Atypical myopathy-associated hypoglycin A toxin remains in Sycamore seedlings despite mowing, herbicidal spraying or storage in hay and silage

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Running head: Hypoglycin A toxin remains in sycamore seedlings despite treatment.

Summary

Background: Several pasture management strategies have been proposed to avoid hypoglycin A (HGA) intoxication in horses, but their efficacy has never been investigated.

Objectives: To evaluate the effect of mowing and herbicidal spraying on HGA content of Sycamore seedlings and the presence of HGA in seeds and seedlings processed within haylage and silage.

Study design: Experimental study.

Methods: Groups of seedlings were mowed (n = 6), sprayed with a dimethylamine-based (n = 2) or a picolinic acid-based herbicide (n = 1). Seedlings were collected before intervention, and at 48 hours and after 1 and 2 weeks. Cut grass in the vicinity of mowed seedlings was collected pre-cutting and after one week. Seeds and seedling (n = 6) samples processed within haylage and silage were collected. HGA concentration in samples was measured using a validated LC-MS-based method.

Results: There was no significant decline in HGA content in either mowed and sprayed seedlings; indeed, mowing induced a temporary significant rise in HGA content of seedlings. HGA concentration increased significantly (albeit to low levels) in grass cut with the seedlings by one week. HGA was still present in Sycamore material after 6-8 months storage within either hay or silage.

Main limitations: Restricted number of herbicide compounds tested.

Conclusions: Neither mowing nor herbicidal spraying reduces HGA concentration in Sycamore seedlings up to 2 weeks after intervention. Cross contamination is possible between grass and Sycamore seedlings when mowed together. Mowing followed by collection of Sycamore seedlings seems the current best option to avoid HGA toxicity in horses grazing contaminated pasture. Pastures contaminated with Sycamore material should

not be used to produce processed hay or silage as both seedlings and seeds present in the bales still pose a risk of intoxication.

Introduction

Equine atypical myopathy (AM) is a toxic rhabdomyolysis caused by the disruption of mitochondrial metabolism, particularly in skeletal and cardiac muscle cells, following ingestion of hypoglycin A (HGA) contained in plant material derived from some Acer tree species [1-4]. Outbreaks of AM are highly variable and seasonal, with most cases occurring in the autumn (when seeds are present on pasture) or in the spring (when seedlings germinate and grow) [5,6]. In Europe, the main source of HGA are the seeds and seedlings of *Acer pseudoplatanus* (Sycamore tree) [1-3,7,8]. Although spring outbreaks are less common than those in autumn (they account for between 4-12% of all cases seen annually [6,9]), Sycamore seedlings have the highest HGA content when compared with seeds and leaves [7,8]. Consequently, horse owners have legitimate concerns about the risk that Sycamore seedlings pose to their grazing horses, particularly given that seedlings often grow in profusion [10].

The removal of toxic material from affected pastures is the optimal preventative measure when trying to minimise risk of intoxication [11], but this is not always practical, particularly in spring pastures. In low-contaminated areas, fencing a particular section of the pasture can reduce exposure to toxic material. In contrast, highly-contaminated pastures require a more wide-ranging approach to decontaminate pastures, to avoid both intoxication of grazing animals and potentially, contamination of processed grass. This remains a huge concern among horse owners who cannot identify the best strategy to reduce AM risk, and equine practitioners struggle to provide evidence-based advice due to the lack of relevant research. Despite this, several strategies are commonly discussed within online blogs and lay equine publications: for seeds, removal with a paddock cleaner/vacuum can be a realistic option in the autumn but picking seedlings, one by one, in the spring is much more challenging and time consuming. As a result, mowing of affected pastures and/or herbicidal spraying is a common practice among horse owners. However, no data regarding the efficacy of these strategies exists. The aim of this study was to evaluate the effect of mowing and herbicide treatment in Sycamore seedlings and to examine the possible persistence of HGA in seeds and seedlings processed with hay or silage.

