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Authors

Bochnia, M
Ziegler, J
Sander, J
et al.

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Hypoglycin A Content in Blood and Urine Discriminates Horses with Atypical Myopathy from Clinically Normal Horses Grazing on the Same Pasture

M. Bochnia[1]*, J. Ziegler[2], J. Sander[3], A. Uhlig[4], S. Schaefer[1], S. Vollstedt[5], M. Glatter[1], S. Abel[2], S. Recknagel[4], G. F. Schusser[4], M. Wensch-Dorendorf[1], A. Zeyner[1]

1 Institute of Agricultural and Nutritional Sciences Martin Luther University Halle-Wittenberg, Halle (Saale), Germany, **2** Department of Molecular Signal Processing, Leibniz Institute of Plant Biochemistry, Halle (Saale), Germany, **3** Screening Labor, Hanover, Germany, **4** Department of Large Animals Medicine, Faculty of Veterinary Medicine, Leipzig, Germany, **5** Traditional Chinese Equine Medicine, Bokholt-Hanredder, Germany

* mandy.bochnia@landw.uni-halle.de

Abstract

Hypoglycin A (HGA) in seeds of *Acer spp*. is suspected to cause seasonal pasture myopathy in North America and equine atypical myopathy (AM) in Europe, fatal diseases in horses on pasture. In previous studies, this suspicion was substantiated by the correlation of seed HGA content with the concentrations of toxic metabolites in urine and serum (MCPA-conjugates) of affected horses. However, seed sampling was conducted after rather than during an outbreak of the disease. The aim of this study was to further confirm the causality between HGA occurrence and disease outbreak by seed sampling during an outbreak and the determination of i) HGA in seeds and of ii) HGA and MCPA-conjugates in urine and serum of diseased horses. Furthermore, cograzing healthy horses, which were present on AM affected pastures, were also investigated. AM-pastures in Germany were visited to identify seeds of *Acer pseudoplatanus* and serum (n = 8) as well as urine (n = 6) from a total of 16 diseased horses were analyzed for amino acid composition by LC-ESI-MS/MS, with a special focus on the content of HGA. Additionally, the content of its toxic metabolite was measured in its conjugated form in body fluids (UPLC-MS/MS). The seeds contained 1.7–319.8 µg HGA/g seed. The content of HGA in serum of affected horses ranged from 387.8–8493.8 µg/L (controls < 10 µg/L), and in urine from 143.8–926.4 µg/L (controls < 10 µg/L), respectively. Healthy cograzing horses on AM-pastures showed higher serum (108.8 ± 83.76 µg/L) and urine concentrations (26.9 ± 7.39 µg/L) compared to control horses, but lower concentrations compared to diseased horses. The range of MCPA-carnitine and creatinine concentrations found in diseased horses in serum and urine were 0.17–0.65 mmol/L (controls < 0.01), and 0.34–2.05 µmol/mmoL (controls < 0.001), respectively. MCPA-glycine levels in urine of cograzing horses were higher compared to controls. Thus, the causal link between HGA intoxication and disease outbreak could be further

substantiated, and the early detection of HGA in cograzing horses, which are clinically normal, might be a promising step in prophylaxis.

Introduction

In horses on pasture, atypical myopathy (AM) and the similar disorder called seasonal pasture myopathy (SPM), are highly fatal forms of non exertional rhabdomyolysis occurring mainly in autumn but also less frequently in the subsequent spring [1–4]. Particular, season-dependent pasture characteristics seem to promote the incidence of AM or SPM [4], [5]. The first cases of AM were reported in 1939 in the North of Wales [6] and since the 1980s, also in various continental European countries [7–10]. The similar disease, SPM, is mainly described in North America [11], [12]. The first continental European outbreak occurred in Northern Germany in 1995 [12], whereby nearly all of the AM-affected horses died (111/115). However, the quantity of affected horses on the respective pasture is not uniform worldwide. In Europe large outbreaks with many affected horses on the same pasture are described, whereas in North America only a few horses with the same conditions develop the disease [2], [11], [13], [14]. Thus, cograzing horses exhibiting no disease symptoms have not been studied alongside affected horses.

