diseases among AQH disciplines varied widely. Within breeds that have highly specialized disciplines, like the AQH, popular lineages may permeate one discipline. A high-performing, disproportionately popular animal can produce an unusually large number of offspring that will likely compete in the same discipline as their parent(s). An unintended consequence of this is that, if the popular parent is a carrier for disease and passes the disease variant on to offspring who also become popular sires or dams, then disease frequency will rise in each discipline.

Allele frequency studies are important because they allow breeders, researchers, and veterinarians to estimate disease burden and carrier risk in an overall population and determine which disease allele/s are relevant to their decisions for breeding and management. Estimated allele frequencies also allow for monitoring of breed- and discipline-specific risk.

In recent years, disease allele frequencies have also provided breed registries with information necessary to make evidenced based policy decisions and communicate with registry members. Monitoring these estimates over time is necessary, to continue to guide genetic testing recommendations, and identify frequencies for newly discovered disease alleles further imparting guidance to veterinary clients, breeders, and owners. In this study, we provide current estimates on allele, carrier, and affected frequencies for the six described inherited diseases in AQHs, with a specific focus on comparing the overall population to top performers in each discipline. Additionally, we provide the first report of allele and carrier frequencies for EJSCA in AQHs using the same samples and cohorts evaluated for the other known AQH diseases.

Methods

The selected categories of AQH performance disciplines ("subdisciplines") evaluated were reining, cutting, barrel racing (barrels), halter, racing, western all-around, and English all-around. The records of the "top-performing" AQHs, as determined by amount of money earned, for all disciplines except racing were pulled from Q-DATA (https://www.qstallions.com/leaders) and University of California Veterinary Genetics

Laboratory (VGL) accession numbers, when applicable, were provided by the AQHA. Similarly, the records of the top-performing racehorses, also defined by money earned, were pulled from Equibase.

Thirty to fifty samples from each of the top performer's list were pulled from the repository at the VGL and were used to isolate DNA. Inclusion criteria were limited to samples from horses registered with the American Quarter Horse Association and with sufficient follicles banked for DNA extraction.

A randomized list of 2000 AQHA registered horses with dates of birth between 2020-2024 were pulled from the UC Davis VGL database. Samples were filtered for one degree of separation and then organized into lists by sex and data of birth. Each list was randomized by VGL case number to reduce the likelihood of any biases related to "time of sample submission". Hair samples from the first 30 males and the first 30 females on each list with sufficient follicles banked as part of the AQH genetic testing program was utilized. A total of 60 randomly selected samples per year, and 300 randomly selected samples overall were utilized for this study (**Supplemental Table 1**).

DNA was extracted from hair from both cohorts following previously published protocols.[31] Extracted DNA was used to genotype each sample for seven inherited diseases (HYPP, GBED, HERDA, PSSM1, MH, MYHM and EJSCA) using the commercially available assays at the UC Davis VGL (https://vgl.ucdavis.edu/panel/quarter-horse-disease-panel and https://vgl.ucdavis.edu/test/equine-juvenile-spinocerebellar-ataxia-ejsca). Samples were also

excluded from either the top performing or randomly selected cohorts for each disease category if genotyping for that particular allele failed repeatedly. For this reason, there were fewer samples tested than the number of records provided for each subdiscipline, and there were fewer than 60 samples for some years of the random cohort (**Supplemental Table 1**). All genotypes obtained from horses in this study remained confidential and were used solely for the purpose of estimating allele frequencies. Therefore, 297 sample results were analyzed for HYPP, GBED, HERDA, PSSM1, MYHM, and EJSCA, and 296 sample results were analyzed for MH.