Materials and methods

Experimental interventions in seedling-contaminated pastures

Seedlings from 9 locations were assigned to 2 groups: mowing (n = 6) and herbicidal treatment (dimethylamine[a] n = 2; picolinic acid[b] n = 1). Dimethylamine herbicide contained 500 g/L 2/4-dimethylamine salt, and the picolinic acid herbicide contained both 240 g/L triclopyr and 60 g/L clopyralid. Mowed seedlings were cut with scissors at mid-stem length (60 seedlings/location) with surrounding grass and left to wither (Fig 1). Thereafter, 15 of the 60 seedlings/location were collected in random fashion and analysed at each time point. Seedlings were visually assessed for the presence of cotyledon leaves and/or true leaves as well as stem and leaf turgor in order to estimate their age. For the herbicide treatment, areas of 3 m by 3 m (9 m^2) containing at least 200 seedlings were sprayed with a portable hand pressure pump spray device according to the manufacturer's instructions (Fig 2).

At each site, 15 seedlings were collected before intervention and at 48 hours, 1 and 2 weeks later and grass cuttings cut alongside the seedlings were sampled pre-intervention and one week later. Seedlings in the treatment areas were always collected from at least 5 equally-dispersed regions, pooled and cryogenically milled (one sample/location/time point). Then, one gram of the obtained homogenate was extracted with methanol and HGA concentrations were measured in each sample by a validated LC-MS based method [8].

Sample pellets after extraction were dried in an oven at 80°C for 3 days, then weighed. HGA results were normalised to dry matter obtained from each sample to account for water loss during the course of the experiment. Experiments were performed in 2 consecutive years: in 2017, the experiment was performed during the first 2 weeks of June with a mowing group (n = 4 locations) and dimethylamine treatment group (n = 2 locations). Average temperature in the area where the experiment was performed was 18°C (14-21°C [range]), humidity 85% and average daily rainfall of 6 mm. In 2018, the experiment was performed during the last 2 weeks of May with a mowing group (n = 2 locations) and picolinic acid herbicide treatment group (n = 1 location). Average temperature in the area where the experiment was performed in 2018 was 15°C (10-18°C [range]), humidity 83% and average daily rainfall of 4 mm.

Presence of HGA in *A. pseudoplatanus* seeds and seedlings preserved with hay and silage

Samples were analysed in this experiment in which seedlings and seeds were maintained for 6 months in processed grass forage (hay and silage). Seedlings were collected from a pasture and sent to lab to be analysed (time zero), then bales of hay and silage were produced from that pasture and some further seedlings from the same source introduced in marked sections of the hay and silage bales (15 seedlings/bale: 2 hay and 2 silage bales) to aid later recovery of the samples. Seedlings from each bale were pooled, cryogenically milled and 1 g of the homogenate was extracted and analysed as described previously. Separately, 2 independent samples, each containing at least 25 seeds, were analysed as homogenates after being identified in hay, 8 months after baling.

Data analysis

In the experimental setting, HGA concentration among the different time points within the same intervention group was assessed using a Friedman's test, followed by Dunnett's multiple comparison test to compare pre-intervention values to those in subsequent measurements. For the grass data set, HGA concentration was compared between pre-intervention values and those at 7 days by Mann-Whitney test. Differences were considered statistically significantly different when $p \leq 0.05$ for all the analyses.

Results

There was no significant reduction in HGA concentrations from seedlings sampled before (272.2 µg/g; 49.7- 422.4 [median and range]) and at 15 days (181.8 µg/g; 92.9-25 1) in the mowing group ($p>0.9$), and before (243 µg/g; 119.4-294.2) and at 15 days (206 µg/g; 139-221.7) in the herbicide treatment groups ($p = 0.4$) (Fig 3A and Fig 4). Indeed, HGA was significantly higher in seedling cuttings after 48 h (801.9 µg/g; 193-1995; $p = 0.04$) in the mowing group compared to before mowing. Seedlings that were younger (locations 2, 3 and 4; cotyledon leaves still prominent, soft stem) had greater increases in HGA concentration at 48 h (6.9 fold ± 3.04; mean ± s.d.) than those that were older (locations 1, 5 and 6; cotyledon leaves decaying, more fibrous stem; 1.04 fold ± 0.15) (Fig 3A). Re-emergence of seedlings was not noticed in the areas from where the seedlings for the mowing experiment were collected.