In addition to the presence of *Acer spp. (Acer negundo, Acer pseudoplatanus)*, other pasture-related risk factors exist [14], [15]. Gusts of wind have been reported to coincide with an outbreak or were discussed to be a forerunner, respectively [4], [11], [16]. Predisposed horses for AM or SPM have been found to be predominantly young males (< 3 years; [14]) that were kept on poorly maintained pastures (often more than 12 hours a day) without supplementary feeding. Previous studies revealed increased prevalence of AM in females, but this is probably due to the more frequent exposure to pastures compared to males [4]. The prevalence in young horses might be due to the fact, that horses until the age of three years spend more time on pastures until horse owners start to work with them [4]. Several hypotheses about the biochemistry underlying disease development have been communicated in the past, but the discovery of the disruption of fatty acid ß-oxidation and amino acid metabolism by an acquired enzymatic deficiency of multiple acyl-CoA dehydrogenases (MADD) was a major step in diagnostic investigation [17]. As a consequence, there is excessive myofiber lipid storage and an abnormal production of blood acylcarnitines and urine organic acids [2], [17]. MADD is also described in humans. Clinical signs of inherited forms in humans range from a fatal neonatal condition to an adult onset mild lipid storage myopathy [18]. After ingestion of unripe Jamaican ackee fruit (*Blighia sapida*), whose seeds contain a nonproteinogenic amino acid called hypoglycin A (HGA, L-α-amino-methylenecyclopropylpropionicacid), an acquired form of MADD in humans can develop, with hypoglycaemia, continuing vomiting, and possible fatal outcome. HGA is metabolized to MCPA (methylencyclopropylacetic acid) and binds to CoA and is consequently a potent inhibitor of Acyl-CoA-dehydrogenases [19]-[20]. In horses with MADD, clinical manifestations include muscular weakness, stiffness, trembling, sweating, and myoglobinuria [1], [17]. Very indicative signs are severe acute myonecrosis of respiratory and postural muscles and less frequently the myocardium [4], [21], [22]. The serum activities of the creatine kinase (CK) and lactate dehydrogenase (LDH) are extremely elevated. The majority of horses developed recumbency and respiratory difficulties. In 75% of all cases the outcome was lethal [4], [7], [12], [15], [23].

Chase et al. [24] determined HGA concentrations in unripe ackee fruit components as high as 939, 711, and 41.6 mg/100 g of seed, aril, and husk components, respectively. Analysis of the ripe fruit components showed a remarkable decrease by about 70% in seeds. A current study conducted by Bowen-Forbes et al. [25] across two different harvest seasons of ackee fruits

shows a strong inverse relationship between HGA and hypoglycin B (HGB), a dipeptide of glutamic acid and hypoglycin, which is also toxic. HGA decreased from 80 mg/100 g in green seeds to 14 mg/100 g in ripened seeds whereas HGB increased from 16 mg/100 g to 118 mg/100 g. The authors reasoned that the rise in HGB content represents a possible detoxification mechanism of the fruit.

HGA was also found in seeds of box elder trees (*Acer negundo*, *Sapindaceae*), which belongs to the same taxonomic plant family as *Blighia sapida*. Interestingly, box elder trees were present on pastures in North America with an increased incidence of SPM in horses [16]. Additionally toxic HGA metabolites were found in urine (MCPA-glycine) and in serum (MCPA-carnitine) of affected horses suggesting the ingestion of seeds of *Acer spp*. [16]. In Europe another *Acer spp*. called *Acer pseudoplatanus* (Sycamore maple) was identified on all pastures with horses affected with AM [26]. Subsequent (> 1 year later) analysis of HGA-content in Sycamore maple seeds of AM-affected farms could detect a HGA content (3.6–252.9 µg/seed [27]; 0.74 and 7.2 mg/g seed [28]) similar to previous studies (3.0–160.0 µg/seed; [16]). In addition to the ackee fruit (*Blighia sapida*) hypoglycin A or hypoglycin-like compounds were also detected in different chestnuts by Bressler et al. [29], for example *Aesculus parviflora*, *Aesculus glabra* as well as in many other species. All studies noticed a high variation of HGA content in different plants according to the level of maturity. Unger et al. [27] also showed a high variation of HGA content in the seeds of *Acer pseudoplatanus*, but a direct link between maturity and measured HGA concentrations is missing. In order to provide toxicological evidence of ackee, Fincham [30] measured HGA in blood and plasma of human patients. However, the detection limit was too high (1.4 mg/L) because HGA is already toxic at lower concentrations. A more sensitive HGA detection method to improve the prevention of intoxication of ackee or maple poisoning was presented approximately 40 years later [28] with a detection limit for HGA of 0.35 µg/L blood. The first quantitative determination of HGA in serum samples of two AM affected horses showed a HGA content of 446.9 and 87.8 µg/L [28]. However, there was no proof between the consumption of HGA containing maple seeds and the disease.

We hypothesized that the outbreak of AM in horses on pastures in Germany was caused by the ingestion of seeds of sycamore maple tree that contained high amounts of HGA. Toxic HGA and its metabolites (MCPA-conjugates) could be identified in the serum and urine of the affected horses. Cograzing healthy horses coexisting on affected pastures were also tested to determine the concentration of HGA in order to establish a link between HGA levels in serum and urine and their clinical disease.

