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Seasonal pasture myopathy/atypical myopathy in North America associated with ingestion of hypoglycin A within seeds of the box elder tree

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Summary

Reasons for performing study: We hypothesised that seasonal pasture myopathy (SPM), which closely resembles atypical myopathy (AM), was caused by ingestion of a seed-bearing plant abundant in autumn pastures.
Objectives: To identify a common seed-bearing plant among autumn pastures of horses with SPM, and to determine whether the toxic amino acid hypoglycin A was present in the seeds and whether hypoglycin metabolites were present in SPM horse serum or urine.
Methods: Twelve SPM cases, 11 SPM pastures and 23 control farms were visited to identify a plant common to all SPM farms in autumn. A common seed was analysed for amino acid composition (n = 7/7) by GC-MS and its toxic metabolite (n = 4/4) identified in conjugated form in serum [tandem mass spectrometry (MS/MS)] and urine [gas chromatography (GC) MS]. Serum acylcarnitines and urine organic acid profiles (n = 7) were determined for SPM horses.
Results: Seeds from box elder trees (*Acer negundo*) were present on all SPM and 61% of control pastures. Hypoglycin A, known to cause acquired multiple acyl-CoA dehydrogenase deficiency (MADD), was found in box elder seeds. Serum acylcarnitines and urine organic acid profiles in SPM horses were typical for MADD. The hypoglycin A metabolite methylenecyclopropylacetic acid (MCPA), known to be toxic in other species, was found in conjugated form in SPM horse serum and urine. Horses with SPM had longer turn-out, more overgrazed pastures, and less supplemental feeding than control horses.
Potential relevance: For the first time, SPM has been linked to a toxin in seeds abundant on autumn pastures whose identified metabolite, MCPA, is known to cause acquired MADD, the pathological mechanism behind SPM and AM. Further research is required to determine the lethal dose of hypoglycin A in horses, as well as factors that affect annual seed burden and hypoglycin A content in *Acer* species in North America and Europe.

Keywords: horse; muscle; rhabdomyolysis; beta oxidation; multiple acyl-coA dehydrogenase

Introduction

A highly fatal muscle disease termed seasonal pasture myopathy (SPM) has been described for many decades in horses on pastures in the Midwestern USA and eastern Canada [1–3]. A similar disorder called atypical myopathy (AM), noted with increasing frequency, exists in the UK and Northern Europe [4–6]. Outbreaks of SPM and AM vary from year to year and are seasonal, with most cases occurring in the autumn and fewer cases occurring in the subsequent spring [5,7]. In North America, only a few horses are affected on a given pasture, whereas in Europe large outbreaks occur with many horses on the same premises being affected [2,5,6]. Horses that develop SPM and AM are usually kept on sparse pastures with an accumulation of dead leaves, deadwood and trees in or around the pastures [5,6], often without being fed supplemental hay or grain [2]. Affected horses develop severe acute myonecrosis involving respiratory and postural muscles, and myoglobinuria, which in at least 75% of cases leads to death within 72 h [2,5,6]. The cause(s) of SPM and AM are unknown; however, proposed contributing factors include low dietary selenium [4], ingestion of *C. sordelli* toxin in the soil [8] and ingestion of maple leaves (*Acer pseudoplatanus*) infested with European tar spot fungus (*Rhytisma acerinum*) [9]. White snake root toxicity has also been proposed but appears unlikely after further evaluation of cases in the Midwestern USA [2].

Horses with SPM and AM have a lipid storage myopathy involving oxidative postural and respiratory muscles and, in some cases, cardiac muscle [2,10]. Studies of AM and, subsequently, SPM identified an acquired deficiency in skeletal muscle multiple acyl-CoA dehydrogenases (MADD), which produces a specific abnormal pattern of accumulation of blood acylcarnitines and urine organic acids [11,12].

Inherited forms of MADD in man are due to mutations in the electron transfer flavoprotein gene, the electron transfer flavoprotein–dehydrogenase gene [13] and riboflavin transporters [14]. Clinical signs range from a fatal neonatal condition to an adult onset mild lipid storage myopathy [15]. An acquired form of MADD also exists in man, arising from ingestion of seeds of the unripe Jamaican ackee fruit (*Blighia sapida*) [16,17]. Ackee seeds contain high concentrations of a nonproteogenic amino acid called hypoglycin A (L-α-amino-methylenecyclopropylpropionic acid) whose metabolite methylenecyclopropylacetic acid (MCPA) is metabolised to MCPA-CoA, which is a potent inhibitor of multiple acyl-CoA dehydrogenases [17]. Ingestion of unripe ackee fruit can be lethal in man, with clinical signs of hypoglycaemia and persistent vomiting [16,17]. Hypoglycin A has also been found in seeds from a tree species, *Acer*, common in the Midwestern USA, which is a member of the same Sapindaceae family as *Blighia sapida* [18]. *Acer* spp. with black tar spot fungus on their leaves have been noted previously in pastures of horses with AM [9].

We hypothesised that SPM in horses was caused by ingestion of seeds plentiful in fall pastures that contained high concentrations of hypoglycin A. The objectives of this study were to determine: 1) whether there was a common abundant seed-bearing plant in pastures of horses affected by SPM in the fall; 2) whether hypoglycin A was present in the fall burden of seeds from that plant; and 3) whether toxic hypoglycin metabolites could be identified in serum or urine from affected horses.

Materials and methods

Horses

Seasonal pasture myopathy cases were identified from muscle biopsy submissions to the Neuromuscular Diagnostic Laboratory at the University

TABLE 1: Demographic data, clinical findings of SPM cases

Horse	EH	FS1	FS2	WI	RI	MY	OS	MC	CL	HE	PP	NE
SPM status	Suspect	CF	CF	CF	CF	CF	CF	CF	CF	Suspect	CF	CF
Breed	SB	BL	BL	QH	APP	MFT	PT	TW	MG	QH	QH	TB
Age (years)	2	12	13	16	2	5	9	4	4	6	8	4
Sex	g	m	g	m	s	g	G	g	g	m	g	g
Serum CK (u/l)	>20,000	>24,000	47,400	813,933	na	>2000	447,308	290,986	>16,000	>2000	>125,000	580,880
Myofibre lipid	na	+++	na	+++	+++	+++	na	+++	++	na	+++	+++
Abnormal UOA	na	na	yes	na	na	yes	yes	yes	yes	na	yes	yes
Date affected	Nov-05	Apr-09	Apr-09	May-09	Oct-09	Nov-09	Nov-09	Oct-11	Oct-11	Nov-11	Nov-11	Nov-11