Grass cuttings, sampled in the vicinity of seedling cuttings were found to be contaminated with HGA (from zero before intervention to 16.6 µg/g; 2.5-19.9 [median and range]) (Fig 3B). Grass surrounding seedlings in the herbicide treatment areas did not contain HGA at any time point, either pre-intervention or one week later.

Seeds and seedlings processed within bales of hay or in silage had detectable HGA. Duplicate samples of seedlings from the same source added to 2 separate hay bales had a HGA concentration of 271 ± 26.2 μg/g, while duplicate samples of seedlings added to silage had a concentration of 473.5 ± 50 μg/g. These values represented a reduction of 75.4% (hay) and 57.1% (silage) when compared with fresh pasture (time zero = 1103 μg/g). Seed samples that had been present in bales of hay for 8 months had appreciable amounts of HGA (105 μg/g and 256 μg/g).

Discussion

Pasture management, and in particular removal of contaminating Sycamore seedlings, is considered a key strategy to reduce incidence of AM [7,11]. In the current study, we investigated the most common practices – mowing and herbicidal spraying. We found that neither method resulted in a significant reduction or removal of intoxication risk in the time frame studied; indeed, mowing resulted in a short-term elevation in HGA content in dying seedlings. We also found that seedlings and seeds that are stored in pasture hay or silage have appreciable HGA content. All of these findings likely have significant implications for horse owners, and private and commercial equine feed manufacturers.

The choice of herbicides for this research was based on information from practices used by local farmers and owners of livery yards and online blogs. Both herbicidal products selected are auxin-mimetics that are absorbed primarily by a plant's foliage and to some extent, by the roots; they have little adverse effect on grasses when administered appropriately [12]. Auxins are hormones that allow development and reshaping of growth, enabling plants to react to the changing environment [13]. They are particularly important in the germination and early establishment of seedlings where the active transport of these molecules between apical parts (where they are produced) to the roots, ensures coordinated development of the plant [14]. The application of auxin-mimetic herbicides triggers unsustainable growth and disturbs metabolic activity [15,16]: imbalance with auxin repressors leads finally to downregulation of cell division, plant death and decay. Stem curvature, tissue swellings and foliage senescence with chloroplast damage and destruction of membrane and vascular integrity of the plant are the most evident physiological effects of these herbicides [15]. Our results showed that spraying with these herbicides did not substantially alter seedling HGA content. Therefore, use of pastures for grazing after application of this family of herbicides is not recommended, at least within the 2-week window studied here. Whether this measure might be suitable to control pastures or in areas dedicated to forage production, provided dead seedlings have fully degraded before harvesting occurs remains unknown. Further, it is unclear whether other herbicides that have a different mechanism of action might be more suitable for seedling control when use of pastures for grazing is required more quickly. Finally, the stable concentration of HGA in seedlings treated with auxin-mimetic herbicides revealed here, provides information about the pathways involved in HGA production, although the latter requires further investigation.

Mowing similarly did not reduce the concentration of HGA in decaying seedlings over a 2-week time interval; instead it resulted in a temporary rise in HGA content. The metabolic pathways that govern HGA production function and dynamics in plants are not fully understood, but our results suggest that stress, here induced by cutting, might upregulate HGA production. Conceivably, this might reflect pathways that reflect defence mechanisms designed to avoid grazing: toxic substances produced by plants are usually complex chemicals that have a deterrent and/or toxic effect [17,18]. Most chemical defences are produced by plants as secondary metabolites, often used in tissues that are vulnerable to grazing over short time spans, for example young leaves or seeds. They tend to be recycled for nitrogen storage for plant growth as the risk of grazing declines [19]. Additionally, in the seedlings that were more mature, the elevation of HGA content after intervention was less marked than in the younger seedlings. Feasibly, this might reflect a plant strategy to regulate the production of HGA as the seedling ages to promote growth to a new tree. Seedlings might conceivably be more palatable during the cotyledon and/or early leaf stages (tender, soft sprouts) and perhaps more likely to be ingested inadvertently with surrounding grass; hence the possible requirement for higher HGA-associated defences at this young age.

