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ORIGINAL ARTICLE

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Rejection behaviour of horses for hay contaminated with meadow saffron (*Colchicum autumnale* L.)

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Abstract

Background: Extensively used grasslands are frequently utilised for hay production for equines. Especially, extensive meadows have a great variety of plant species, which may include plants that are poisonous for equines such as meadow saffron (*Colchicum autumnale* L.). To authors' knowledge investigations about horses` avoidance behaviour towards dried meadow saffron in hay are missing. Reports of farmers are contrary to clinical symptoms described in case reports and associated with meadow saffron in hay.

Objectives: The aim of this study was to determine the rejection behaviour of horses for hay contaminated with meadow saffron (MS) when fed ad libitum.

Study design: An 18-day feeding trial with six adult geldings to observe the rejection behaviour for hay contaminated with MS.

Methods: The horses were fed a basal diet containing hay ad libitum and a mineral supplement during the feeding trial. At six different daytimes, hay contaminated with 1% or 2% dried MS was provided to the horses over a duration of 1 h. The rejection behaviour was observed personally and by video recordings. If a horse ingested more than two plants of MS during one observation period, the observation was stopped and repeated at another day. When the observation period had to be stopped twice, the horse was excluded from the experiment.

Results: Five of six horses ingested MS during the first feeding periods. One horse rejected leaves and capsules at the beginning of the study, but it showed repeated ingestion of MS after the seventh observation period.

Main limitations: Lack of knowledge about secondary plant metabolites affecting taste and their variability between fresh and dried plants.

Conclusions: The intake of MS in hay by horses could not be ruled out with certainty. Therefore, feeding hay contaminated with MS should be avoided for equids.

KEYWORDS
ad libitum, colchicine, horse, poisonous plants, selection

1 | INTRODUCTION

Meadow saffron (*Colchicum autumnale* L., family: Colchicaceae) is a common plant species in extensively used grasslands (Jung et al., 2010; Winter, et al., 2011a, 2011b). In spring, its leaves develop with a tulip-like capsule that contains seeds (Buch & Jagel, 2010; Jung et al., 2011). Due to this, leaves and capsules of meadow saffron (MS) are usually found in hay. Late harvesting favours the distribution of seeds and hence the dispersal of MS.

The alkaloid colchicine and its derivatives are found in MS and increasingly in maturing seeds (Seither & Elsäßer, 2014). Colchicine inhibits mitosis in tissues and causes a wide range of symptoms, such as colic, haematuria or coughing, which cannot be exclusively attributed to the uptake of MS (the exact data for toxicological dose in equines are missing) (Kamphues & Meyer, 1990; Wolf et al., 2009).

Although horses have been observed to avoid MS in pastures (Buch & Jagel, 2010; Jung et al., 2011; Seither & Elsäßer, 2014), equivocal observations have been reported on rejection behaviour in horses fed hay-based diets. On one hand, Austrian farmers have reported that horses avoid the plants in hay (Winter et al., 2011b). On the other hand, other case reports have retrospectively associated clinical symptoms in horses with MS contamination in hay (Kamphues & Meyer, 1990; Wolf et al., 2009). Therefore, this study aims to assess the ability of horses to reject MS in hay. To the authors' knowledge, this study is the first of its kind.

We hypothesised that adult horses that are fed hay ad libitum can reject MS in the contaminated diet.

2 | MATERIALS AND METHODS

2.1 | Animals/ husbandry

Six adults (aged 11–17), clinically healthy warmblood geldings with an average body weight (BW) (± standard deviation) of 674 kg (± 85 kg), were kept in individual boxes on straw bedding. Horses had daily free access to a paddock for several hours. Hay was fed ad libitum, except for the time horses spent on the paddock. In addition, 50 g of a commercial mineral feed (Reformin Plus, Hoeveler) was provided on daily basis. Water was freely available at all times.

2.2 | Hay and plant material

Non-contaminated hay from the region of Saxony was used as the basal ration. MS was harvested at the end of May from extensively used grassland areas in the region of Hessen. After cutting, MS were dried by hanging in a well-ventilated barn for at least 6 weeks.

2.3 | Test design

At different times of the day, each horse was fed 1–1.5 kg of hay previously mixed with MS to achieve a contamination rate of 1% and 2%. Therefore, the day was divided into six periods of 4 h each (Figure 1). The horses were randomly divided into three groups ($n = 2$) to ensure close observation.

According to time periods, the observation periods were performed in an 18-day randomised feeding schedule.

A preliminary feeding test was carried out to determine the amount of non-contaminated hay each horse consumed within 1 h (Table 1). The test ensured that sufficient hay was available during the observation period.

2.4 | Observation period

Each horse was observed by one person, and the feeding behaviour was documented and video recorded (Camera: Sony FDR-AX53, Sony Europe B.V.).