SPM status: suspected cases = abnormal serum creatine kinase activity or pigmenturia with no exercise history in horses kept on pasture in the fall. CF = confirmed cases based on myodegeneration, abnormal myofibre lipid content and/or characteristic accumulation of acylcarnitines and urine organic acids (UOA). SB = Saddlebred; BL= Belgian Draught; QH = Quarter Horse; APP = Appaloosa; MFT = Missouri Fox Trotter; PT = American Paint; TW = Tennessee Walking Horse; MG = Morgan Horse; TB = Thoroughbred; na = not available; ++ = moderate; +++ = marked.

of Minnesota between 2005 and 2011 and from an online survey of seasonal pasture myopathy instituted in 2011 by the University of Minnesota Neuromuscular Diagnostic Laboratory. All medical records were reviewed to obtain information from necropsy reports, muscle biopsy reports, serum biochemistry profiles, serum acylcarnitine profiles and urine organic acids. Inclusion criteria for suspected cases were: unexercised horses on pasture with acute clinical signs of muscle pain, weakness and recumbency as well as high serum creatine kinase (CK) activity [>2000 for undiluted samples, >45,000 when dilution performed] or pigmenturia (Table 1). Inclusion criteria for confirmed cases were, in addition to the above criteria, abnormal elevations in MADD-specific urine organic acids and serum acylcarmitines or demonstration of a lipid storage myopathy in at least one of the muscles sampled. Excessive lipid storage was judged as a score of >6 using the lipid scoring system of Schultz *et al*. [19]. The score is the sum of number of fibres staining for lipid (scale 1–3) + density of lipid droplets/fibre (0–3) + (size of lipid droplets (0–3). The study met the guidelines provided by the University of Minnesota Animal Use and Care Committee.

Muscle samples

Available fresh muscle tissues were frozen in isopentane chilled in liquid nitrogen within 24 h of sampling. Cryosections were stained with haematoxylin and eosin (H&E), periodic acid–Schiff (PAS), amylase PAS and Oil red O [20]. Formalin fixed muscle samples were stained with H&E and PAS. The amount of muscle degeneration was graded as mild, moderate or severe from H&E stains and the amount of neutral lipid accumulation was graded similarly from Oil red O stains.

Serum and urine samples

After identification of MADD as the metabolic defect causing the lipid storage myopathy in AM [11] and SPM [12], submission of serum and urine samples was requested from all potential cases (beginning in 2008). Serum and urine samples were either frozen upon arrival at the Neuromuscular Diagnostic Laboratory for later shipment or shipped directly on ice packs to the Institute of Metabolic Disease of Baylor Research Institute. Serum acylcarnitine concentrations were determined by a modification of flow injection analysis tandem mass spectrometry (MS/MS) [21]. Urine organic acids were identified and quantified by gas chromatography–mass spectrometry (GC-MS) [22]. Reference ranges, derived from serum samples from 35 healthy mature horses and urine samples from 5 healthy mature Quarter Horse mares, have been previously published [12]. Blood samples were obtained in the spring of 2011 from horses that had daily pasture access on farms in the same regions as affected horses; farms did not necessarily contain box elder trees. Reference urine samples were obtained in January 2010 from horses on the university campus that were turned out daily on a drylot that had no trees.

Questionnaires

Owners of SPM affected horses were asked to answer questions with regard to the date the horse was first introduced to the pasture where it developed SPM; date the horse first showed signs of SPM; presence of other horses on the same pasture; hours on pasture; whether horses received any supplemental feeding in addition to grazing pastures; topography of pastures; rainfall during the affected season; whether horses had access to trees while on the pasture; and whether there was fallen wood or trees located inside the pasture.

Site visits: In October and November of 2011 at least one investigator performed a site visit of SPM pastures located in Minnesota and Iowa. Digital images of the pasture, plants and trees were obtained, and notes were made with regard to the presence of dead wood, pasture topography, standing water and overgrazed pastures (grass growth <8 cm with no allocated rest/re-growth time). A horticultural specialist was enlisted to identify plant species based on images or fresh samples of leaves or seeds. For farms outside Minnesota and Iowa where a site visit was not possible, digital images of the pastures and trees were requested. After visiting the first 4 farms, focus with regard to plant species was directed toward a common tree that had abundant seeds on branches and on the pastures, the box elder (*Acer negundo*). Owners of all farms were provided with digital images of the fibrous winged bearer of seeds called samaras from the box elder tree (Fig 1), and a sample of samaras was requested.

Control farms

The Extension service at the University of Minnesota offers a pasture management programme to owners who are interested in improving the quality of their horse's pastures. Owners of farms within Minnesota who had enrolled in a University of Minnesota Extension pasture management programme were contacted and asked to serve as control farms and to answer a written questionnaire. Control farms did not have a history of SPM occurrences in the preceding years. A site visit had been performed for these 7 control farms in the spring and summer of 2011. In the autumn of 2011, owners were sent pictures of box elder trees with samaras and asked if these were present on their horse's pastures. Because horses of the local Fox Hunt are kept on pastures for many hours a day, club members were also contacted in the spring of 2012 and asked to participate as control farms. Site visits for all 16 farms that agreed to participate were performed in May 2012 and a trained investigator determined whether box elder trees with samaras were present in horse pastures.

Weather information

Information on maximum wind gusts and wind speeds as well as daily precipitation and maximum and minimum temperatures was obtained for 5 days preceding the onset of clinical signs of SPM. Data were obtained from Weather Underground (http://www.wunderground.com) or the Farmer's Almanac (http://www.farmersalmanac.com) using the closest weather station to each farm. Information on wind speed, temperature and precipitation matched for dates and locations was also collected for the control year of 2010, a year in which no cases of SPM were identified by the Neuromuscular Diagnostic Laboratory. In addition, the historical averages of rainfall and maximum and minimum temperature for matching dates and locations were obtained for comparison. Historical averages for wind speed were unavailable.