Material and Methods

Case selection

In autumn of 2013 and 2014, 16 horses (7 Warmbloods, 4 Haflinger, 5 ponies; comprising 7 geldings, 2 stallions, 7 mares) were studied, which were turned out on pastures in Germany and exhibited acute clinical signs of muscle pain and weakness. The farm veterinarians conducted the initial treatment and diagnosed AM based on the clinical signs and clinicopathological results. Five horses were admitted to the equine clinic of the Department of Large Animals Medicine, Faculty of Veterinary Medicine Leipzig, Germany, for intensive care where they died or were euthanized. In addition to the preliminary report, clinical signs included the sudden onset of indicator signs of acute rhabdomyolysis (e.g. myoglobinuria, stiffness, trembling, sweating), weakness, recumbency and depression, with rapid progression of signs or even unexpected death. Of the investigated cases 15 horses died and one survived. Eight serum samples and 6 ante-mortem urine samples were collected from these affected horses during the

disease. Additionally 12 horses that were clinically normal and cograzing pastures with AM cases were included in the study.

Ethics statement

Animal Care and Use Committee approval was not obtained for this study because no animals were handled specifically for this experiment. Blood and urine samples were collected by qualified veterinarians through their routine practice, in the framework of official programs aimed at the diagnosis and a possible treatment. Total duration of the blood sampling procedure did not exceed 1 min, and non-painful sampling was confirmed by the absence of any retreat behavior of the horses during the procedure. Urine samples were collected as free-catch urine. The detection of the contents of HGA and MCPA-conjugates was conducted with the owner's consent. An experienced botanist and a veterinarian executed the botanical rating and collection of seed samples on affected pastures.

Therefore, the legal restrictions do not apply, as they are waived in the case of non-experimental procedures and routine veterinary practices with livestock species (no laboratory animals).

Botanical inspection and owner interview

The AM and cograzing horses originated from 11 different pastures (pasture A-L, Fig 1). Nine affected pastures were visited directly after the occurrence of AM or at latest 14 days after the outbreak to collect seed samples. An experienced botanist and a veterinarian, with the owner´s consent, conducted the botanical rating. Digital images were taken and available trees and plants were obtained and cataloged. Samples of grass, leaves and seeds were collected. The focus was directed towards *Acer spp*., especially sycamore maple trees (*Acer pseudoplatanus*) to determine the presence of the seeds and the availability of those to the horses. Individual seed sampling in 2013 from soil was conducted according to the protocol of Valberg et al. [16] (pasture B, C, E, F). In 2014 the sampling method was adapted according to the protocol of Unger et al. [27] with pooled samples from the trees (pasture G, H, J, K, L). Pasture A and D were not visited, because

AM-pasture	A		B	C				D	E	F		G	H	J		K		L
TN	2		2	4				2	2	3		2	2	5		5		5
time on pasture[1]	24*		24	12*				6*	12*	10*		6*	6*	10*		12*		12*
AH	A1	A2	B1	C1	C2	C3	C4	D1	E1	F1	F2	G1	H1	J1		K1		L1
breed	WB		GRP	HF				GRP	WB	GRP	WB	GRP	GRP	WB		WB		WB
sex	g	g	g	m	m	m	s	g	g	m	m	m	m	s		g		g
age [yrs.]	2		6	2	2	3	3	7	1.5	4	16	5	2	10		1.5		5
date of death	9th Oct 2013		22nd Oct 2013	29th Oct 2013	30th Oct 2013	1st Nov 2013		6th Nov 2013	11th Nov 2013	16th Nov 2013	**	6th Apr 2014	22nd Apr 2014	11th Oct 2014		29th Oct 2014		Oct 2014
CG	-		B2	-				D2	E2	F3		G2	H2	J2	J3	J4	J5	K2	K3	K4	K5	L2	L3	L4	L5
breed	-		WB	-				GRP	GRP	WB		GRP	GRP	WB				WB				WB			
sex	-		m	-				m	m	m		m	m	m	m	m	m	g	g	s	m	g	g	g	m
age [yrs.]	-		***	-				***	***	***		***	***	5	20	5	5	12	16	10	15	20	9	19	17

Fig 1. Specific characteristics of AM-affected and cograzing horses dedicated to the individual AM-pasture. TN = total number of horses on individual pasture; [1] in hrs/d, *additional food stuffs (hay, concentrate); AH = affected horses, CG = cograzing horses: the denomination is made up of the letter of pasture (A-L) and the number of affected or cograzing horses on this pasture; WB = Warmblood, GRP = German Riding Pony, HF = haflinger; g = gelding, m = male, s = stallion; **survived, affect date 17th Nov 2013, ***not available.

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it was too late for seed sampling (information about these cases more than 4 weeks later). Additionally the owners were asked about the development of AM (first admission to the pasture, date of first clinical signs, composition of the herd, hours on pasture, additional food stuffs).

MCPA-conjugates analysis

Samples of affected horses obtained immediately before demise and within 3 days of the onset of AM disease were available for analysis. Acylconjugates concentrations were analyzed by ultra performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) to identify MCPA-carnitine and -glycine in equine serum and urine in a special screening laboratory in Hannover, Germany [31]. Samples of cograzing horses were tested as well. Owners gave permission for measuring MCPA-conjugates in available body fluids. Serum samples of 14 horses without any symptoms of AM and without contact to seeds of *Acer spp.* on pastures were used as controls. In these cases blood was drawn as part of routine screening or in the diagnostic work up of cases unrelated to AM. Five urine samples of horses were collected as free-catch urine from healthy horses as negative controls. All samples were frozen at -20°C until they were sent to the laboratory for analysis.