Allele frequencies, carrier frequencies, affected frequencies, and respective standard error values were calculated using the following formulas:

Allele frequency:

$$f_D = (H_{D/N} + 2H_{D/D}) / 2n$$

$$SE_{fD} = \sqrt{((f_D * (1 - f_D)) / 2n)}$$

Where:

f_D represents the frequency of the disease allele

SE_{fD} represents the standard error of f_D

$H_{D/N}$ represents the number of horses with one disease allele (heterozygous)

$H_{D/D}$ represents the number of horses with two disease alleles (homozygous)

n represents tested population number

Carrier frequency (recessive disorders only):

$$Cf_D = (H_{D/N}) / n$$

$$SE_{Cf\mathrm{D}} = \sqrt{((Cf_{\mathrm{D}} * (1 - Cf_{\mathrm{D}})) / n)}$$

Where:

Cf_{D} represents the frequency of carriers for the disease

$SE_{Cf\mathrm{D}}$ represents the standard error of Cf_{D}

$H_{\mathrm{D/N}}$ represents the number of horses with one disease allele (heterozygous)

n represents tested population number

Affected frequency (dominant disorders only):

$$Af_{\mathrm{D}} = (H_{\mathrm{D/N}} + H_{\mathrm{D/D}}) / n$$

$$SE_{Af\mathrm{D}} = \sqrt{((Af_{\mathrm{D}} * (1 - Af_{\mathrm{D}})) / n)}$$

Where:

Af_{D} represents the frequency of horses affected by the disease

$SE_{Af\mathrm{D}}$ represents the standard error of Af_{D}

$H_{\mathrm{D/N}}$ represents the number of horses with one disease allele (heterozygous)

$H_{\mathrm{D/D}}$ represents the number of horses with two disease alleles (homozygous)

n represents tested population number

The frequency values for the AQH random population and all performance subdisciplines were analyzed individually. HYPP, PSSM1, GBED, and HERDA allele frequencies from the AQH random population cohort were cross-examined with previous data from Tryon et al.[30] To detect significant differences between the AQH random population and performance subdiscipline allele frequencies in this study, the z-test for proportionality was performed on each pair of

compared values and standard errors. P-values were determined using a two-sided test for significance ($p=2\times P(Z>|z|)$).

Results

Genotyping was 99.5% successful, with one AQH random cohort horse failing testing for MH, two top performing racing horses failing testing for all diseases, one top performing reining horse failing testing for ESJCA and MYHM, and one top performing barrel horse and one top performing English all-around horse both failing testing for PSSM1. Allele, carrier and disease frequencies for the random AQH cohort and specific AQH subdisciplines are reported in **Table 1**.

For the autosomal dominant diseases, HYPP and PSSM1 were only identified in the AQH random cohort and the halter horse subgroup. The HYPP allele frequency in halter horses ($f_{HYPP}=0.318 \pm 0.050$) was significantly higher ($p<0.00001$) than in the AQH random cohort ($f_{HYPP}=0.013 \pm 0.005$). The PSSM1 allele frequency in halter horses ($f_{PSSM1}=0.386 \pm 0.052$) was also significantly higher ($p<0.00001$) than in the AQH random cohort ($f_{PSSM1}=0.012 \pm 0.004$). When evaluating corresponding disease frequencies, the prevalence of HYPP and PSSM1 in our study was highest in halter horses (63.6 ± 5.1% and 61.4 ± 5.2%, respectively). Thirty-nine percent of halter horses had at least one copy of both PSSM1 and HYPP. MYHM was identified in all cohorts, except barrels, racing and English all-around (**Table 1**), and was significantly higher ($p=0.022$) in reining horses ($f_{MYHM}=0.156 \pm 0.038$) than in the AQH random cohort ($f_{MYHM}=0.066 \pm 0.010$). Consistent with the corresponding allele frequency, the highest affected

frequency in the AQH random cohort was MYHM at 12.8 ± 1.4%. The MYHM affected frequency was also high in top performing reining horses (28.9 ± 4.8%) and halter horses (22.7 ± 4.5%) (**Table 1**). The AQH random cohort had a low MH allele prevalence (f_{MH}=0.002 ± 0.002). MH is not observed in any top performer subdiscipline (**Table 1**).