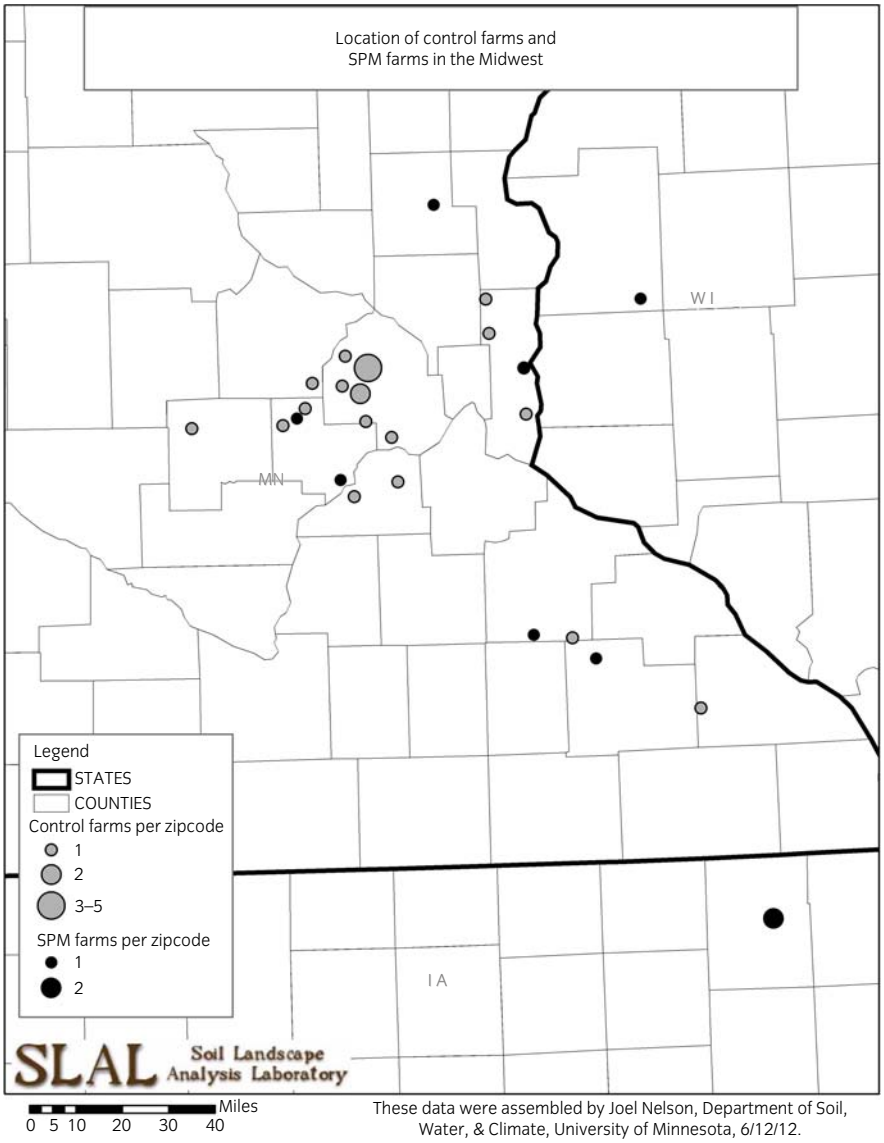

Fig 1: Map depicting the location of seasonal pasture myopathy (SPM) farms in Midwestern USA (dark circles) and the location of control farms (grey circles). Grey circle size reflects the number of farms in a location and black circle size reflects the number of SPM affected horses in a location. MN = Minnesota, WI = Wisconsin, IA = Iowa. SPM farms from southeastern WI, New York, and Alberta, Canada, are not depicted.

Hypoglycin A analysis in seeds

Box elder samaras were collected from a random sampling of trees on 7/11 SPM farms in the fall of 2011. Samaras were collected within a few weeks to 3 months of 4 SPM horses becoming affected (MC, CL, HE, PP), within 18 months of 3 horses on 2 farms (FS1&2, WI) becoming affected and 6 years after one horse became affected (EH). A concurrent control sample of samaras from an ash tree on the pasture of horse HE was also obtained. Individual seeds were dissected from the samara husks, pulverised and extracted in dilute HCl for methyl chloroformate derivatisation and amino acid analysis by GC-MS [23]. The electron impact ionisation fragmentation pattern for derivatised hypoglycin A was manually assigned by comparison to NIST08 database electron impact ionisation spectra of underivatised hypoglycin A and other methyl chloroformate amino acid derivatives. The elemental composition of hypoglycin ($C_7H_{11}NO_2$ calculated mass = 141.078979 u) was confirmed by accurate mass measurement (M-H [m/z] = 140.0712; + proton 1.0073 = 141.0785 u) to within 3.5 ppm mass error using an LTQ-Orbitrap mass spectrometer (Thermo Scientific) in negative ion electrospray ionisation mode.

Synthesis of MCPA standards

A 2-step synthesis involving MCPA, *N*-hydroxysuccinimide (NHS), and either carnitine or glycine was used to obtain an MCPA-glycine or MCPA-carnitine standard. Successful synthesis of the compounds was confirmed by MS and NMR analysis of the crude product.

Serum and urine MCPA conjugates

Serum and urine samples from 4 horses confirmed to have SPM and abnormalities in serum and urine consistent with MADD were available for analysis of MCPA conjugates. Nine samples from the same healthy horses used for reference serum acylcarnitines were used as controls. UPLC-MS/MS was used to identify MCPA-carnitine in equine serum by derivatisation to the butyl ester and similarity of the retention times of the peak of the precursor ion at *m/z* 312 to that of synthesised standard. The amount of MCPA-carnitine was calculated from the peak area of *m/z* 312 compared to the peak area of *m/z* 350 for the internal standard of

TABLE 2: Serum acylcarnitines and urine organic acids from horses with seasonal pasture myopathy

Acylcarnitine (μmol/l)	SPM horses mean ± s.d.	Normal equine reference range [12]
Free carnitine (n = 4)	94.25 ± 32.52	4.3–31.3
C-2 (n = 7)	61.42 ± 31.57	≤18.96
C-4 (n = 7)	43.75 ± 32.46	≤1.06
C-6 (n = 7)	7.63 ± 5.88	≤0.12
C-14 (n = 7)	0.19 ± 0.15	≤0.02
C-16 (n = 7)	0.44 ± 0.34	≤0.02
C-18:2 (n = 7)	0.13 ± 0.06	≤0.02
Urine organic acids (mmol/mol creatinine)	n = 7	
Ethylmalonic acid	251.57 ± 87.52	≤3.14
Methylsuccinic acid	71.00 ± 29.65	≤9.14
Lactic acid	330.33 ± 247.56	≤14.71
Adipic acid	102.43 ± 94.59	0
Glutaric acid	130.14 ± 119.22	0
Butyrylglycine	4164.00 ± 2391.61	≤7.71
Isovalerylglycine	165.67 ± 52.56	≤16.8
Hexanoylglycine	196.60 ± 101.36	0

d_6-octanoyl-carnitine, assuming the same detector response for both acylcarnitines.

