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Hypoglycin A Concentrations in Maple Tree Species in the Netherlands and the Occurrence of Atypical Myopathy in Horses

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Background: Atypical myopathy (AM) in horses is caused by the plant toxin hypoglycin A, which in Europe typically is found in the sycamore maple tree (*Acer pseudoplatanus*). Owners are concerned about whether their horses are in danger if they graze near maple trees.

Hypothesis/Objectives: To measure hypoglycin A in the most common maple tree species in the Netherlands, and to determine whether concentration of toxin is a predictor of AM in horses.

Methods: A total of 278 samples of maple tree leaves, sprouts, and seeds were classified by species. Mean concentrations of hypoglycin A were compared for the type of sample, the season and the occurrence of AM in the pasture (non-AM versus AM). Statistical analysis was performed using generalized a linear model (SPPS22).

Results: Almost all *Acer pseudoplatanus* samples contained hypoglycin A, with concentrations differing significantly among sources ($P < .001$). Concentrations were significantly higher in seeds from the AM group than in seeds from the non-AM group (856 ± 677 and 456 ± 358 mg/kg, respectively; $P = .039$). In sprouts and leaves this was not the case. *Acer platanoides* and *Acer campestre* samples did not contain detectable concentrations of hypoglycin A.

Conclusions and clinical importance: *Acer platanoides and campestre* seem to be safe around paddocks and pastures, whereas almost all A*cer pseudoplatanus* samples contained hypoglycin A. In all AM cases, *Acer pseudoplatanus* was found. Despite significantly higher concentration of hypoglycin A in seeds of pastures where AM has occurred, individual prediction of AM cannot be made by measuring these concentrations because of the high standard deviation.

Key words: Acer; Atypical myopathy; Horse; Pasture-associated myopathy.

Atypical myopathy (AM), the European counterpart of seasonal pasture myopathy (SPM) in the United States, was first described in Europe as an outbreak of pasture-related myopathy in 1939.[1] The first large outbreak occurred in the Autumn of 1995 in Northern Germany,[2] and in following years outbreaks were described by the Atypical Myopathy Alert Group, an epidemiological surveillance network. Established by the University of Liege, the network collects data from all over Europe for epidemiological studies and gives advice to practitioners. The underlying biochemical defect of AM, acquired multiple acyl-CoA dehydrogenase deficiency (MADD), was first described in 2007.[3,4] Since then, risk factors for AM have been identified, such as full-time pasture access, no food supplementation, changing weather conditions, and the presence of maple leaves.[5–12] Maple leaves were suggested to be a cause of AM in 2010, but at that time it was thought that European tar spot (*Rhytisma acerinum)* on the leaves was responsible.[13] In 2012, AM and SPM were identified as being the same disease.[3,14]

Abbreviations:

AM	atypical myopathy
SPM	seasonal pastern myopathy
LC–MS/MS	liquid chromatography–tandem mass spectrometry
AMAG	atypical myopathy alert group
MADD	multiple Acyl-CoA dehydrogenase deficiency
MCPA	methylenecyclopropylacetic acid

The first evidence that hypoglycin A causes AM/SPM came in 2013, when the presence of hypoglycin A was reported in the seeds of the Box elder tree (*Acer negundo*) and its toxic metabolite methylenecyclopropylacetic acid (MCPA) was identified in the serum and urine of horses with confirmed AM/SPM.[15] Because MCPA is a specific inhibitor of multiple acyl-CoA dehydrogenases, it is a probable causal agent of AM/SPM.[15] Since then, studies have confirmed the presence of MCPA in horses in Europe with AM and the existence of *Acer pseudoplatanus* in or around pastures grazed by horses with AM.[16,17] However, the concentration of hypoglycin A appears to be highly variable in the seeds of *Acer pseudoplatanus*.[17]

The genus *Acer* has over 100 species and some species have over 1000 cultivars, but these are rarely grown in or around pastures in the Netherlands. In the Netherlands, although most veterinarians, horse owners, land owners, and landscape designers are aware that maple trees may be related to AM, there is uncertainty about whether maple trees can be safely retained or planted

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around paddocks or pastures, because it is not known whether there is a difference in toxicity among different *Acer* species. The first objective of our study was to measure the concentration of hypoglycin A in the 3 most common maple tree species in the Netherlands. The second objective was to evaluate whether the occurrence of AM can be predicted by the concentration of hypoglycin A in the leaves, seeds, and sprouts of trees present in or around pastures and paddocks.