Grass in contact with cut Sycamore seedlings became contaminated with HGA, although the contamination was low when compared with the amount of HGA contained in Sycamore seedlings. Contamination most likely occurs through sap leakage subsequent to seedling cutting. Additional research is required to establish the persistence of this contamination for extended periods or after hay or silage manufacture. However, our data supports the need for collection and disposal of both seedlings and grass, when they are mowed together.

Our findings challenge the clinical algorithm presented by Van Galen *et al*. [6], in which horses off grass were considered at low probability of being affected by AM. Presumably dependent on relative degrees of contamination, AM might occur in stabled horses or those supplemented with feed with hay/haylage or silage from contaminated pastures. Feed producers manufacturing feeds that originated from pastures (such as forage cubes, silage and hay) should ensure that there is no contaminating Sycamore material during initial harvesting.

In summary, neither mowing nor herbicidal spraying with specific auxin-mimetic herbicides reduced HGA concentration in Sycamore seedlings up to 2 weeks after intervention. Cross contamination is possible between grass and Sycamore seedlings when mowed together. Our data suggests that currently, best practice is collection and disposal of both grass and seedling cuttings to minimise risk of intoxication. Pastures contaminated with

Sycamore material should not be used to produce hay/haylage or silage as both seedlings and seeds processed within grass remain potentially toxic.

Authors' declaration of interests

No competing interests have been declared.

Ethical animal research

Not applicable.

Owner informed consent

Not applicable.

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Authorship

S. González-Medina contributed to the study design, field and laboratory work, interpretation of results and preparation of the manuscript. F. Montesso contributed to the fieldwork. Y.-M. Chang performed statistical analysis. C. Hyde contributed to some laboratory work and critical revision of the manuscript. R. Piercy contributed to the study design, interpretation of results, preparation of the manuscript and obtained funding. All authors have reviewed the manuscript and approved it for submission.

Figure legends

Fig 1: Mowing experiment in a controlled setting. Notice the change in colour induced by the desiccation process in both seedlings and grass.

Fig 2: Picolinic acid treatment area. Notice how the seedlings steadily decayed over the course of the experiment.

Fig 3: Effect of mowing intervention in Sycamore seedlings and grass. Both graphs represent the results of individual HGA concentrations obtained at different time points in the seedlings and grass from 6 different locations (Loc.) A = seedlings and B = grass. Variation of HGA among the time points was assessed statistically by non-parametric methods, HGA variation between pre-intervention and 48 h was significant, p = 0.04. Grass data set (B) was analysed by Mann Whitney test, grass tested positive for HGA after being 7 days in contact with seedling cuttings p = 0.002. Younger seedlings (circles) and older seedlings (triangles) are represented in the graph. Notice the different HGA response to intervention at 48 h between both groups.