Hypoglycin A analysis

Authentic *S*-Hypoglycin A was from Toronto Research Chemicals (Toronto, ON). Plant tissue (between 100 and 300 mg) were ground to a fine powder with a mortar and pestle under liquid nitrogen and subsequently further homogenized with a mixer mill MM301 (Retsch, Haan, Germany) at a frequency of 25/s for 50 s with a single 5 mm diameter steel bead in a 2 ml Eppendorf cup. The resulting powder was extracted with 750 µl 70% (v/v) methanol containing 10 nmol of norvaline with vigorous shaking and the slurry was centrifuged twice at 10,000 g for 5 min.

HGA was analyzed by LC-ESI-MS/MS and quantified as its Fmoc-derivative according to the protocol for amino acid determination published by Ziegler et al. [32] using 25 µl of plant extract or 5 µl of serum and urine samples, respectively. HGA contents in affected and cograzing horses were determined. Owners gave permission for measuring HGA in available body fluids. Serum (n = 5) and urine (n = 4) samples of horses without any symptoms and without contact to *Acer pseudoplatanus* were used as controls. Blood in these cases was drawn as part of routine screening or in the diagnostic work up of cases unrelated to AM. Four urine samples of horses were collected as free-catch urine from healthy horses as negative controls. All samples were frozen at -20°C until analysis.

MS parameters describing the MRMs for the HGA are shown in the Supporting information (S1 Table).

Hypoglycin A calculation in mg HGA/horse

For calculation of HGA concentration in mg HGA/horse to reveal the maximal tolerated dosage (MTD) the following parameters were used:

1. measured HGA concentration in serum (µg/L) of affected, cograzing and control horses

2. estimated total blood volumes ([33], 101 ml/kg bwt), which was calculated with the bwt.

For example :
$$500 \; kg \; bwt \; * \; 101 ml \; blood/kg \; bwt = 50.50 \; L \; blood$$
$$50.50 \; L \; blood \; * \; detected \; concentration \; \mu g \; HGA/L = \mu g \; HGA/horse$$
$$(\mu g \; HGA/horse) \; / \; 1000 \; = \; mg \; HGA/horse$$

Statistical analysis

For the subsequent multiple comparison post hoc test a SAS based macro according to the protocol of Elliott and Hynan [34] was used to reveal differences between the concentrations of HGA in serum and urine of affected and cograzing horses in comparison to the controls. The macro takes into account different group sizes according to Dunn's multiple comparison procedure [34]. Since the data from HGA in urine and serum are not normally distributed, ANOVA could not be used. However, since the data are independent and continuous, and the null hypothesis of Levene's Test to test the homogeneity of variance could not be rejected, the nonparametric Kruskal-Wallis Test to test for difference among groups was applied. The null hypothesis of the Kruskal-Wallis Test was rejected for both traits, i.e. that at least 2 groups are different.

Results

Case history, outcome of owner interview and clinical findings

The affected horses comprised 3 different breeds (Warmblood, Haflinger, German Riding Pony) and included 7 geldings, 2 stallions and 7 mares aged from 1.5–16 years (Fig 1). All affected horses had a good body condition (ranged from 5.0–5.5/9) and the turnout time onto the pasture was variable between the cases (6–24 hrs/d, Fig 1). Trees were present on or around all pastures and horses had access to dead wood and leaves. Pastures were poorly maintained and the grassland was in most cases very sparse, the turf was often damaged with soil showing through and a lot of dead leaves (Fig 2).

All horses (except horse No. B1) got additional feed such as hay and cereals (oat grains) or a mixed feed. Hay was offered ad libitum at stable with free access to the pastures (personal communication with the owner) or was additionally provided at pasture with a hayrack. Cereals or mixed feeds were portioned once or twice a day. All had access to a salt or mineral block and all were routinely dewormed. A total of three horses were used for riding (B1, J1; 2–3 time per week) or driving (D1; 1–2 times per week). The other horses had not been exercised, mainly because of their age (< 3 years).