Urine organic acids including glycine conjugates were isolated from inorganic anions and amino acids using liquid partition chromatography (LPC) on acidified silicic acid. GCMS of the trimethylsilyl (TMS) derivatives was used to identify MCPA-glycine in horse urine by comparison of the retention time and spectrum to the retention time and spectrum of the synthesised MCPA standard. The standard gave primarily the monoTMS derivative with molecular ion *m/z* 241 eluting at 34.83 min, overlapping a small amount of the diTMS derivative with molecular ion *m/z* 313 eluting at 34.96 min. The quantity of diTMS MCPA-glycine in the horse urine was estimated from the total ion chromatogram peak area relative to the total ion chromatogram peak area of diTMS hexanoylglycine and the amount of diTMS hexanoylglycine calculated from a standard curve.

Data analysis

Categorical data comparing cases and control horses were analysed using a Chi-square test. Cells with <5 were compared using Fisher's exact test. Parametric data from weather parameters were compared among the dates in the affected year to the mean for rolling average of the same dates in 2010 using a one-way analysis of variance blocked for disease state or paired *t* test where appropriate. Significance was set at P<0.05.

Results

SPM horses

A total of 12 horses from 11 farms fit the inclusion criteria for SPM. Of these, 2 horses were considered suspect cases and 10 horses were considered confirmed SPM cases (Table 1). All but two cases were fatal. The SPM group comprised 9 different breeds, included 8 geldings, one stallion and 3 mares that ranged in age from 2 to 16 years (median 5.5 years; Table 1). Horses presented with clinical signs of muscular weakness and stiffness of <3 days' duration, dark urine, periods of recumbency, colic-like signs and muscle trembling. In the SPM group, serum CK activity was measured in 11 horses, lipid storage myopathy was documented in 8, elevations in serum acylcarnitines were documented in 6/7 and abnormal urine organic acids were documented in 7/7 horses (Table 2).

Muscle histopathology

Fresh skeletal muscle samples were available from 8 horses, only formalin fixed samples were available from one horse (EH) and 3 horses had no muscle available to examine. Cryostat sections contained a moderate to marked amount of neutral lipid accumulation in myofibres from the diaphragm (MY, MC, WI, RI), intercostal (PP, NE), *sacrocaudalis* muscle (CL) or *vastus lateralis* (FS1) muscle (Table 1). A moderate to marked number of myofibres in all samples of respiratory muscle had Zenker's necrosis, an absent to mild degree of macrophage infiltration, and all muscle samples had normal to decreased periodic acid–Schiff staining for glycogen. The formalin fixed samples showed normal PAS staining and acute severe Zenker's necrosis in postural and respiratory muscles.

Serum and urine samples

Serum acylcarnitine profiles resembled that of MADD in 6/7 analysed samples, with a generalised elevation of short (C2–C5), medium (C6–C12) and long chain (C14–C20) acylcarnitines (Table 2). The surviving horse (FS2) had only mild elevations of acylcarnitines C5–C10 and minor elevations of C12 and C16. The urine organic acid and glycine conjugate profiles measured in 7 horses revealed markedly elevated ethylmalonic acid, methylsuccinic acid, lactic acid, adipic acid, butyrylglycine, isovalerylglycine and hexanoylglycine, consistent with MADD (Table 2). Glutaric acid was elevated in all cases except the surviving horse FS2 (Table 2).

SPM and control farms

Six SPM horses were located on 6 farms in Minnesota, 2 on one farm in Iowa, 2 were from 2 farms in Wisconsin, one was from Alberta, Canada, and one was from New York. The distribution of control farms was concentrated around the Minneapolis/St Paul, Minnesota area (Fig 1). The 11 SPM farms housed between one and 4 horses and the 23 control farms housed between 2 and 20 horses/farm (median 3.5 horses/SPM farm; control farm 9.0 horses/farm). Eight out of 12 SPM horses were in their first autumn or spring season on the pastures when they became ill and 4 horses had been on the pastures for more than one year. Box elder trees with samaras were a consistent feature of all SPM pastures (11/11) and were present in significantly fewer control pastures (61%; 14/23 – Chi-square 5.85, P<0.02; Fig 2). Horses on 91% (10/11) of SPM farms and 48% (11/23) of control horse farms had access to box elder trees or branches (Chi-square = 5.85, P<0.02; Fig 3). Fallen tree trunks or branches were present in 91% (10/11) of SPM pastures and 39% (9/23) of control pastures (Chi-square = 8.1, P<0.01). Areas in the pasture that were muddy or under water were not a consistent feature of SPM pastures, with 25% (3/12) and 65% (15/23) of SPM and control pastures, respectively, having wet areas in the autumn. All SPM horses spent 24 h/day on pasture, which was significantly longer than horses on control farms. Eight of (35%) 23 control horses spent 24 h/day on pasture (Chi-square 13.7, P<0.001). All of the SPM pastures were overgrazed compared to 44% (10/23) of control horse pastures (Fig 4). It was significantly less common for horses on SPM farms to receive supplemental hay or concentrate than for controls. Only 25% (3/12 horses) of SPM horses received supplemental feed on pasture vs. 74% (17/23) of control horses (Chi-square = 7.7, P<0.01).

