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# Hypoglycin A absorption in sheep without concurrent clinical or biochemical evidence of disease

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## Abstract

**Background:** Hypoglycin A (HGA) intoxication after ingestion of *Acer* spp. tree material has never been confirmed in domesticated ruminants despite their similar grazing habitats.

**Objectives:** To investigate whether sheep have low HGA bioavailability caused by rumen HGA breakdown.

**Animals:** Stomach and rumen fluid samples from 5 adult horses and 5 adult sheep respectively. Residual serum samples from 30 ewes and lambs.

**Methods:** Experimental and retrospective cohort study. Hypoglycin A concentration was quantified in horse gastric and sheep ruminal samples after in vitro incubation with *Acer pseudoplatanus* seeds. Serum samples from grazing sheep (n = 20) and nursing lambs (n = 10) obtained before and after their release onto pastures with and without Sycamore seedlings were analyzed for HGA and methylenecyclopropylacetic acid carnitine, and serum biochemistry.

**Results:** Neither ovine rumen nor equine gastric fluid affected HGA content in samples incubated for up to 2 hours. Despite HGA's detection in serum from sheep (n = 13/15; median, 23.71 ng/mL; range, 5.62-126.4 ng/mL) grazing contaminated pastures and in their nursing lambs (n = 2/5; median, 12.5 ng/mL; range, 8.82-15.67 ng/mL), there was no apparent clinical or subclinical disease.

**Conclusions and Clinical Importance:** Any reduced sensitivity to HGA intoxication in sheep seems unrelated to ruminal degradation. Serum HGA concentrations in sheep were similar to those of subclinically affected atypical myopathy horses. Any reduced sensitivity of sheep to HGA might be related to greater metabolic resistance rather than selective grazing habits or lower bioavailability. Hypoglycin A was found in nursing lambs, suggesting that HGA is excreted in milk.

KEYWORDS

atypical myopathy, MCPA-carnitine, seasonal pasture myopathy, sycamore seedlings, toxic myopathy

**Abbreviations:** AM, atypical myopathy; AST, aspartate aminotransferase; CK, creatine kinase; GF, gastric fluid; GGT, gamma-glutamyl transferase; HGA, hypoglycin A; MCPA-carn, methylenecyclopropyl-acetic acid carnitine; MeOH, methanol; RF, ruminal fluid.

## 1 | INTRODUCTION

Atypical myopathy (AM) is a toxic rhabdomyolysis caused by the ingestion of hypoglycin A (HGA)—contained in seeds, seedlings and leaves of some *Acer* tree species.[1-5] Hypoglycin A's principal metabolite (methylenecyclopropyl-acetic acid [MCPA]) has various metabolic effects, of which the best characterized is the inhibition of mitochondrial acyl-CoA dehydrogenase enzymes.[6,7] In the United Kingdom and Northern Europe, the principal source of HGA is Sycamore trees (*Acer pseudoplatanus*), a species found commonly adjacent to and within grazing pasture land.

Hypoglycin A is toxic in several species. In humans (unlike horses), hepatopathy and encephalopathy occur.[8-10] However, reports of HGA-associated disease in ruminants that share grazing habitats with horses) are lacking, although recently AM was confirmed in exotic deer (*Elaphurus davidianus*) kept at a zoo in Germany.[11] In the 1970s, unusual outbreaks of severe rhabdomyolysis in cattle were encountered in the United Kingdom[12-14]: these animals displayed stiffness, myoglobinuria, and recumbency with increased serum creatine kinase (CK) activity after several days grazing new pasture. A plant intoxication was suggested as the cause, but was not confirmed.[14] Nevertheless, the lack of additional or confirmed reports of domesticated ruminant intoxication, despite enhanced recognition and possible increased incidence of AM in horses,[15,16] suggests that ruminants might be less sensitive to the toxin. Consequently, and because HGA content of Sycamore seedlings is not reduced by either mowing or herbicidal spraying,[17] some horse owners resort to co-grazing or prior grazing of Sycamore seedling-contaminated pastures with ruminant species. To our knowledge, however, reduced sensitivity in ruminants has not been established.