Materials and Methods

Collection of Leaves, Seeds, and Sprouts

After an information campaign in horse journals and at meetings, 278 samples of maple tree leaves, sprouts, and seeds collected by horse owners in 2014 were sent to Utrecht University (UU) and RIKILT-Wageningen UR (Figs 1, 2, 3). The samples were taken from the ground, packed in plastic, and immediately sent to the laboratory where they were stored at -20°C within 24 hours of collection. They were classified by an experienced botanist at the species level (*Acer pseudoplatanus*, *Acer platanoides,* or *Acer campestre*) on the basis of the samples and accompanying photographs sent by owners. Sprouts were only used in this study upon positive classification by means of accompanying leaves, seeds or both. The minimum amount of material was 50 g for seeds and sprouts and 100 g for leaves. Owners were asked to complete a questionnaire with information about date of collection, address of the pasture, species of maple tree (in their opinion), signalment (name, breed and age) of the horse(s), and presence of disease (AM) in horses that were grazed in the pastures where the material was collected. If the pasture had contained horses with AM, details about the disease (including date of onset and the final outcome), laboratory results and name of the veterinarian were provided. The named veterinarian was contacted to confirm the diagnosis and provide more details. Inclusion criteria to confirm AM were recumbency, dark colored urine, high serum activity of creatine kinase (CK, either too high to measure in practice, or >100,000 IU/L in a laboratory) and postmortem examination, which was performed in 4 cases. Exclusion criterion for AM was lack of CK analysis unless postmortem examination was performed.

Fig 1. The geographic location of the pastures where the Acer seeds, sprouts and leaves were sampled.

Chemical Analysis

Hypoglycin A was measured using a newly validated method based on liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS).[a]

Seed samples (50 g, as received) were ground in a Retsch mill, and leaf samples (50–100 g) were cryogenically milled into a powder, using liquid nitrogen. One gram of homogenized sample material was extracted with 10 mL of milliQ water (mechanical shaker, 1 h). After centrifugation (5000 g, 10°C, 10 min), 10 μL of the clear extract was injected into an LC–MS/MS system, acquiring 2 transitions (m/z 142.1→74.1 and 142.1→96.0). Seed sample extracts typically were diluted 10- to 100-fold. Hypoglycin A was identified based on matching retention time and ion ratio of the 2 transitions, against an analytical reference standard (s-hypoglycin A[b]) dissolved in milli-Q water. Quantification was performed by multilevel calibration. A consistent linear relationship (residuals <20%) between the concentration of hypoglycin A and the MS-response was obtained in the range 0.01–2 mg/L, corresponding to 0.1–20 mg/kg in nondiluted extract and 10–2000 mg/kg for 100-fold diluted extracts.

The accuracy of the method was determined as the recovery obtained after spiking known amounts of hypoglycin A to blank leaf (1 mg/kg) and seed (10 mg/kg) samples in 5-fold dilutions. The average recoveries were 72% for leaf and 82% for seeds. The corresponding repeatabilities (relative standard deviation, RSD) were 11% and 7%, respectively. The RSD obtained for repetitive analysis (n = 5) of a homogenized seed sample containing hypoglycin A at a concentration of 194 mg/kg was 8%. Recoveries obtained for hypoglycin A in spiked seed samples (10 mg/kg) concurrently analyzed with the study samples were consistent with those of the validation and in the range of 73–95%.

Selection and Grouping of Samples

Samples with incomplete questionnaire data on relevant facts, such as address and obtainable data about the disease if present,

Fig 2. **A, B, C** Leaves of *Acer pseudoplatanus*, *Acer campestre* and *Acer platanoides*.

were excluded from further statistical analysis (9 samples). If multiple samples from 1 pasture were submitted, which happened often, the mean result of the analyses per sample type (sprout, seed or leaf) were counted as 1 result in further statistical analysis. This approach resulted in a reduction in sample numbers by 69. Samples that came from outside of the Netherlands also were excluded (24). Samples that could not be unambiguously assigned to an *Acer* species (6), samples that were not fresh (17), and samples that contained material other than seeds, sprouts and leaves (10) also were excluded from the study. This approach resulted in 143 usable samples, 30 from the spring and 113 from the autumn of 2014. These samples originated from 81 locations in the Netherlands. The samples were grouped by whether they came from pastures grazed by healthy horses or horses with AM (non-AM versus AM pastures). Mean concentrations of hypoglycin A were compared for the type of sample (seed versus sprout versus leaf), season (spring versus autumn) and disease status for the pasture (non-AM versus AM).

Statistical Analysis

Statistical analysis was performed using univariate generalized linear models with multiple comparisons, corrected with a posthoc Bonferroni test.[c]

Control for normal distribution was performed. A P value of .05 was set as statistically significant.