Weather

Maximum wind speed and maximum wind gusts (mean ± s.d.) for the 5 days preceding illness were significantly higher in the period before horses became ill (speed 29.73 ± 4.41 km/h; gusts 40.25 ± 6.42 km/h, P<0.05) compared to the same days in 2010 (speed 24.79 ± 3.78; gusts 34.27 ± 3.77 km/h, P<0.01). There was no difference in daily precipitation between the 5 days preceding horses becoming ill (1.30 ± 1.66 mm) and the 2010 data (1.29 ± 1.49 mm). There was no difference in minimum daily temperature and maximum daily temperature between the dates preceding horses becoming ill (min 2.00 ± 2.62°C; max 12.61 ± 2.69°C) and the historical averages (min 0.33 ± 2.49°C; max 10.74 ± 2.82°C). The year 2010 had significantly warmer temperatures (min 4.26 ± 2.62°C; max 14.44 ± 339°C) than the historical averages over time and than the same dates preceding the horse's illness (P<0.01).

Hypoglycin assay in seeds

Hypoglycin A was not identified in the seeds from an ash tree but was the major amino acid component of the box elder seeds, often dwarfing

Fig 2: A box elder tree bearing numerous seed containing samaras (inset) in early November within an SPM pasture (CL).

Fig 3: Two SPM pastures, (a) Horse CL and (b) Horse MC, located in a hollow with numerous box elder trees and felled trees present as well as (c) the closest unaffected horse pasture to MC showing flat pastures with an absence of trees and fallen trunks and branches.

Fig 4: Example of the sparse pastures in early November that contain numerous box elder samaras from SPM farms (a) EH and (b) WI.

alanine, proline and glutamate, which are usually the most abundant amino acids in plant tissues (Fig 5). Hypoglycin A concentrations varied significantly from seed to seed within and between SPM farms, with the highest measurement (~160 μg/seed) approximately 50 times greater than the lowest (~3 μg/seed; Table 3). The average hypoglycin A content for all seeds from SPM farms was ~40 μg/seed.

Serum and urine MCPA conjugates

MCPA-carnitine was below the limit of detection (approximately 0.001 nmol/l) in serum of 9 reference control horses. MCPA-carnitine concentrations for the 4 SPM serum samples ranged from 4.8 nmol/l to the remarkably high level of 102.4 nmol/l in Horse CL (Fig 6; Table 3). MCPA-glycine in equine urine was found only as the diTMS derivative with molecular ion *m/z* 313 eluting at 35.11 min just after the very large peak of diTMS hexanoylglycine at 34.97 min. The small difference in retention time for the diTMS MCPA-glycine in the standard and the horse urine may be caused by the large preceding peak of diTMS hexanoylglycine in the horse urine. Glycine conjugated MCPA in urine ranged from 0.79–3.97 mmol/mol creatinine in 6 of the 7 SPM horses compared to one SPM and 5 reference control horses, where it was below the limit of detection (<0.01 mmol/mol creatinine; Table 3). The lack of MCPA-glycine in the urine of one SPM horse may reflect variation in the time of sampling in relation to disease onset, or variable sensitivity of this toxic metabolite in urine.

Discussion

Although MADD is known to be the underlying defect of both SPM in the USA [12] and AM in Europe [11], the cause of this acquired metabolic disorder has remained elusive [2,8]. In the present study we identified a

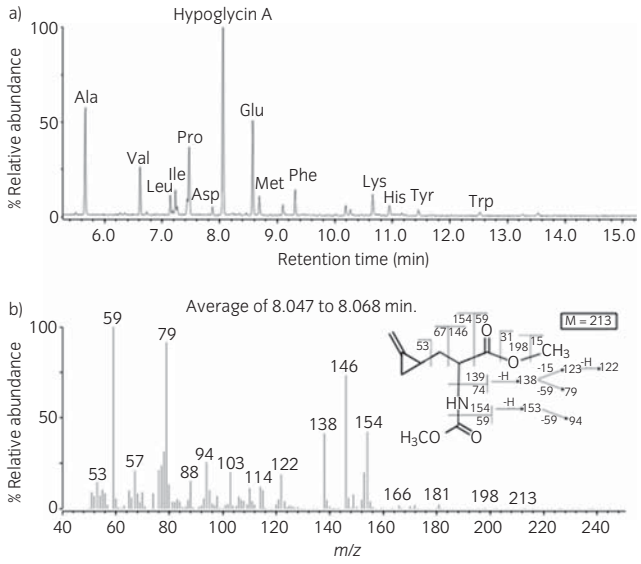

Fig 5: GC-MS detection and identification of hypoglycin A in box elder tree seeds. (a) Total ion chromatogram of methylchloroformate derivatives of amino acids extracted from seeds showing a higher content of hypoglycin A than any other amino acid. (b) Electron impact ionisation (EI) spectrum of the methylchloroformate derivative of hypoglycin A with annotated structure (inset).

toxic branched chain amino acid called hypoglycin A in the seeds from the box elder tree, which was common to all SPM pastures, and also found high concentrations of its toxic metabolite MCPA-carnitine in serum from SPM horses. The fact that another acquired form of MADD in rats and man is caused by hypoglycin A ingestion [24] strengthens our conclusion that hypoglycin A is the likely toxic cause of SPM in horses.

The criteria used to diagnose hypoglycin A toxicity in SPM horses were the same as those used in human patients [17,24]. Criteria include the accumulation of short, medium and long chain acylcarnitines in serum,

TABLE 3: Hypoglycin A content of box elder seeds as well as serum MCPA-carnitine and urine MCPA glycine concentrations in individual SPM-affected and control horses

	Hypoglycin in box elder seeds	Serum MCPA-carnitine		Urine MCPA-glycine	
Horse	μg/seed ± s.d.	UPLC-MS/MS retention time (min)	nmole/l	GCMS retention time (min)	mmol/mole creatinine
EH	31 ± 19	na	na	na	na
FS2	56 ± 46	na	na	35.08	2.16
WI	44 ± 25	na	na	na	na
MY	na	na	na	35.15	1.52
OS	na	na	na	35.12	1.53
MC	68 ± 27	1.58	4.8	35.13	0.87
CL	40 ± 14	1.57	102.4	35.11	3.97
HE	77 ± 66	na	na	na	na
PP	9 ± 4	1.65	47.2	35.08	0.79
NE	na	1.64	22.5	na	<0.01
Controls	Ash seed = 0	na	<0.01	na	<0.01
			n = 9		n = 5

na = not available. Box elder seeds were obtained in November. Seeds from Horses MC, CL and HE were obtained within a month of clinical signs. Other samples were obtained months to years after horses were affected. SPM cases in which urine was not available were suspected but not confirmed to have MADD.