Not all domesticated animals are equally deterred by toxic compounds present in plants. Indeed, some have developed adaptive responses that counteract toxins or prevent intoxication.[18-20] Differences in gastrointestinal physiology among species might play an important role in HGA absorption and metabolism. In domestic ruminants and equids, amino acid-like compounds are absorbed in the small intestine,[21] however, differences in stomach anatomy and physiology might influence their bioavailability. In ruminants, most protein contained in feedstuffs is degraded in the rumen and consumed by its resident microorganisms. Only a small proportion (rumen-undegradable protein) bypasses the rumen microbiota and reaches the small intestine.[21] The long gastric emptying time of the ruminant forestomach as well as the metabolic and chemical reactions triggered by the existent microflora negatively affects the viability of drugs administered orally in these species.[22,23] These physiological differences might play a major role in degradation of ingested HGA, feasibly reducing toxicity. The initial aim of this study was to investigate the hypothesis that HGA is degraded in the ovine rumen, such that it fails to be absorbed systemically after ingestion. If correct, this would confirm and explain reduced sensitivity to HGA in sheep compared to horses and add credibility to certain grazing practices to reduce incidence of AM in horses. Consequently, with access to residual samples from grazing sheep, we were able to measure HGA concentrations in ovine serum and examine any corresponding clinical or subclinical evidence of disease.

## 2 | METHODS

In vitro experiments and retrospective serum testing were performed with approval from the Royal Veterinary College's Clinical Research Ethical Review Board who assigned the following unique reference number: 2017 1708-2.

### 2.1 | Exposure of Sycamore seed homogenate to gastric fluids: Stomach and rumen

Gastric fluid (GF) from 5 adult horses and rumen fluid (RF) from 5 adult sheep was obtained immediately after each animal's euthanasia at a local abattoir (for reasons unrelated to this study). Samples were maintained at temperatures <10°C for up to 2 hours while being transferred to the laboratory. Subsequently, they were shaken and incubated at 38°C for 15 minutes in a water bath and then filtered through a 70-μm cell strainer filter (BD-Falcon) in order to remove particulate plant matter while preserving microbiota.

Seed homogenate for the experiment was prepared by grinding 400 g of seeds from a single Sycamore tree. Equine GF and RF were used as test fluids and water and methanol as controls. Each gastric and rumen fluid aliquot was sampled before addition of Sycamore homogenate. Thereafter, technical duplicates were prepared for each animal or control as follows: 4 mL of the stomach/rumen/water/methanol fluid were incubated with 1 g of seed homogenate in a water bath at 38.5°C (stomach/rumen/water) or 50°C (methanol-standard extraction)[1] and aliquots were analyzed after 1 and 2 hours. Aliquots were processed and analyzed for HGA content as previously described.[1]

### 2.2 | Serum samples

Serum from 3 groups of North Country mule ewes and lambs used in these experiments were residual samples obtained following routine (unrelated) health checks and retrospectively analyzed for HGA and MCPA-carnitine (MCPA-carn) using a technique previously described.[24] Samples were available from group 1 animals 1 day before and 2 and 7 days after their release onto pasture in the spring (2017) and from groups 2 and 3 animals, 1 day before and 2, 4, and 7 days after their release onto pasture in the spring (2018). Full biochemistry panels (urea, creatinine, magnesium, calcium, albumin, globulin, alanine aminotransferase, alkaline phosphatase, total bilirubin, gamma-glutamyl transferase [GGT], triglycerides, CK, and aspartate aminotransferase [AST]) analyzed within 1 hour of collection were available for group 1, and remaining serum was separated and kept frozen (−20°C) for a maximum of 1 week before toxicological analysis. No chemistry was available for groups 2 and 3. Animals in groups 1

and 2 had been turned out onto pastures contaminated with Sycamore seedlings and animals in group 3 had been turned out onto a Sycamore seedling-free pasture. The 3 pastures were of similar sizes (around 0.5 acres) on the same premises and the land and the sheep were under the same ownership. Group 1 was composed of 10 ewes (aged 2-4 years) and 10 lambs (aged 4 days) and serum samples were only available from ewes. Group 2 and 3 were each composed of 5 ewes and 5 lambs (aged 2-4 years and 4 days, respectively) from which serum samples were available from all animals.