All horses exhibited typical clinical symptoms of AM, as it was described in several previous studies [1–5], [13–16]. Interestingly the horses C1, C2, C3 and C4 from the same pasture developed AM consecutively within 48 hours, although access to pasture was constrained after the first horse showed the onset of disease. Hence, although the seeds containing toxic amino acid HGA were no longer available, the horses succumbed to disease within two days (Fig 1). Horse C4 was the last horse from this pasture becoming ill and the blood sample showed a moderate increase in CK. However, this horse was euthanized at a very early stage of the disease while it was still standing. One horse died within 6 hours (F1), owners found the horse dead on pasture. The second and oldest (16 years) horse on this pasture (F2) survived, and the concentrations of CK and LDH were only moderately increased. After giving the horse intensive care (treatment included intravenous administration of fluids 70–100 mL/kg bwt/day, flunixin-meglumin over 7 days) a paralysis of the tongue and a slightly lameness was observed, but this was reversible after 4–6 weeks. Available blood samples from all horses showed a marked increase in CK (range 6,154–1,215,987 U/L, normal: Warmblood 146–346 U/L, Haflinger and Pony 181–652 U/L [35]) and LDH (range 816–43,802 U/L, normal: Warmblood 223–536 U/L, Haflinger and Pony 293–822 U/L [35]).

MCPA-carnitine and acylcarnitines in serum and urine

Serum and urine acylcarnitines profiles resembled that of MADD in all analysed samples (Tables 1 and 2). Concentrations of medium chain acyl conjugates in serum were at least ten

times higher compared to controls, whereas urine samples of affected horses exhibited a 1,00–1,000 times higher level compared to control samples. High MCPA-carnitine was found in all sera of severely diseased horses, but MCPA-glycine was present at very low concentrations. However, in urine the MCPA-glycine concentrations were high in all samples obtained from diseased animals. In all cases, high concentrations of acylcarnitines (e.g. Butyryl-, Isovaleryl-, Valeryl-carnitine) and MCPA-conjugates (MCPA-carnitine and–glycine) in serum corresponded to elevated levels in urine. Especially horse C4 showed a moderate increase beyond all parameters. However, this horse was euthanized at a very early stage of the disease and therefore detected concentrations are much lower in comparison to the other affected horses.

In healthy, cograzing horses increased concentrations of the glycine-fraction (Valerylglycine, Hexanoylglycine, MCPA-glycine) in urine were observed. The levels of Valeryl-carnitine in body fluids were higher than the concentrations in controls, but much lower compared to the concentrations of affected horses. MCPA-carnitine in serum was detectable only in very low concentrations.

Hypoglycin A assay in seeds and bodyfluids

There was a strong relationship between the presence of Sycamore maple trees (*Acer pseudoplatanus)* and the incidence of AM. Trees were either found right on the pasture or as in the

Fig 2. Pasture A: a typical AM-pasture in autumn with many trees and very sparse grassland.

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Table 1. Measured concentrations of MCPA-conjugates and acylcarnitines (C4:1 –C10:1) in serum of affected and cograzing horses compared to controls determined by UPLC-MS/MS.

Acylcarnitine and MCPA-conjugates [µmol/L]	AH						CG	Controls
	A1	A2	B1	C4	D1	E1		
MCPA-glycin	0.023	0.010	0.014	0.016	0.008	0.024	*)	*)
Valerylglycin	1.74	1.7	1.86	0.88	6.32	3.75	*)	*)
Hexanoylglycin	2.72	3.7	3.95	2.23	4.5	5.83	*)	*)
Isobutyryl-carnitine	13.00	15.88	3.57	3.0	1.75	6.73	0.63±0.09	0.54
Butyryl-carnitine	100.64	121.87	60.8	43.95	37.23	58.51	0.78±0.18	0.67
Isovaleryl-carnitine	43.1	44.9	24.32	12.38	16.46	25.78	0.22±0.04	0.18
Valeryl-carnitine	1.57	2.24	1.07	0.39	0.44	0.94	0.01±0	*)
MCPA-carnitine	0.459	0.652	0.508	0.166	0.320	0.459	0.002±0.001	*)
Hexanoyl-carnitine	29.24	39.9	34.4	11.62	8.87	25.08	0.29±0.09	0.24
Octanoyl-carnitine	2.01	2.80	2.51	0.68	0.88	2.22	0.04±0.09	*)
Decenoyl-carnitine	0.47	0.84	0.4	0.11	0.16	0.42	0.02±0	*)

AH = affected horses

CG = cograzing horses (means±sd)

*) below limit of detection (< 0.001µmol/L).

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case of pasture B the tree was about 50 meters away. However, HGA containing seeds were consistently found on all pastures (Fig 3).

Seed sampling was performed on 9 pastures with AM occurrence and the HGA concentrations of the seeds were analysed. HGA concentrations in sycamore maple seeds varied highly from seed to seed between 0.7–111.6 µg/seed. The average weight of the collected seeds was 314 mg (range from 191–428 mg). Both chosen sampling methods showed a very high variation of HGA contents (1.7–319.8 µg/g seed; Fig 4).

Table 2. Measured concentrations of MCPA-conjugates and acylcarnitines (C4:1 –C10:1) in urine of affected and cograzing horses compared to controls determined by UPLC-MS/MS.