Fig 3. Acer sprouts.

Results

In total, 143 of the collected samples (93 seed samples, 14 sprout samples, and 36 leave samples) from a total of 81 locations were included (Fig 1). None of the *Acer platanoides* (n = 11) and *Acer campestre* (n = 13) samples (29 samples of seeds, 11 of leaves and 1 of sprouts) contained detectable concentrations of hypoglycin A. In contrast, most *Acer pseudoplatanus* leaves, seeds, and sprouts contained the toxin. In 2 samples of seeds and 6 samples of leaves, no hypoglycin A was found (ie, concentration below limit of quantification [1 mg/kg for leaves and 10 mg/kg for seeds]). All samples of sprouts were positive. In all AM cases, *Acer pseudoplatanus* was found. The factor season was not statistically significant ($P = .841$) and was excluded from further statistical analysis.

The mean ± SD concentrations of hypoglycin A in the samples are presented in Table 1. Hypoglycin A concentrations differed significantly among the sample types (seeds and sprouts, $P < .001$; seeds and leaves, $P < .001$; sprouts and leaves, $P = .000$). Concentrations were significantly higher in seeds from the AM group than in seeds from the non-AM group (seeds, 856 ± 677 versus 456 ± 358 mg/kg; $P = .039$). In sprouts, (1365 ± 795 mg/kg versus 1097 ± 310, $P = .493$) and leaves (31 ± 49 versus 37 ± 34 mg/kg, $P = .819$) this was not the case and when values for all seeds, sprouts, and leaves were combined the difference between non-AM and AM was not significant either ($P = .127$); (Table 1).

There were 16 horses (providing 29 samples) with AM of which 11 died and 5 survived. There was no relationship between the concentration of hypoglycin A in the samples and outcome of the disease ($P = .250$).

Discussion

Maple trees belonging to the genus *Acer* are extensively present worldwide. There are >100 species of *Acer*. To date, *Acer negundo* (box elder tree) and *Acer pseudoplatanus* (sycamore maple) have been associated with AM.[15,17]

Table 1. The concentration (mg/kg) of hypoglycin A in samples of *Acer pseudoplatanus* taken from pastures or paddocks grazed by healthy horses and horses with atypical myopathy (AM).

	Seeds		Sprouts		Leaves	
	Healthy	AM	Healthy	AM	Healthy	AM
Acer pseudoplatanus spring						
N	3	4	7	5	3	4
Mean ± SD	37 ± 64	1156 ± 1697	1094 ± 407	1365 ± 980	42 ± 33	68 ± 136
Range	0–110	146–3683	484–1584	97–2539	5–66	0–271
Acer pseudoplatanus autumn						
N	47	10	1	0	12	6
Mean ± SD	482 ± 493	736 ± 610	1119		36 ± 47	7 ± 14
Range	8–2518	25–1804			0–148	0–35
Acer pseudoplatanus total						
N	50	14	8	5	15	10
Mean ± SD	456 ± 358[a]	856 ± 677[a]	1097 ± 310	1365 ± 795	37 ± 34	31 ± 49
Range	0–2518	25–3683	484–1584	97–2539	0–148	0–271

[a]Concentration significantly different between pastures used by healthy and AM horses ($P = 0.039$).

The species *Acer campestre* (hedge maple) and *Acer platanoides* (Norway maple) did not contain detectable hypoglycin A concentrations, and thus it is probably safe, in terms of AM, to grow these species in or around pastures and paddocks. In 1973, semiquantitative measurements (not-detected, trace, weak, moderate, and strong) also indicated that these tree species were free of hypoglycin A.[18]

The most common maple tree in the Netherlands is *Acer pseudoplatanus*. We found that the leaves, sprouts, and seeds contained measurable concentrations of hypoglycin A. Although hypoglycin A concentrations were significantly lower in seeds collected from pastures grazed by healthy horses than in samples from pastures grazed by AM horses, concentrations were too variable to allow predictions to be made as to whether or not the presence of an individual tree would be a threat to horses grazing in its vicinity. Our results are consistent with a study that found hypoglycin A concentrations in seeds to be highly variable among trees on the same or different farms.[17] Hypoglycin A concentrations in our study were similar to those previously reported.[17] In the previous study, a mean concentration of 690 mg/kg was found with variation between 40 and 2810 mg/kg and in our study a mean of 856 mg/kg with variation between 25 and 3683 mg/kg was found.