### 2.3 | Seedling and grass collection and analysis

Four hundred grams of seedlings from group 1 and group 2 pastures and 400 g of grass from group 3 pasture were collected. Of this, 1 g of seedlings or grass for each group was analyzed by liquid chromatography-mass spectrometry.[1]

### 2.4 | Statistics

All statistical analyses and graphical representations were performed using commercial software (SPSS21) and/or GraphPad Prism 7.2. The Shapiro-Wilk test was used to determine the normality of the distribution in data sets, and data were Log10 transformed to achieve normality where necessary. Differences in HGA extraction among gastric and rumen fluid as well as controls (methanol and water) at 1 and 2 hours were assessed by 2-way repeated measurement analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Changes in serum HGA and MCPA-carn, AST, CK, and GGT over time were evaluated by repeated measurement ANOVA. AST, GGT, and CK enzyme activities were compared between group 1 sheep with detectable serum MCPA-carn and those without detectable MCPA-carn by Mann-Whitney *U* test. Changes in HGA concentrations within individual animals were correlated with biochemistry data for group 1, using a multivariate linear mixed-effects model with unstructured (co)variance.[25-27] Both correlation within individuals (biochemistry values in an individual associated with changes in serum HGA concentration in the same individual) and between subjects (whether individuals with higher biochemistry measurements also tend to have higher/lower value for HGA) were evaluated. In all analyses $P < .05$ was regarded as statistically significant.

## 3 | RESULTS

### 3.1 | Incubation of Sycamore seed homogenate with gastric/rumen fluid

Hypoglycin A was not detectable in gastric and rumen fluid before addition of the Sycamore seed homogenate. Neither incubation with equine gastric nor ovine rumen fluid at either time point influenced the HGA content of mixtures with Sycamore homogenate in comparison with standard methanol extraction ($P = .99$; Figure 1). Indeed, both equine GF and ovine RF enhanced the soluble HGA content of the mixture in comparison with water alone after 1 hour ($P < .01$; Figure 1).

### 3.2 | Serum investigation

All sheep in all groups remained apparently healthy over the time course of the study. Contaminated pastures had a HGA measurement in seedlings of 1368 μg/g (group 1) and 728 μg/g (group 2). No HGA was found in grass consumed by group 3. All sheep had undetectable HGA and MCPA-carn before release onto pasture. All Sycamore seedlings on the pastures were ingested within 7 days.

In group 1, HGA was detected at 48 hours in all sheep (32.2 ng/mL; 5.6-126.4 [median and range], declining significantly by day 7 [16.3 ng/mL; 5.2-22.0; $P = .03$]) (Figure 2A). MCPA-carn in this group was identified in a single ewe (11.75 ng/mL) on day 2, which also had the highest serum HGA concentration (126.4 ng/mL); trace levels of MCPA-carn (0.075 ng/mL) were also detected in 4 other ewes on day 2. GGT and AST activities were significantly higher in group 1 ewes after being released onto pasture ($P < .05$) while no significant differences were encountered in CK activity over time ($P = .09$) (Figure 2). There was no significant correlation between HGA and CK ($P > .99$), AST ($P = .82$), and GGT ($P = .93$). There was no