Acylcarnitine and MCPA-conjugates [µmol/mmoL creatinine]	AH				CG	Controls
	A1	B1	D1	E1		
MCPA-glycin	0.28	1.23	1.97	1.03	0.015±0.012	*)
Valerylglycin	72.68	208.10	286.80	159.75	3.99±1.28	0.50
Hexanoylglycin	133.32	256.81	192.85	305.83	0.29±0.09	*)
Isobutyryl-carnitine	55.74	50.06	57.46	47.21	0.78±0.36	0.31
Butyryl-carnitine	105.72	83.07	59.02	207.92	0.03±0.01	0.01
Isovaleryl-carnitine	34.1	23.39	29.23	75.35	0.01±0	0.01
Valeryl-carnitine	7.8	11.72	14.74	9.31	*)	*)
MCPA-carnitine	0.34	2.05	1.98	0.92	*)	*)
Hexanoyl-carnitine	35.77	55.20	38.22	78.87	*)	*)
Octanoyl-carnitine	7.21	15.56	11.46	17.3	*)	*)
Decenoyl-carnitine	4.56	14.87	13.04	11.37	*)	*)

AH = affected horses

CG = cograzing horses (means±sd)

*) below limit of detection (< 0.0001 µmol/mmoL creatinine).

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Fig 3. Pasture B. Numerous seeds found on pasture where the closest sycamore maple tree was 50 meters away from the pasture.

Body fluids of all affected horses contained the toxic amino acid HGA (Table 3). Measured concentrations in serum ranged from 387.8–8,493.8 µg/L and in urine from 143.8–926.4 µg/L. Cograzing horses showed HGA concentrations between the controls and diseased horses, ranging from 37.8 and 328.5 µg/L (n = 12; p < 0.01,) in serum, and between 18.9 and 35.1 µg/L (n = 4; p < 0.01,) in urine. HGA content in serum and urine of control horses (no access to AM-pastures) was below the limit of detection (< 10 µg/L). The results with statistical analysis are summarized in Table 4, wherein significance is indicated by p-values < 0.05.

Hypoglycin A in mg HGA/horse

Referring measured serum HGA concentration to the bwt of the horses and their estimated total blood volumes revealed a minimal MTD in horse A1 with 17.47 mg HGA/horse and a maximal MTD in horse G1 with 128.68 mg HGA/horse (Table 5). Within the set of cograzing horses the mean of the determined HGA concentrations was used for comparison. Cograzing horses showed HGA concentrations between the controls and diseased horses with a mean of 5.99 mg HGA/horse, thereby in close proximity to the minimal MTD of horse A1. The HGA content in serum of control horses was calculated with the detection line (< 10 µg HGA/ L) and revealed amounts of < 0.5 mg HGA/horse.

Discussion

In the present study acquired MADD was diagnosed in AM horses by increases in short and medium chain serum acylcarnitines [2], [17]. In addition to the presence of MCPA-conjugates in blood or urine of affected horses, HGA, as the possible cause for developing a MADD, was measured in the seeds of *Acer pseudoplatanus* from affected pastures and in body fluids of AM horses. To our knowledge HGA levels in body fluids of affected horses during an outbreak of AM have not been reported yet. In previous studies the presence of serum MCPA-carnitine was a consistent feature [16] and was absent in horses without contact to *Acer spp.* on pastures. In this study MCPA-carnitine concentrations in serum of affected horses were highly variable and ranged from 166–652 nmol/L. The highest concentration (4.8–102.4 nmol/L) reported by Valberg et al. [16] is within the range of our results. Votion et al. [13] measured lower concentrations of 20.4 μ 17.2 nmol/L. Since the use of the same internal standard for analysis (octanoylcarnitine) allows the comparison of the results between the studies these differences are most likely due to the amount of ingested toxic material and metabolism. The lowest concentration of MCPA-carnitine in this study was found in a horse (C4) exhibiting the lowest concentration of short and medium chain acylcarnitines (Valeryl-, Hexanoyl-, Isovaleryl-, Valeryl-, Octanoyl- and Decenoylcarnitine), but a high concentration of HGA in serum, respectively. Possibly the early euthanasia of this horse led to an immediate stop of metabolism of the toxic amino acid right after the beginning of intoxication. At this pasture C with 4 Haflinger horses, one after another developed an AM, although the horses did not have access to AM affected pasture after the first horse became ill. Within 3 days all horses died (C1-C3) or were euthanized (C4), respectively. Possibly different diet preferences of the horses on pastures increased the toxicity of HGA. Previous studies revealed different amounts of HGA as an acute toxic dose (130–150 mg HGA/kg bwt [36], 100 mg HGA/kg bwt [37]) or as the MTD (1.5 mg HGA/kg bwt [38]) leading to the conclusion that the susceptibility of animals to HGA is influenced by carbohydrate/protein ratio in their diet or the composition of the administered hypoglycin preparation.
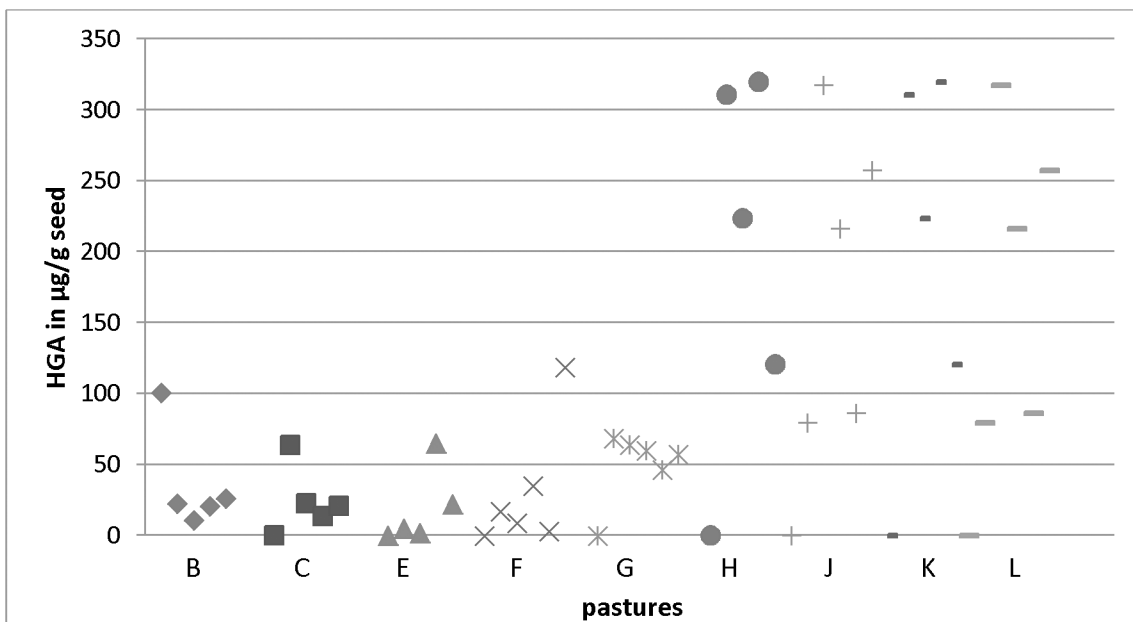