Within the autosomal recessive diseases, the GBED allele was identified across all disciplines, except English all-around (**Table 1**), and was significantly higher (p=0.018) in reining horses (f_{GBED}=0.109 ± 0.032) than in the AQH random cohort (f_{GBED}=0.030 ± 0.007). Accordingly, GBED carrier frequency was lower in the AQH random cohort (6.1 ± 1.0%), but high in top performing reining (21.7 ± 4.3%), cutting (14.6 ± 3.6%), and western all-around horses (20.7 ± 5.3%) (**Table 1**). The HERDA allele was identified in all disciplines except barrels, halter and racing (**Table 1**), and was significantly higher (p=0.007) in cutting horses (f_{HERDA}=0.135 ± 0.035) than in the AQH random population (f_{HERDA}=0.039 ± 0.008). Accordingly, HERDA carrier frequency was highest in cutting horses (27.1 ± 4.5%) and Western all-around horses (13.8 ± 4.5%) (**Table 1**). The EJSCA allele was identified in the AQH random population (f_{EJSCA}=0.012 ± 0.004), reining horses (f_{EJSCA}=0.011 ± 0.011) and cutting horses (f_{EJSCA}=0.010 ± 0.010), with no significant differences observed between these three groups. Accordingly, the EJSCA carrier frequency was 2.4 ± 0.6% in the AQH random cohort, 2.2 ± 1.6% in the top performing reining population, and 2.1 ± 1.5% in the top performing cutting population (**Table 1**).

Tryon et al.[30] evaluated allele frequencies for HYPP, PSSM1, GBED and HERDA in top performing AQH subgroups using a similar methodology. A random AQH cohort was also included in that

study, however, these horses were randomly selected by year of birth and geographic location, which differs from the methodology used in our AQH random cohort selection. Regardless, comparisons can be made from Tryon's study of AQHs born from 2001-2004 and the current study (**Table 2**).

The HYPP allele frequency in our AQH random cohort was not statistically different as compared to the HYPP allele frequency reported in 2001-2004 random cohort of AQH horses (f_{HYPP}= 0.008 ± 0.004; p=0.44).[30] Within specific AQH subdisciplines, the HYPP allele frequency remains highest in halter horses, but was also statistically not different between time periods (p=0.74) (**Table 2**). In our AQH random cohort, the PSSM1 allele frequency is significantly lower than the 2001-2004 study random AQH cohort (f_{PSSM1}=0.055 ± 0.012, p=0.0007)[30]. Accordingly, the PSSM1-affected frequency is lower in AQH Control groups from 11.3% in 2000-20024 to 2.4 ± 0.6% from 2020-2024 (**Table 2**). Within specific AQH subdisciplines, the PSSM1 allele frequency in our study was significantly higher in halter horses (p=0.00005), but lower in reining and cutting horses (p=0.046 and p=0.006, respectively), as compared to previous estimates (**Table 2**).[30]

Our GBED allele frequency in the AQH random cohort was not significantly different from the previously reported frequency (f_{GBED}=0.054 ± 0.011, p=0.07)[30]. However, our GBED carrier frequency in top performing reining horses (0.109 ± 0.032) is greater than previous reports (0.016 ± 0.009, p=0.006) (**Table 2**). In the AQH random cohort, the HERDA allele frequency trended upwards as compared to the previous time point (p=0.09, **Table 2**). Within specific

AQH subdisciplines, the HERDA allele frequency remains high in cutting horses, but is not significantly different from the previous study (p=0.87).[30]

MH, MYHM and EJSCA allele frequencies were not reported in the previous Tryon, et al. study.[30] However, MH prevalence was recently reported in large sample of over 147,000 QH (f_{MH}=0.0013) and our random sampling data were similar to this large cohort study (**Table 1**).[32]

Discussion

Genotyping the top-performing AQHs revealed a high HYPP and PSSM1 frequency in halter horses (0.318 ± 0.050 and 0.386 ± 0.052, respectively). While none of the top performing reining or cutting horses were observed to have the PSSM1 allele, there was a higher GBED frequency in reining horses (0.109 ± 0.032), and a higher HERDA frequency in cutting horses (0.135 ± 0.035), as compared to the other disciplines. Genotyping a population of randomly selected AQHs identified low PSSM1 frequency (0.012 ± 0.004), and that MYHM frequency (0.066 ± 0.010) was significantly higher than any other disease allele in this population. These results demonstrate that disease allele frequencies in AQHs can be highly variable between performance subgroups.