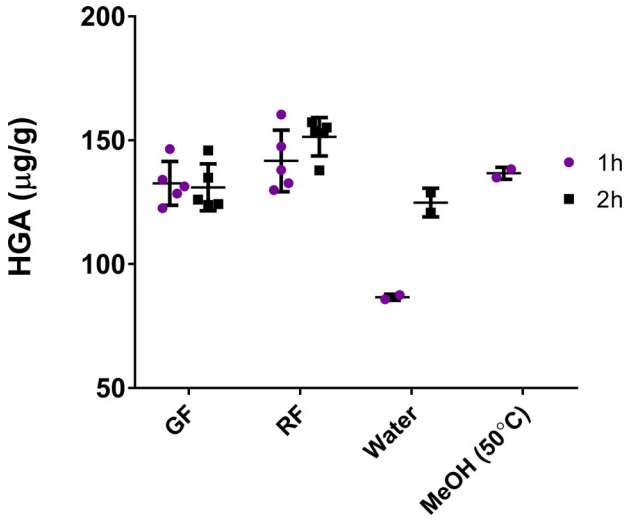


**FIGURE 1** Effect of (ex vivo) equine gastric fluid and ovine rumen fluid in a Sycamore seed homogenate extraction. Scatter plots represent individual values derived from fluid obtained from each animal at 2 time points (purple circles = 1 hour) and (squares = 2 hours). Error bars show mean and SD of 1 g seed homogenate incubated with different gastric fluid (GF) and rumen fluid (RF) samples (n = 5). Control conditions were water and standard seed extraction (methanol [MeOH] at 50°C). No significant differences were found between the gastrointestinal fluids from the different species or with methanol extraction. Both gastric fluid and rumen fluid had enhanced HGA extraction after 1 hour compared with water (*$P < .01$)

significant within subject correlation between HGA and CK ($P$ = .78), AST ($P$ = .83), and GGT ($P$ = .26) (Figure 2B1,C1,D1). Serum activities of CK ($P$ > .99), AST ($P$ = .80), and GGT ($P$ = .96) were not significantly different between sheep with detectable MCPA-carn and sheep without detectable MCPA-carn. The group 1 ewe with the highest HGA and MCPA-carn did not have elevated activity of serum enzymes in