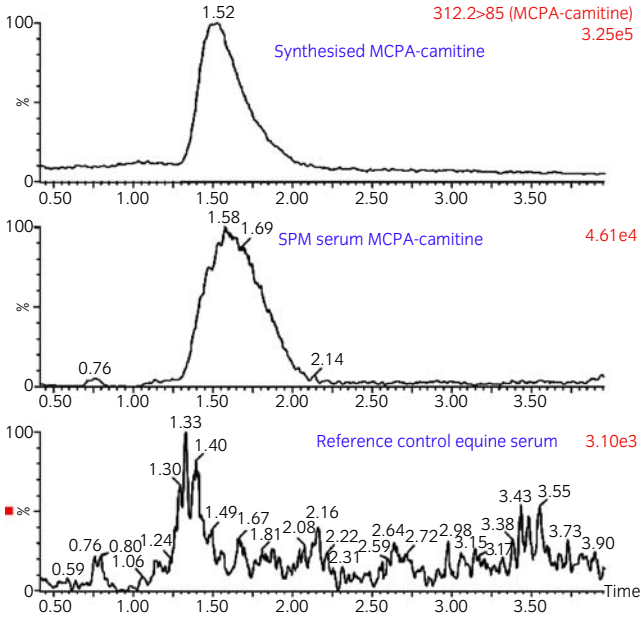

Fig 6: MCPA-carnitine UPLC-ESI-MS/MS chromatograms for transition from protonated molecular precursor ion *m/z* 312 to product ion *m/z* 85 of butyl esters. The exponent numbers at the upper right of each panel indicate the intensity of the peak in each panel. Note that MCPA-carnitine standard (top) represents the same large peak in a SPM horse (MC) (middle) but is absent in the reference control serum (bottom).

abnormal organic acids in urine and, if possible, detection of the active metabolite MCPA-CoA, its carnitine ester or glycine conjugate. The serum acylcarnitine and urine organic acid profiles of 7/7 SPM horses were consistent with MADD [11,12]. Since demonstrating MCPA-CoA esters or conjugates in serum/plasma or urine is difficult owing to the rapid elimination of these compounds [17], it is highly significant that MCPA-carnitine and MCPA-glycine were identified in 4/4 and 6/7 SPM horse samples, respectively. Variation in concentrations of MCPA-carnitine and MCPA-glycine between SPM horses may be due to variation in sample collection time relative to disease onset or differences in the amount of toxin ingested. An ideal control group would have included serum and urine from unaffected horses on the same pastures as affected horses, as well as samples obtained in October from horses housed on the control farms. Unfortunately, owing to restrictions on timing and the retrospective nature of much of this study, this was not possible.

Following ingestion, hypoglycin A is rapidly metabolised to MCPA in the mitochondrial matrix [25] through the same pathways used for other branched chain amino acids [26]. The initial step is catalysed by an aminotransferase that has relatively high activity in skeletal muscle [26]. MCPA-CoA serves as a substrate for, and irreversibly inhibits, short and medium chain acyl-CoA dehydrogenases [27] essential for β-oxidation of fatty acids [27,28]. In addition, through sequestration of CoA and carnitine, MCPA also inhibits the carnitine-acyl-CoA transferase system required for transportation of long chain fatty acids into the mitochondria, impairing mitochondrial β-oxidation of long chain fatty acids [29]. Amino acid metabolism (isovaleryl-, 2-methylbutyryl-, glutaryl-CoA dehydrogenases) is disrupted by MCPA through covalent modification of the flavin adenine dinucleotide [30]. The MCPA found in afflicted horses' serum therefore would have a major impact on energy generation, particularly when horses are mobilising fat because of a negative energy balance. A negative energy balance was probably promoted in SPM horses by offering little supplemental feed while horses were housed on overgrazed pastures, in some cases during inclement weather conditions.

Although lipid storage myopathy and severe rhabdomyolysis were the predominant clinical features of purported hypoglycin A-induced MADD in horses, human hypoglycin A toxicity is characterised by severe vomiting followed by seizures, loss of consciousness, and possibly death [16,17]. Rats, like man, develop severe hypoglycaemia and hypothermia after

ingestion of hypoglycin, and susceptibility to the toxin is reported to decrease from guinea pigs, rabbits, dogs, cats, rats to mice [31]. Scarce human clinical laboratory data with unripe ackee fruit hypoglycin toxicity describe profound hypoglycaemia, severe metabolic acidosis, and less than a 3-fold increase in aspartate transaminase (AST) activity with normal alkaline phosphatase and γ-glutamyl transferase activities [26,27]. Thus, in contrast to the primary myopathy in horses, in man, the reported site of action of MCPA-CoA appears to be the liver, with histopathological evidence of microvesicular steatosis and clinical evidence of hypoglycaemia potentially arising from increased reliance on glucose owing to reduced lipid metabolism, depletion of liver glycogen reserves, and impairment of gluconeogenesis from amino acids [28,32]. Glutamate peptides in unripe ackee fruit are thought to contribute to neurological signs in humans [30].

The maximum tolerated dose for hypoglycin A fed to rats in ackee seeds is 1.5 mg/kg bwt/day [33]. Conversion of this dose to the horse using body surface area and the equation 1.5 mg/kg bwt x $500^{0.75}/6$ (rat km value) yields a maximum tolerated dose of 26.5 mg/horse. This would correspond to as few as 165 seeds at the highest concentration of hypoglycin/seed (160 μg/seed) and over 8000 at the lowest concentration. The maximal fecundity for box elders has been estimated at 5×10^5 samaras per tree per year [34]; thus horses on pastures could easily consume more than this maximum tolerated daily dose. These numbers are highly speculative, as it is very possible that horses succumb to hypoglycin A toxicity at levels very different from the rat. Further studies are needed to determine the lethal dose of hypoglycin A and factors that affect the highly variable hypoglycin A seed concentrations, including effects of seed maturity, temperature fluctuations, rainfall, sunlight and more [34,35]. Hypoglycin A concentrations were not measured in box elder seeds from the control farms because sample collection occurred in May when seeds were immature.