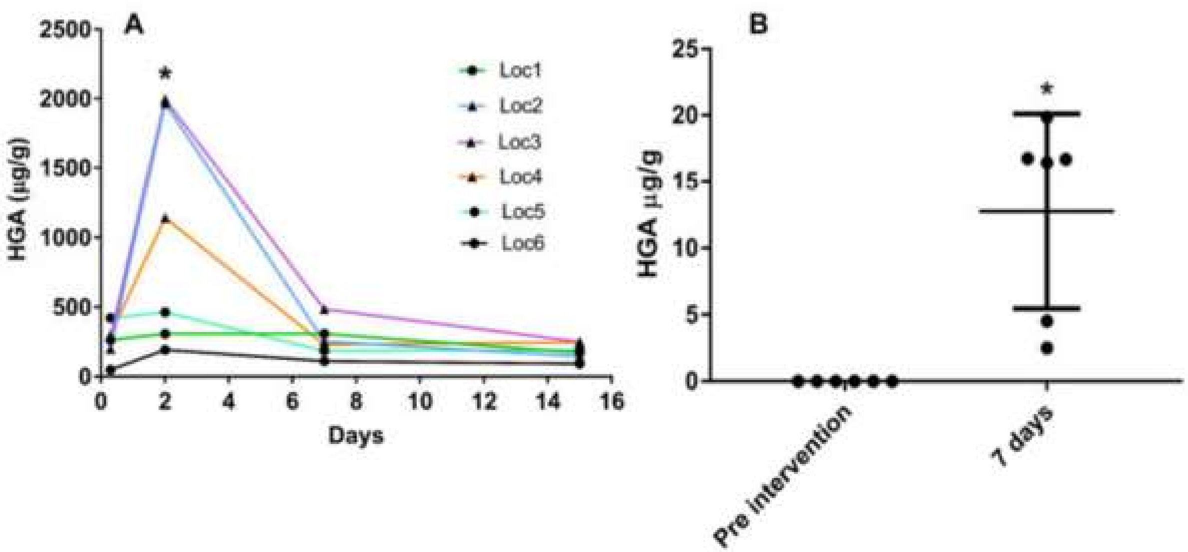

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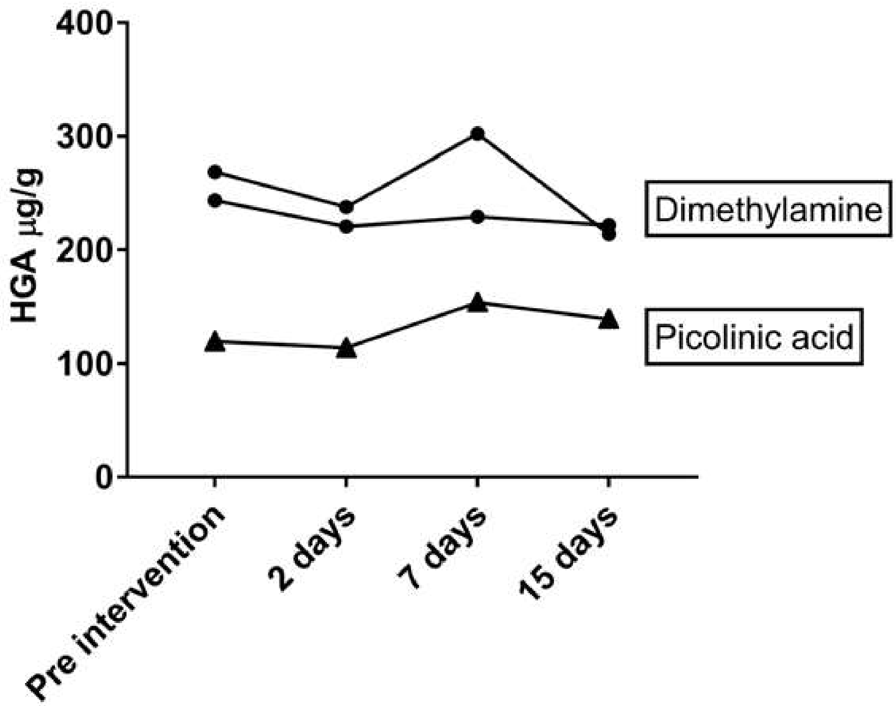
Fig 4: Effect of herbicide spraying. Two different herbicides were tested (dimethylamine n = 2 locations; picolinic acid n = 1 location). Individual results are shown. HGA concentration pre-intervention was compared with the subsequent time points by Friedman test. There was no significant difference between pre-intervention HGA contents and subsequent time points in herbicide-treated seedlings; p = 0.4.



Manufacturers' addresses

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References

[1] Baise, E., Habyarimana, J.A., Amory, H., Boemer, F., Douny, C., Gustin, P., Marcillaud-Pitel, C., Patarin, F., Weber, M. and Votion, D.M. (2016) Samaras and seedlings of Acer pseudoplatanus are potential sources of hypoglycin A intoxication in atypical myopathy without necessarily inducing clinical signs. *Equine Vet. J.* **48**, 414-417.