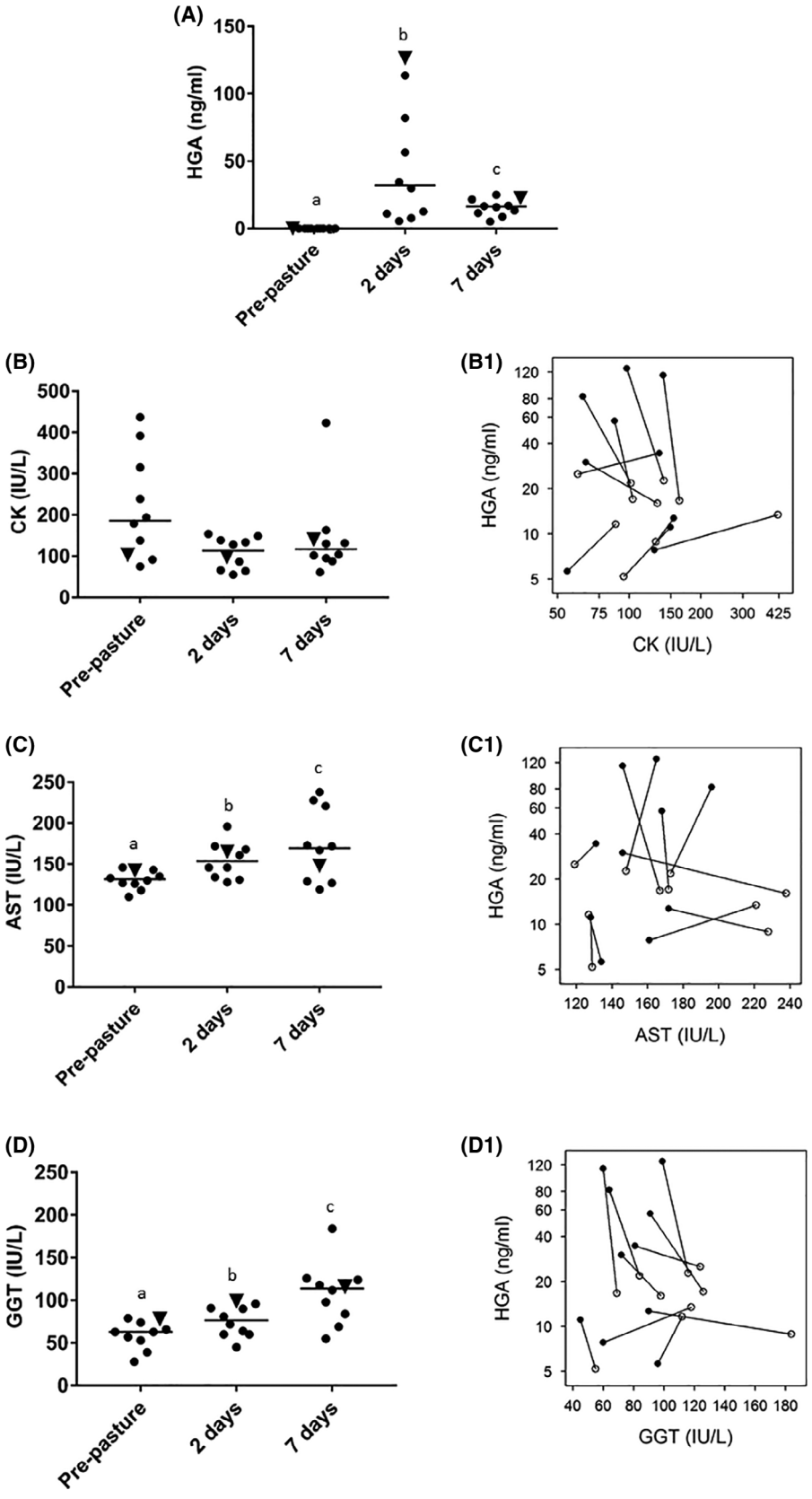


**FIGURE 2** Hypoglycin A (HGA) concentration and relevant biochemistry activity in group 1 sheep (left) and correlation between relevant biochemistry parameters and HGA concentration (right). Individual animal data is over the study period (n = 10 ewes) with the median (horizontal line). Data sets with different lower case letters are statistically significantly different. Ewe with elevated methylenecyclopropyl-acetic acid carnitine (MCPA-carn) concentration is represented with an inverted triangle. A, Serum HGA concentration was undetectable before turnout and then was significantly higher at 2 days post turnout than at 7 days; a < b $P$ = .02; a < c $P$ < .001; c < b $P$ = .08. B, Serum creatine kinase (CK) activity was not significantly different in sheep before and after grazing contaminated pasture; $P$ = .09. C, Aspartate aminotransferase (AST) activity was significantly higher 2 days after turn out and was then further increased at 7 days; a < b $P$ = .02; b < c; $P$ = .37. D, Gamma-glutamyl transferase (GGT) activity was significantly higher 2 days after turn out and was then further increased at 7 days; a < b $P$ = .0001; a < c $P$ = .002: c > b $P$ = .009. Absence of significant within subject correlation between $\log_{10}$ HGA concentration and CK ($P$ = .78); B1), AST ($P$ = .83; C1) and GGT activity ($P$ = .26; D1) at day 2 (filled circles) and 7 (open circles) time points. Each line joins data from an individual animal (n = 10 ewes)