When compared to the allele frequencies reported by Tryon et al.[30], our findings show some significant differences in the estimates of allele frequencies in the AQH population. While sampling bias should always be considered when comparing longitudinal allele frequency studies, the methods of selection for top performing AQHs in each subgroup were similar

between the two studies. Therefore, for our top performing subgroup data, these changes may reflect evolving breeding practices by increased use of genetic screening. For example, the PSSM1 allele frequency shows a lower frequency in our random cohort, as compared to those reported by Tryon et al, which suggests a potential decrease in PSSM1 prevalence over time. The PSSM1 allele frequency was significantly lower in the top performers of the reining and cutting subdisciplines than previously reported, further supporting appropriate use of genetic testing with regard to this variant specifically in these subdisciplines.

On the other hand, a higher PSSM1 allele frequency in the halter subdiscipline was noted as compared to the previous study.[30] Additionally, our study revealed relatively high PSSM1 and HYPP allele frequencies as compared to all other disease alleles in all other subdisciplines. Notably, PSSM1 allele frequency was *higher* than HYPP allele frequency in top performing halter horses, which is the opposite of what the previous study reported.[30] While this may reflect different breeding practices specifically in halter horses over time, it is important to consider that, when the Tyron et al study was conducted, the PSSM1 allele had only been recently identified, and the control population was selected from horses submitted for parentage testing only. Mandated disease panel genetic testing for stallions began in 2014, after the Tryon, et al. study was published. Therefore, these differences may also reflect a sampling difference, despite the random sampling methodology performed in both studies. Additional longitudinal studies of estimated allele frequencies are needed to continue to monitor trends and draw further conclusions.

The affected frequencies of HYPP and PSSM1 in top performing Halter horses were 63.6 ± 5.1% and 61.4 ± 5.2%, respectively, suggesting that over half of all elite halter AQHs are estimated to be affected with HYPP or PSSM1. In the elite halter horses that were tested in this study, 38% had one HYPP allele *and* one or two PSSM1 alleles. Interestingly, a previous study investigating 296 horses with muscle disease identified 15 horses with more than one muscle disease allele, but none of these had both PSSM1 and HYPP.[33] Out of those halter horses in our study with both alleles, one is also a carrier for GBED, and two also have one MYHM allele. Since MYHM is also dominant disease, these two horses have the potential to be affected with a total of three dominant muscle disorders, and yet they are performing at the top of their discipline. Evaluating management strategies and clinical phenotypes in a well-designed study that includes metadata related to performance would help determine successful management strategies and investigate if other genetic variants are rescuing phenotypes. The consistently high frequency of the HYPP allele, as well as the greater frequency of the PSSM1 allele in the halter subdiscipline, supports the possibility that the use of genetic testing may differ by discipline. It is possible that selection for the exaggerated musculature associated with HYPP is still a priority in selection of breeding halter horses, while diseases, such as PSSM1, are not selected against. Halter horses are judged in competition based on conformation, so further data collection and evaluation are needed to evaluate the overall health and best management practices of horses with one or more deleterious alleles despite high placements in show based on their conformational traits.