The question arises as to why not all horses grazing pastures containing or surrounded by *Acer pseudoplatanus* develop AM. The most likely explanation might relate to differences in the amount of seeds, sprouts, and leaves ingested, other components of the diet, and the sensitivity of the individual horse. The availability of seeds, leaves, and sprouts largely is determined by the season and weather, with changes in wind and temperature loosening the attachment of leaves and seeds.[6,10,15,19] Indeed, inclement weather or high winds have been reported to precede clinical signs of AM.[6,12,15] Strong winds can disperse leaves and seeds over a long distance, perhaps depositing them in pastures that do not contain *Acer* trees. Seeds not consumed by horses in autumn could sprout in the spring. All of these factors could explain why *Acer* trees are not always found in the paddock or pastures grazed by horses with AM.[8]

Our results suggest that, per kilogram, sprouts are most dangerous to horses, followed by seeds, with leaves being potentially least harmful. Data show that there are more cases of AM in Autumn than in Spring.[8] Because sprouts are more common in Spring and seeds are more common in Autumn, it would appear that horses eat more seeds than sprouts either because more seeds are available or because they prefer seeds to sprouts. However, whether horses eat seeds or sprouts is to a large extent determined by the availability of other feedstuff.[20] In general, Spring pasture contains more and better grass than Autumn pasture, and thus horses may have less reason to eat other feedstuff.[20]

The finding that the concentration of hypoglycin A is high in sprouts suggests that horse owners should be alert to the presence of *Acer* sprouts in pastures. Owners should be advised to mow areas of pasture with Maple sprouts and to remove the mown material. A factor that needs further investigation is tree stress.[20,21] Tree stress or abiotic stress may increase the hypoglycin A concentration in seeds.

There are several limitations of our study. Several owners who sent in samples did not return the questionnaire or did not complete it fully. Thus, not all analyzed data were used. Moreover, if there was doubt (after consultation with the owner's veterinarian) about whether a horse suffered from AM or not, data for samples collected from that horse's paddock or pasture were not used. Many horse owners enthusiastically provided more than 1 sample. For proper statistical analysis, results of the samples of 1 type (seed, sprout, or leaf) originating from 1 pasture were pooled and the mean concentration of these was treated as 1 sample. All of these factors decreased the number of usable samples from 278 to 143. Better instructions for owners beforehand might have prevented these problems.

Another limitation is sample size. Because toxin concentrations are highly variable among trees on the same farm, it would have been better to have taken more samples of all materials. Doing so might have made the statistical outcome stronger. Reasons for not having done so were mainly financial.

The seeds were fresh frozen and not desiccated before freezing and changes in toxin concentrations as a result of artificial freezing could have occurred in the spring samples in the same fashion as occurs naturally in autumn.

Ideally all cases would have been confirmed by analyzing blood or urine for MADD or toxin or toxin metabolites. Because doing so was not always possible, the described inclusion criteria were used to make the diagnosis.

In conclusion, it is important to know the tree species when giving advice regarding the toxicity of maple trees and the potential risk for horses, because some *Acer* species contain hypoglycin A and others do not. Unfortunately, the amount of hypoglycin A in seeds is so variable that a reliable prediction of the occurrence of AM cannot be made for individual farms. Therefore, it is not worthwhile to measure hypoglycin concentrations in individual seed samples in order to predict AM risk. However, in all cases in which no hypoglycin A was found, no clinical signs of AM were observed.

Thus, the most important recommendation is that owners should prevent horses from eating seeds, sprouts, leaves, or any combination of these from *Acer pseudoplatanus* (sycamore maple), whereas the seeds of *Acer campestre* (hedge maple) and *Acer platanoides* (Norway maple) appear to be safe for horses. Steps to prevent the ingestion of toxin include moving the horse to a safer pasture, decreasing the size of the pasture (away from the trees), blowing away seeds and leaves, or mowing and removing sprouts. Adequate roughage supplementation in sparse pastures during the high-risk season also may prevent AM.

Footnotes

[a] Acquity UPLC I-class LC system coupled to a Micromass Quattro Ultima Pt triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) interface operated in positive mode (Waters, Manchester UK). Separation was achieved on a Obelisc-R, 5 μm 2.1x150mm column (Crawford Scientific Ltd, Strathaven, Lanarkshire, UK)

[b] US Biological, Salem, MA, USA

[c] IBM SPSS Statistics 22

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Conflict of Interest Declaration: Authors declare no conflict of interest.

Off-label Antimicrobial Declaration: Authors declare no off-label use of antimicrobials.

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Supporting Information

Additional Supporting Information may be found online in Supporting Information:

Figure S1. Box plot graph of the results.