The range of the box elder tree native to North America overlaps the cases of SPM we have identified, extending from California across Alberta and Ontario to New York in the north and southern Texas to central Florida in the south [34]. It is also found locally in other states and provinces and grows preferentially in hollows along streams. The box elder tree was introduced into Europe (Eschen-Ahorn, Germany; Vederesdoorn, The Netherlands) as early as 1688 and is found as a wind-break and shelter-belt tree [34]. The female tree bears large numbers of seed-containing samaras in the autumn, many of which remain on the tree through the winter into the spring. Because of weak wood, fallen branches and trunks are common. The common epidemiology of SPM and AM and the presence of other hypoglycin-containing *Acer* spp. in Europe (*Acer pseudoplatanus*) [18] lead to the possibility that SPM and AM are both caused by ingestion of hypoglycin A.

While the results of this study implicate seeds of the box elder tree in the pathogenesis of SPM, it is important to note that horses on over half of the control pastures had access to box elder tree seeds. Whether horses ingest a toxic dose of hypoglycin A is probably affected by the number of box elder trees near a pasture; the number of samaras on a given tree; the amount of hypoglycin A in seeds; the degree of negative energy balance in the horse; and the predisposition of horses to eat seed-containing samaras. Subjectively, more box elder trees were present on SPM vs. control farms, high winds may have increased samaras on pasture, and the lack of other supplemental feeds may have promoted ingestion of box elder seeds by affected horses. It is important to note, however, that in this paper the characteristics of pastures where affected horses were housed were described retrospectively by 7/12 owners, which could have introduced some recall bias. With these preliminary data, SPM and AM outbreaks could be diminished by removing access to box elder seeds and providing supplemental feed while horses are on pasture. Clearly, further research is necessary to confirm this association between SPM and hypoglycin A in seeds from box elder trees, and to determine in Europe whether AM is also correlated to exposure to hypoglycin A in box elder or other *Acer* spp.

In conclusion, this study supports hypoglycin A, present in the seeds of box elder trees, as the cause of SPM based on the abundance of hypoglycin A in box elder seeds from SPM pastures and the presence of the toxic metabolite of hypoglycin A, MCPA, in the serum and urine of affected horses. Since MCPA is a specific inhibitor of multiple acyl-CoA dehydrogenases, the pathophysiological mechanism responsible for SPM and AM, box elder seeds are strong candidates as the causal agent of SPM.

Authors' declaration of interests

No conflicts of interest exist for any of the authors.

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Authorship

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References

1. Pope, D.C. and Heslop, C.H. (1960) An outbreak of myoglobinuria in light horses. *Can. Vet. J.* **1**, 171-174.
2. Finno, C.J., Valberg, S.J., Wunschmann, A. and Murphy, M.J. (2006) Seasonal pasture myopathy in horses in the midwestern United States: 14 cases (1998-2005). *J. Am. Vet. Med. Ass.* **229**, 1134-1141.
3. Bowen, J.N. and Craig, J.F. (1942) Myoglobinuria in horses. *Vet. Rec.* **35**, 354.
4. Whitwell, K.E., Harris, P. and Farrington, P.G. (1988) Atypical myoglobinuria: an acute myopathy in grazing horses. *Equine Vet. J.* **20**, 357-363.
5. van Galen, G., Marcillaud, P.C., Saegerman, C., Patarin, F., Amory, H., Baily, J.D., Cassart, D., Gerber, V., Hahn, C., Harris, P., Keen, J.A., Kirschvink, N., Lefere, L., McGorum, B., Muller, J.M., Picavet, M.T., Piercy, R.J., Roscher, K., Serteyn, D., Unger, L., van der Kolk, J.H., van Loon G., Verwilghen, D., Westermann, C.M. and Votion, D.M. (2012) European outbreaks of atypical myopathy in grazing equids (2006-2009): spatiotemporal distribution, history and clinical features. *Equine Vet. J.* **44**, 614-620.
6. Votion, D.M., Linden, A., Delguste, C., Amory, H., Thiry, E., Engels, P., van Galen, G., Navet, R., Sluse, F., Serteyn, D. and Saegerman, C. (2008) Atypical myopathy in grazing horses: a first exploratory data analysis. *Vet. J.* **180**, 77-87.
7. Votion, D.M. and Serteyn, D. (2008) Equine atypical myopathy: a review. *Vet. J.* **178**, 185-190.
8. Unger-Torroledo, L., Straub, R., Lehmann, A.D., Graber, F., Stahl, C., Frey, J., Gerber, V., Hoppeler, H. and Baum, O. (2010) Lethal toxin of *Clostridium sordellii* is associated with fatal equine atypical myopathy. *Vet. Microbiol.* **144**, 487-492.
9. van der Kolk, J.H., Wijnberg, I.D., Westermann, C.M., Dorland, L., de Sain-van der Velden, M.G., Kranenburg, L.C., Duran, M., Dijkstra, J.A., van der Lugt, J.J., Wanders, R.J. and Gruys, E. (2010) Equine acquired multiple acyl-CoA dehydrogenase deficiency (MADD) in 14 horses associated with ingestion of Maple leaves (*Acer pseudoplatanus*) covered with European tar spot (*Rhytisma acerinum*). *Mol. Genet. Metab.* **101**, 289-291.