Other important allele frequency differences include the greater GBED allele frequency in the reining subdiscipline, which may suggest that, like in the case of halter horses, selection for performance traits may be taking precedence over disease allele reduction. However, unlike HYPP and PSSM1, GBED is an autosomal recessive disease, so carriers are unaffected. This might allow breeders to select away from producing affected horses while still prioritizing desired traits. Given that GBED carrier frequency is higher in in the top performing reining horses as compared to the previous time point, it is important to ensure that breeders are appropriately using marker assisted selection to avoid producing GBED affected foals, especially for those breeders using intense inbreeding for selection. It is equally important to continue to monitor this frequency. While not reaching statistical significance, the HERDA allele frequency was higher in the random cohort studied here, therefore pointed education on genetic testing across the breed may help eliminate a further increase in this allele frequency overall. For example, since it is also an autosomal recessive disease, it is possible that breeders are consistently using popular sires, despite being carriers for HERDA because of their performance success. There is also speculation among certain disciplines, such as cutting, that having one HERDA allele gives horses a competitive advantage. A well-designed study investigating the functional consequence of horses with one copy of HERDA is needed to develop an evidence-based recommendation and protect the health of the breed.

The AQH diseases that were not compared to the Tyron et al. study are MH, MYHM, and EJSCA. MH was not observed in any top performer discipline and, out of 296 AQH Control horses, only 1 had an MH allele. This resulted in a low allele frequency of 0.002 ± 0.002, which is consistent

with a recently reported MH allele frequency in a cohort of 147,046 AQHs (0.0013).[32] The MYHM allele frequency, however, was significantly higher than any other frequency in the AQH random cohort, and it was also relatively high in the reining and halter subdisciplines. This is also consistent with previously reported studies, including those looking specifically at muscle disease.[33] Lastly, the EJSCA allele was present in the AQH random cohort and the reining and cutting subdisciplines. While the frequency of the EJSCA allele is relatively low, it could become more prevalent over time since it is a new autosomal recessive disease that is being propagated through top performing individuals in two subdisciplines. All AQHA disease alleles require reassessment over time, as these data from this study show differences from the two time points evaluated, suggesting that some disease alleles are being selected against while others in certain disciplines may be under positive selection.

This study highlights discipline-dependent allele frequencies within the AQH population. We have also shown that there are differences that can be gleaned from previous studies, and that these differences may reflect differences in breeding practices or popular lineages within each performance discipline. Additionally, this study evaluated cohorts for all 7 known AQH diseases. Three were included in this type of study for the first time, which lays a foundation moving forward for continued reassessment. Accurate, up-to-date knowledge of the frequency and distribution of disease alleles in the AQH population not only allows breeders to make informed decisions but also helps veterinarians and industry stake holders monitor the effectiveness of intervention efforts (genetic testing, etc.). It also allows for the identification of emerging risks, such as new diseases like EJSCA or diseases that may be increasing in allele frequency by

discipline like HYPP. It also can help direct scientists to develop relevant, evidence-based studies to address where additional knowledge for appropriate recommendations are needed. . The reporting of these results supports widespread knowledge, from owners and competitors to veterinarians and researchers, that will allow the community to coordinate and refine efforts to inform and educate toward a healthy breed standard.

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Tables

Table 1—Allele, carrier, and disease frequencies for all diseases from this study in the random AQH cohort and all top performance subdiscipline groups. The highest allele frequency for each group is indicated in red font. *=significantly different allele frequency between AQH random cohort and subdiscipline groups at ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05.