Fig 4. Hypoglycin A concentration in seeds of *Acer pseudoplatanus* collected from affected pastures detected by LC-ESI-MS/MS analysis. pasture B,C,E,F sampled in 2013; pasture G,H,J,K,L sampled in 2014.

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Table 3. Measured concentrations of HGA in serum and urine of affected and cograzing horses.

Horse group and No.		HGA [µg/L]	
		serum	urine
AH	A 1	387.8	157.9
	A 2	468.1	*
	B 1	1734.3	143.8
	C 4	878.4	*
	D 1	4032.6	531.6
	E 1	2058.6	338.4
	G 1	8493.8	191.8
	H 1	4014.3	926.4
CG	J 2	328.5	30.8
	J 3	95.3	22.8
	J 4	197.5	35.1
	J 5	81.8	18.9
	K 2	76.8	*
	K 3	56.5	*
	K 4	46.4	*
	K 5	84.6	*
	L 2	37.8	*
	L 3	66.7	*
	L 4	66.9	*
	L 5	166.4	*

AH = affected horses
CG = cograzing horses
*sample not available.

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Concentrations of HGA in seeds of *Acer pseudoplatanus* from AM pastures ranging from 0.7 to 111.6 µg/seed are similar to previously reported values with 4–56 µg/seed [16] and 3.6–252.9 µg/seed [27]. The latter showed high variation of HGA content in individual seeds collected from a single tree. Even though pooled samples (five seeds, triplicated samples) were used, the interpretation of the results remains difficult. A direct link between measured HGA

Table 4. Statistical analysis according to Dunn's multiple comparison procedure within the measured HGA parameters.

HGA in µg/L	Kruskal-Wallis test p-value	group comparison	Dunn's test p-value
urine	0.0038	AH vs. CG	<0.0001
		AH vs. controls	<0.0001
		controls vs. CG	>0.05
serum	<0.0001	AH vs.CG	0.00734
		AH vs. controls	<0.0001
		controls vs. CG	>0.05

AH = affected horses
CG = cograzing horses
significance is indicated by p-values < 0.05, results show that the groups CG and controls did not differ among each other, but both groups (CG, controls) are different from AH.

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Table 5. HGA concentration in mg HGA/horse subjected to the blood volumes of the horsesHGA.

HGA	AH								CG	Controls
	A1	A2	B1	C4	D1	E1	G1	H1		
[µg/L]serum	387.8	468.1	1734.3	878.4	4032.6	2058.6	8493.8	4014.3	108*	< 10
bwt in kg	446	450	368	300	350	450	150	130	550	500
BV** [L]	45.04	45.45	37.17	30.30	35.35	45.45	15.15	13.13	55.55	50.50
[mg]/horse	17.47	21.28	64.46	26.62	142.55	93.56	128.68	52.71	5.99	< 0.5

AH = affected horses

CG = cograzing horses

*mean of CG (n = 12)

**BV = total blood volume: calculated with 101 ml/kg bwt [33].

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amounts in seeds and the number of affected horses cannot be drawn. Farms without reported AM cases showed the highest concentrations of HGA in the collected seeds from these pastures [27].