[2] Bochnia, M., Ziegler, J., Sander, J., Uhlig, A., Schaefer, S., Vollstedt, S., Glatter, M., Abel, S., Recknagel, S., Schusser, G.F., Wensch-Dorendorf, M. and Zeyner, A. (2015) Hypoglycin A Content in Blood and Urine Discriminates Horses with Atypical Myopathy from Clinically Normal Horses Grazing on the Same Pasture. *PLoS One* **10**, e0136785.

[3] Unger, L., Nicholson, A., Jewitt, E.M., Gerber, V., Hegeman, A., Sweetman, L. and Valberg, S. (2014) Hypoglycin A concentrations in seeds of Acer pseudoplatanus trees growing on atypical myopathy-affected and control pastures. *J. Vet. Intern. Med.* **28**, 1289-1293.

[4] Valberg, S.J., Sponseller, B.T., Hegeman, A.D., Earing, J., Bender, J.B., Martinson, K.L., Patterson, S.E. and Sweetman, L. (2013) Seasonal pasture myopathy/atypical myopathy in North America associated with ingestion of hypoglycin A within seeds of the box elder tree. *Equine Vet. J.* **45**, 419-426.

[5] Van Galen, G., Amory, H., Busschers, E., Cassart, D., De Bruijn, M., Gerber, V., Keen, J., Lefere, L., Pitel, C.M., Marr, C., Muller, J.M., Pineau, X., Saegerman, C., Sandersen, C., Serteyn, D., Torfs, S., Unger, L., Verwilghen, D. and Votion, D.M. (2010) European outbreak of atypical myopathy in the autumn 2009. *J. Vet. Emerg. Crit. Care (San Antonio)* **20**, 528-532.

[6] van Galen, G., Marcillaud Pitel, 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.

[7] Westermann, C.M., van Leeuwen, R., van Raamsdonk, L.W. and Mol, H.G. (2016) Hypoglycin A Concentrations in Maple Tree Species in the Netherlands and the Occurrence of Atypical Myopathy in Horses. *J. Vet. Intern. Med.* **30**, 880-884.

[8] Gonzalez Medina, S., Hyde, C., Lovera, I. and Piercy, R.J. (2018) Detection of equine atypical myopathy-associated hypoglycin A in plant material: Optimisation and validation of a novel LC-MS based method without derivatisation. *PLoS One* **13**, e0199521.

[9] Gonzalez-Medina, S., Ireland, J.L., Piercy, R.J., Newton, J.R. and Votion, D.M. (2017) Equine atypical myopathy in the UK: Epidemiological characteristics of cases reported from 2011 to 2015 and factors associated with survival. *Equine Vet. J.* **49**, 746-752.

[10] Jones, E.W. (1945) Biological flora of the British Isles. Acer L. *J. Ecol.* **32**, 215-252.

[11] Votion, D.-M. (2016) Atypical myopathy: an update. *In Practice* **38**, 241-246.

[12] Willoughby I, D.J. (1995) *The use of herbicides in the forest*, 4th edition edn., HMSO Publications London. p 317.

[13] Friml, J. (2003) Auxin transport — shaping the plant. *Curr. Opinion in Plant Biology* **6**, 7-12.

[14] Benková, E., Michniewicz, M., Sauer, M., Teichmann, T., Seifertová, D., Jürgens, G. and Friml, J. (2003) Local, Efflux-Dependent Auxin Gradients as a Common Module for Plant Organ Formation. *Cell* **115**, 591-602.

[15] Grossmann, K. (2007) Auxin Herbicide Action: Lifting the Veil Step by Step. *Plant Signaling & Behavior* **2**, 421-423.

[16] Sterling, T. (1997) *Mechanism of action of natural auxins and the auxinic herbicides*. pp 111-141.

[17] Laycock, W.A. (1978) *Coevolution of Poisonous Plants and Large Herbivores on Rangelands*. University of Arizona. p 335.

[18] Hulme, P., and C. W. Benkman (2002) Granivory. In: *Plant-animal interactions*, Ed: C.M.H.a.O. Pellmyr. Oxford Univ. Press, Oxford, UK. pp 132-154.

[19] Poulton, J.E. (1990) Cyanogenesis in Plants. *Plant Physiology* **94**, 401-405.