Population	HYPP		PSSM1		MYHM		MH		GBED		HERDA		EJSCA	
	Allele Frequency	Disease Frequency	Allele Frequency	Disease Frequency	Allele Frequency	Disease Frequency	Allele Frequency	Disease Frequency	Allele Frequency	Carrier Frequency	Allele Frequency	Carrier Frequency	Allele Frequency	Carrier Frequency
AQH random	0.013 ± 0.005	0.027 ± 0.007	0.012 ± 0.004	0.024 ± 0.006	0.066 ± 0.010	0.128 ± 0.014	0.002 ± 0.002	0.003 ± 0.002	0.030 ± 0.007	0.061 ± 0.010	0.039 ± 0.008	0.064 ± 0.010	0.012 ± 0.004	0.024 ± 0.006
Reining	0	0	0	0	0.156 ± 0.038 *	0.289 ± 0.048	0	0	0.109 ± 0.032 *	0.217 ± 0.043	0.022 ± 0.015	0.043 ± 0.021	0.011 ± 0.011	0.022 ± 0.016
Cutting	0	0	0	0	0.021 ± 0.015	0.042 ± 0.020	0	0	0.073 ± 0.027	0.146 ± 0.036	0.135 ± 0.035 **	0.271 ± 0.045	0.010 ± 0.010	0.021± 0.015
Barrels	0	0	0	0	0	0	0	0	0.011 ± 0.011	0.022 ± 0.015	0	0	0	0
Halter	0.318 ± 0.050 ****	0.636 ± 0.051	0.386 ± 0.052 ****	0.614 ± 0.052	0.114 ± 0.034	0.227 ± 0.045	0	0	0.011 ± 0.011	0.023 ± 0.016	0	0	0	0
Racing	0	0	0	0	0	0	0	0	0.021 ± 0.015	0.042 ± 0.020	0	0	0	0
Western all-around	0	0	0	0	0.017 ± 0.017	0.034 ± 0.024	0	0	0.103 ± 0.040	0.207 ± 0.053	0.069 ± 0.033	0.138 ± 0.045	0	0
English all-around	0	0	0	0	0	0	0	0	0	0	0.017 ± 0.017	0.034 ± 0.024	0	0

Table 2—Allele frequencies and standard errors of HYPP, PSSM1, GBED, and HERDA in the AQH control and top performer subdiscipline cohorts, grouped by the results from Tryon et al. (2001-2004) and this study (2020-2024). The year ranges corresponding to each study refer to the range of birth years of the horses included in each study. If a disease allele frequency was not reported in the previous study, it is reported here as "UA" for "unavailable". *=significantly different between Tryon et al. study and current study at ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05.

Population	No. of Horses Tested		HYPP Allele Frequency		PSSM1 Allele Frequency		GBED Allele Frequency		HERDA Allele Frequency	
	2001-2004	2020-2024	2001-2004	2020-2024	2001-2004	2020-2024	2001-2004	2020-2024	2001-2004	2020-2024
AQH random	200	297	0.008 ± 0.004	0.013 ± 0.005	0.055 ± 0.012	0.012 ± 0.004 ***	0.054 ± 0.011	0.030 ± 0.007	0.021 ± 0.007	0.039 ± 0.008
Reining	97	46	0	0	0.022 ± 0.011	0 *	0.016 ± 0.009	0.109 ± 0.032 **	0.046 ± 0.015	0.022 ± 0.015
Cutting	113	48	0	0	0.033 ± 0.012	0 **	0.068 ± 0.017	0.073 ± 0.027	0.142 ± 0.023	0.135 ± 0.035
Barrels	82	46	0.006 ± 0.006	0	0.007 ± 0.007	0	0.006 ± 0.006	0.011 ± 0.011	0.006 ± 0.006	0
Halter	118	44	0.299 ± 0.030	0.318 ± 0.050	0.155 ± 0.024	0.386 ± 0.052 ****	0.026 ± 0.010	0.011 ± 0.011	0.004 ± 0.004	0
Racing	106	48	0	0	0.010 ± 0.007	0	0	0.021 ± 0.015	0	0
Western all-around	UA	29	UA	0	UA	0	UA	0.103 ± 0.040	UA	0.069 ± 0.033
English all-around	UA	29	UA	0	UA	0	UA	0	UA	0.017 ± 0.017

Supplemental Table 1—The number of AQH random horses successfully genotyped for each disease allele.

Random AQH Birth Year	Number of horses genotyped for HYPP, GBED, HERDA, PSSM1, MYHM, and EJSCA	Number of horses genotyped for MH
2020	59	59
2021	60	60
2022	59	58
2023	59	59
2024	60	60
Total	**297**	**296**