The level of HGA in affected horses on AM-pastures calculated with their bwt and the estimated total blood volume is similar to the MTD in rats as determined by Blake et al. [38] using ackee as the source. Valberg et al. [16] converted the MTD for rats to a MTD for horses by using body surface area and the equation 1.5 mg HGA/kg bwt * bwt $^{0.75}$/6 (rat km value) and determined a MTD of 26.5 mg HGA/500 kg horse. This calculation can be confirmed by our results because the level of HGA in the present study ranged from 17.47 mg HGA/horse and 128.68 mg HGA/horse and especially the minimal dosage is in the field of the calculation of Valberg et al. [16]. Extrapolating 26.5 mg HGA/horse on the highest measured HGA concentration (820.8 µg/seed, [27]) 32 seeds would be sufficient to poison a 500 kg horse. Especially in autumn, when the first seeds drop to the ground, for example after a windstorm, the percentage of seeds with high HGA content available on the surface of the pasture is very high, and horses would probably not hesitate or even would not notice the ingestion of a limited number of seeds. However, to evaluate the HGA concentrations in the horse it is important to also look at the measured concentrations of the MCPA-conjugates. Since HGA is rapidly metabolized to MCPA, both values comprise essential information about HGA ingestion. Thus, looking at only body fluid HGA levels possibly underestimates the amount of HGA ingested by the horse.

Valberg et al. [16] included horses without clinical symptoms cograzing on the same pasture as an "ideal" control group. However, these horses incorporate HGA, although at lower concentrations (~5.99 mg HGA/horse, Table 5). The ingestion of seeds of *Acer pseudoplatanus* from cograzing horses on pastures was demonstrated [39]. Since the seeds have no characteristics which make them unpalatable to horses, there is a chance that they will be consumed during grazing. If these consumed seeds contain a high concentration of HGA, it could result in poisoning [40]. An ideal control group consists of horses without access to seeds of *Acer spp*. This distinction is necessary because both groups are clinically unaffected, but the likelihood of developing an AM-disease is higher within the cograzing horses because of detectable concentrations of HGA and MCPA-conjugates.

Currently we are unable to standardise the MTD of HGA for horses, but suggestions about possible quantities can be deduced based on our results. Concentrations of HGA in cograzing horses were higher than in controls but always lower compared to affected horses, although some of these horses (Table 3) showed HGA-contents in close proximity to affected horses. This assumption leads to the conclusion that the detected HGA concentrations in horses and

therefore a possible intoxication mainly depends on i) the high variation of HGA levels in seeds and thus ii) the availability of highly toxic seeds and iii) the different feed preferences of the individual horse as well as iv) the time of blood sampling (before or after a peak). Obviously, cograzing horses as well as affected horses ingested HGA containing material (most likely *Acer pseudoplatanus* seeds, [39]), but the cograzing group did not acquire the necessary toxic amount of HGA at the time of sampling. The detection of low but elevated HGA concentration in blood samples of cograzing horses should result in a complete closing off the pasture to all horses. Still, clinical symptoms may develop shortly afterwards, but an early initiated therapy may decelerate the metabolism of HGA to the toxic MCPA-conjugates and prognosis could be improved.

Conclusions

This study correlated an outbreak of AM with high contents of HGA in seeds of *Acer pseudoplatanus* on affected pastures and in body fluids of diseased horses. Although the horses in our study obtained additional foodstuffs (hay, mixed feeds) and had in most cases only limited access to the pasture, they ingested enough HGA to be poisoned. The recommendation of preventing horses from grazing on affected pastures with *Acer pseudoplatanus* is indispensable, however, the analysis of HGA in horses grazing on pastures with *Acer pseudoplatanus* without reported cases allows a quick survey, whether or not HGA was ingested. We suggest that the availability of a test for early detection of HGA in clinically normal horses might represent a valuable diagnostic tool for prophylaxis, providing information on the HGA-status of horses at risk, similar to a diagnostic marker.

The causal chain of HGA availability by *Acer pseudoplatanus* seeds, HGA ingestion and HGA as well as toxic metabolites in body fluids of affected horses, was further substantiated by our study. Although sycamore maple seeds were the likely source of toxin in all horses in the present study other related trees or plants as a cause for the disease cannot be excluded.

Supporting Information

S1 Table. MS parameters for MRM-transitions.
(DOCX)

Acknowledgments

Author Contributions

Conceived and designed the experiments: MB SS AZ. Performed the experiments: MB AZ. Analyzed the data: JZ JS. Contributed reagents/materials/analysis tools: AU SR GFS SA MG. Wrote the paper: MB JZ SV MW AZ.

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