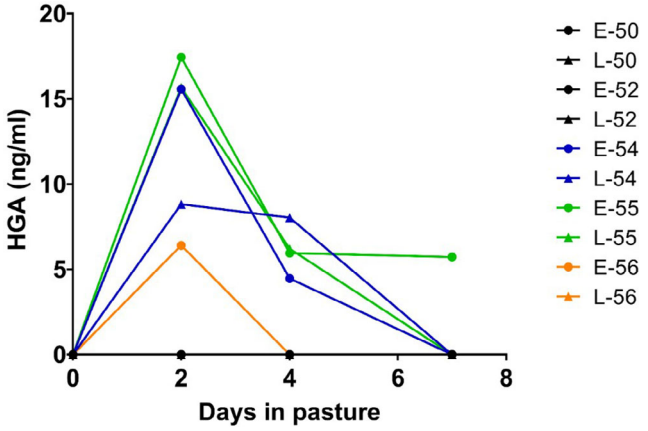


**FIGURE 3** Hypoglycin A (HGA) concentration in sheep from group 2. Graph shows results obtained in serial sampling over 7 days. Hypoglycin A was detectable in 3 ewes (E-54, E55, E56) and 2 lambs (L54, L55) after 48 hours grazing in contaminated pasture. Hypoglycin A was undetectable at all time points in a lamb (L-56) from a ewe (E56) with relatively low serum HGA concentrations. Animals 50, 52, and L-56 had undetectable HGA concentrations: their data are superimposed on the *x*-axis

comparison with other sheep in the group with undetectable HGA (Figure 2).

In group 2, HGA was detectable in 3 ewes and 2 corresponding lambs at 48 hours (Figure 3). The HGA concentration found in this group (13.15 ng/mL ± 5.9 (mean ± SD) was significantly lower than that in group 1 (48.03 ng/mL ± 45; $P < .001$). There was no detectable HGA and MCPA-carn at all time points evaluated in group 3.

## 4 | DISCUSSION

Sheep are sometimes used in pasture management strategies to graze certain plants that have greater toxicity in other species.[28] As such, if sheep are less susceptible to HGA toxicity, they might be suitable to mitigate risk in horses grazing pastures contaminated with HGA-containing plant material. In this study, we tested the hypothesis that (unlike in equine GF), HGA is degraded by ovine RF. These data reveal no evidence of rumen degradation of HGA: indeed, both rumen and stomach fluids enhanced HGA release from seeds in comparison with water, as shown previously for other solvents,[1] perhaps due to the compound's polarity. Furthermore, our work reveals that HGA is absorbed systemically by sheep after ingestion of *Acer* spp. material.

Herbivores can overcome toxicity from plants by both behavioral and physiological adaptations.[29,30] Selective grazing is probably the first mechanism to account for interspecies' differences in grazing patterns: these patterns might be influenced by the nutritional value of the toxic plant and nontoxic forage availability. Metabolism of the toxin can also differ considerably between species as a result of production of binding proteins in the saliva[18,19] or chemical degradation by foregut microbes; these are well-established mechanisms that prevent ruminants from deleterious effects of phytotoxins.[31,32] In this work, the effect of saliva in HGA was not investigated, but these data reveal no degradation when HGA-containing seed homogenate was incubated in rumen fluid for up to 2 hours. However, rumen retention time is considerably longer than that tested in the described experiment (24-48 hours)[33-35]; consequently, we cannot be certain that a longer incubation would not have resulted in degradation, but given that sheep in this study had appreciable serum HGA concentrations within 48 hours of grazing contaminated pasture, rumen protection in this species seems unlikely. Indeed, serum HGA concentrations in these groups of sheep were similar to those in horses with subclinical disease (5-81 ng/mL)[36] (with mildly raised muscle enzyme activities) or slightly lower than those in horses without clinical signs that were co-grazing with AM-affected horses (37.8-328.5 ng/mL).[37] The HGA serum concentrations from these sheep were lower than those detected typically in horses with either severe or mild clinical signs[36]; hence, we cannot rule out the possibility that disease would not have occurred if the animals had absorbed more toxin. Furthermore, we do not have data from co-grazing horses so direct comparison between the species is not possible. A recent study described a myopathy associated with HGA ingestion in an exotic deer species[11] in which an association between increased serum activity of muscle derived enzymes and serum HGA concentration was found. However, the serum HGA and MCPA-carn concentration was lower in the deer with severe myopathy (35 and 1.64 ng/mL, respectively)[11] than the single ewe with detectable MCPA-carn in our study; these results suggest that there might be differences between ruminant species' relative susceptibilities to HGA intoxication and metabolism.