10. Cassart, D., Baise, E., Cherel, Y., Delguste, C., Antoine, N., Votion, D., Amory, H., Rollin, F., Linden, A., Coignoul, F. and Desmecht, D. (2007) Morphological alterations in oxidative muscles and mitochondrial structure associated with equine atypical myopathy. *Equine Vet. J.* **39**, 26-32.
11. Westermann, C.M., Dorland, L., Votion, D.M., de Sain-van der Velden, M.G., Wijnberg, I.D., Wanders, R.J., Spliet, W.G., Testerink, N., Berger, R., Ruiter, J.P. and van der Kolk, J.H. (2008) Acquired multiple acyl-CoA dehydrogenase deficiency in 10 horses with atypical myopathy. *Neuromuscul. Disord.* **18**, 355-364.
12. Sponseller, B.T., Valberg, S.J., Schultz, N.E., Bedford, H., Wong, D.M., Kersh, K. and Shelton, G.D. (2012) Equine multiple acyl-CoA dehydrogenase deficiency associated with seasonal pasture myopathy in the Midwestern United States. *J. Vet. Intern. Med.* **26**, 1012-1018.
13. Olsen, R.K., Andresen, B.S., Christensen, E., Bross, P., Skovby, F. and Gregersen, N. (2003) Clear relationship between ETF/ETFDH genotype and phenotype in patients with multiple acyl-CoA dehydrogenation deficiency. *Hum. Mutat.* **22**, 12-23.
14. Bosch, A.M., Abeling, N.G., Ijlst, L., Knoester, H., van der Pol, W.L., Stroomer, A.E., Wanders, R.J., Visser, G., Wijburg, F.A., Duran, M. and Waterham, H.R. (2011) Brown-Vialetto-Van Laere and Fazio Londe syndrome is associated with a riboflavin transporter defect mimicking mild MADD: a new inborn error of metabolism with potential treatment. *J. Inherit. Metab. Dis.* **34**, 159-164.
15. Frerman, F.E. and Goodman, S.I. (1985) Deficiency of electron transfer flavoprotein or electron transfer flavoprotein : ubiquinone oxidoreductase in glutaric acidemia type II fibroblasts. *Proc. Natl. Acad. Sci. U.S.A.* **82**, 4517-4520.
16. Meda, H.A., Diallo, B., Buchet, J.P., Lison, D., Barennes, H., Ouangre, A., Sanou, M., Cousens, S., Tall, F. and Van de, P.P. (1999) Epidemic of fatal encephalopathy in preschool children in Burkina Faso and consumption of unripe ackee (*Blighia sapida*) fruit. *Lancet* **353**, 536-540.
17. Joskow, R., Belson, M., Vesper, H., Backer, L. and Rubin, C. (2006) Ackee fruit poisoning: an outbreak investigation in Haiti 2000-2001, and review of the literature. *Clin. Toxicol. (Phila)* **44**, 267-273.
18. Fowden, L. and Pratt, H.M. (1972) Cyclopropylamino acids of the genus *Acer*: distribution and biosynthesis. *Phytochemistry* **12**, 1677-1681.
19. Schultz, N. (2010) Assessment of intramyofiber lipid content in horses. ACVIM Forum: 2010; Anaheim, California: Journal of Veterinary Internal Medicine: 780-781.
20. Cumming, W.J.K., Fulthorpe, J.J., Hudgson, P. and Mahon, M. (1994) *Color Atlas of Muscle Pathology*, Mosby-Wolfe, London.
21. Rashed, M.S., Bucknall, M.P., Little, D., Awad, A., Jacob, M., Alamoudi, M., Alwattar, M. and Ozand, P.T. (1997) Screening blood spots for inborn errors of metabolism by electrospray tandem mass spectrometry with a microplate batch process and a computer algorithm for automated flagging of abnormal profiles. *Clin. Chem.* **43**, 1129-1141.
22. Sweetman, L. (1991) Organic acid analysis. In: *Techniques in Diagnostic Human Biochemical Genetics: A Laboratory Manual*, Ed: F.A. Hommes, WileyLiss Inc, New York. pp 143-176.
23. Chen, W.P., Yang, X.Y., Hegeman, A.D., Gray, W.M. and Cohen, J.D. (2010) Microscale analysis of amino acids using gas chromatography-mass spectrometry after methyl chloroformate derivatization. *J. Chromatogr. B. Analyt Technol Biomed Life Sci.* **878**, 2199-2208.
24. Tanaka, K., Isselbacher, K.J. and Shih, V. (1972) Isovaleric and -methylbutyric acidemias induced by hypoglycin A: mechanism of Jamaican vomiting sickness. *Science* **175**, 69-71.
25. Salaun, J. (2000) Cyclopropane derivatives and their diverse biological activities. *Top. Curr. Chem.* **207**, 2-20.
26. Kean, E.R. (1989) Hypoglycin. In: *Toxicants of Plant Origin*, Ed: P.R. Cheeke, CRC Press, Boca Raton, Florida. pp 229-262.
27. Saraya, A. and Madan, K. (2008) Toxic liver injury. In: *Tropical Hepatogastroenterology*, Ed: B.N. Tandon, Elsevier, New Delhi, India. pp 344-353.
28. Ikeda, Y. and Tanaka, K. (1990) Selective inactivation of various acyl-CoA dehydrogenases by (methylenecyclopropyl)acetyl-CoA. *Biochim. Biophys. Acta* **1038**, 216-221.
29. Gregersen, N. (1985) The acyl-CoA dehydrogenation deficiencies. Recent advances in the enzymic characterization and understanding of the metabolic and pathophysiological disturbances in patients with acyl-CoA dehydrogenation deficiencies. *Scand. J. Clin. Lab. Invest. Suppl.* **174**, 1-60.
30. Barceloux, D.G. (2008) Akee fruit and Jamaican Vomiting Sickness (*Blighia sapida* Köenig). In: *Medical Toxicology of Natural Substances: Foods, Fungi, Medicinal Herbs, Toxic Plants, and Venomous Animals*, Ed: D.G. Barceloux, John Wiley & Sons, Hoboken. pp 34-38.
31. Wenz, A., Thorpe, C. and Ghisla, S. (1981) Inactivation of general acyl-CoA dehydrogenase from pig kidney by a metabolite of hypoglycin A. *J. Biol. Chem.* **256**, 9809-9812.
32. Sherrat, H.S.A. (1986) Hypoglycin, the famous toxin of unripe Jamaican ackee fruit. *Trends Pharm. Sci.***7**, 186-191.
33. Blake, O.A., Bennink, M.R. and Jackson, J.C. (2006) Ackee (*Blighia sapida*) hypoglycin A toxicity: dose response assessment in laboratory rats. *Food Chem. Toxicol.* **44**, 207-213.
34. Mêdrzycki, P. (2012) Invasive alien species fact sheet – *Acer negundo*. Online Database of the European Network on Invasive Alien Species – NOBANIS. http://www.nobanis.org/files/factsheets/Acer_Negundo.pdf. Accessed 6-10-2012.
35. Bowen-Forbes, C.S. and Minott, D.A. (2011) Tracking hypoglycins A and B over different maturity stages: implications for detoxification of ackee (*Blighia sapida* K.D. Koenig) fruits. *J. Agric. Food Chem.* **59**, 3869-3875.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig S1: MCPA-glycine spectra. A. Spectrum of diTMS derivative of synthesised MCPA-glycine with molecular ion at *m/z* 313 and M-15 at *m/z* 298 with a large *m/z* 247 typical of diTMS derivatives. B. Spectrum of diTMS derivative of MCPA-glycine from urine of an SPM horse (CL).