Toxin clearance patterns, detoxification mechanisms, and toxin tolerance might differ considerably between herbivore species.[31] Urine was not available for analysis; therefore, differences in renal clearance could not be established in this study. Although, strong conclusions cannot be drawn from a single animal with detectable serum MCPA-carn, differences in toxin tolerance between species seem possible. HGA is not toxic per se, but needs intracellular conversion to its toxic metabolite, MCPA-CoA. This bioactivation is believed to be initiated by the branched amino acid transferase enzyme[38-40] that has species-specific activities[41,42] being significantly lower in ruminants compared to monogastrics.[42-44] As a result, HGA metabolism might be slower in some ruminants, resulting in reduced biosynthesis of toxic MCPA and perhaps higher renal clearance of unmetabolized HGA.

We detected (clinically irrelevant) raised GGT and AST (though not CK) activities in (group 1) sheep turned out onto Sycamore-contaminated pasture. However, without biochemical data from a corresponding control group that was not exposed to HGA, we cannot rule out the possibility that the higher serum activities of liver-derived enzymes were a result of metabolic changes in the postpartum ewes or turnout.[45-48] MCPA-carn was detected in a single (clinically normal) ewe that correspondingly had the highest serum HGA concentration (126.4 ng/mL); to our knowledge, MCPA-carn has only been detected in horses with signs of AM and elevated serum CK activities.[3,37,49] Given the lack of association between either GGT, AST, or CK enzyme activities and HGA concentration in individual animals, and the absence of raised CK, AST, or GGT in this 1 ewe with

elevated MCPA-carn (and highest serum HGA), or in all ewes with any detectable MCPA-carn, it seems unlikely that there was HGA-associated subclinical hepatopathy or myopathy in these animals.

Elimination of phytotoxins in milk by lactating animals is considered a minor route of excretion, but has been demonstrated for several other plant toxins.[50-52] This work provides some supportive evidence of HGA elimination through milk as nursing lambs from 2 HGA serum-positive ewes in group 2 had detectable serum HGA. However, the lamb belonging to the third-positive ewe (that had the lowest HGA concentration) had no detectable serum HGA. The transfer of many toxins via milk depends on plasma concentrations (among other factors)[50] and these data suggest this might also be true for HGA. However, direct ingestion of seedlings by lambs cannot be ruled out, though we believe it is unlikely as these animals were under 2 weeks of age. Potential milk excretion of HGA and/or its metabolite as the cause of AM in a neonatal foal has been suggested.[53] Because of the increasing evidence of this excretion route, toxicity of HGA via ingestion of ruminant milk should be considered a possible risk for human health and warrants further research.

Significant differences between groups of ewes grazing contaminated pastures were found in this study. Seedlings on group 2's pasture had lower mean concentration of HGA than those of group 1, which likely accounts for the differences in serum concentrations, assuming similar intake in both groups of animals. Differences in animal density or grazing habits might also have contributed significantly to these results.

In summary, our work reveals that HGA is unaffected by exposure to ovine rumen fluid (at least for 2 hours) and that sheep absorb and metabolize HGA after ingestion of Sycamore material. Despite ingestion of the compound, and despite evidence for its metabolism in at least 1 animal, we found no evidence for associated toxicosis. Whether disease develops in sheep that absorb higher amounts of HGA remains to be seen. Future study of the relative differences in susceptibility of horses and sheep to the toxin might best be made in vitro. Such work might help establish whether sheep can be used safely for Sycamore decontamination of pastures.

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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.

## INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

In vitro experiments and retrospective serum testing were performed with approval from the Royal Veterinary College's Clinical Research Ethical Review Board, reference number: 2017 1708-2.

## HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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