Following each observation period, the crib residues were removed and hay and MS were separated, weighed again and stored for further analysis.

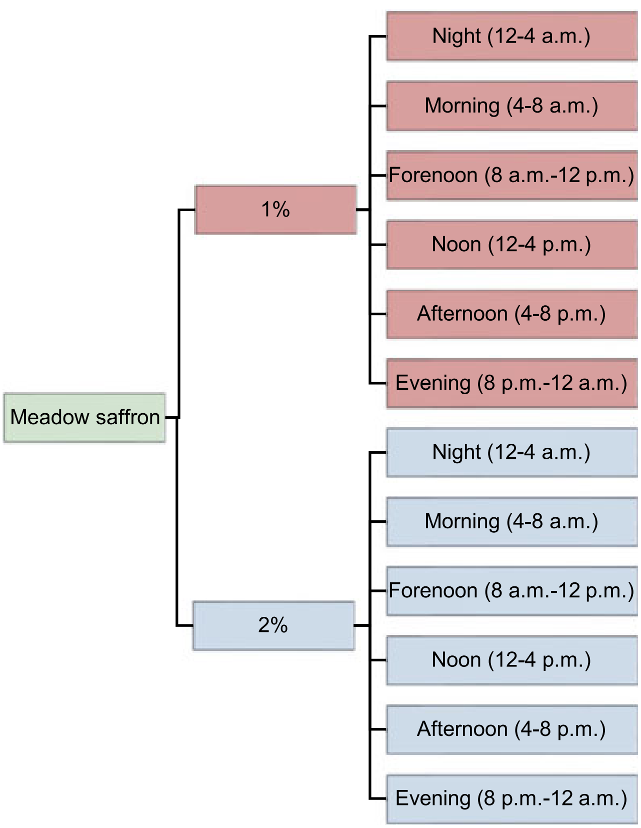

FIGURE 1 Subdivision of 24 h in six periods per contamination rate of meadow saffron (1% or 2%) used per horse; groundwork for the randomised feeding schedule

TABLE 1 Amounts of hay (kg) and calculated amounts of meadow saffron (g) provided to horses during the 1-h observation periods

Horse	Amount of hay	1% Meadow saffron	2% Meadow saffron
1 & 2 5 & 6	1.5	15	30
3 & 4	1.0	10	20

2.5 | Termination criteria

As the study was not designed to induce intoxication, strict termination criteria were defined prior to the trial. If a horse ingested two MS plants during one observation period, the session was stopped, and the observation was repeated at another day at a different time. In the case of the second intake of two MS plants, the horse was excluded from the experiment.

2.6 | Medical monitoring

Throughout the trial period, clinical health examinations, such as general behaviour, heart and respiratory rate, intestinal motility, colour of mucous membranes and conjunctiva, limb pulsation and skin alterations, were performed every second day.

In addition, blood samples were taken before the start and after the end of the trial by venepuncture of the external jugular vein.

2.7 | Measurements

The amounts of hay presented in Table 1 were used to provide hay contaminated with correct quantities of MS.

MS and hay were individually weighed by a scale, mixed and provided in clean, large feeding troughs for a duration of 1 h.

2.8 | Analysis

2.8.1 | Blood analysis

Serum liver parameters [albumin, total protein, triacylglyceride, cholesterol, lactate dehydrogenase, glutamate dehydrogenase, aspartate aminotransferase and gamma-glutamyl transferase], bilirubin and bile acids were analysed using an automated chemistry analyser (Roche Cobas C311, Roche Diagnostic GmbH). White blood cells (WBC) were count using ADVIA 120 (Siemens Healtheneers).

2.8.2 | Hay analysis

Four hay samples were analysed for crude nutrients and fibre fractions.

Dry matter (DM) was determined after oven-drying (103°C). Crude nutrients were assayed by the Weende system (Naumann & Bassler, 1999). Crude fibre (CF), neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) were analysed by ANKOM A220® (ANKOM Technology) according to Van Soest et al. (1991). The content of nitrogen-free extractives (NFE) was determined mathematically:

$$NFE = DM - (CA + CP + CFAT + CF)$$

(CA = crude ash, CP = crude protein, CFAT = crude fat).

2.8.3 | Analysis of MS

To determine crude nutrients and fibre fractions Weende analysis was performed in leaves and capsules by the methods described above.

Mixed samples of MS ($n = 9$) were sent to the German Federal Institute for Risk Assessment, Department Safety in the Food Chain, Berlin, to determine the colchicine content by liquid chromatography–tandem mass spectrometry.

Liquid chromatographic analysis

All measurements were conducted on an Agilent 1290 Infinity Series UHPLC System (Agilent Technologies). Chromatographic reversed-phase separation with 2 µl injection volume was performed on a C18 Hypersil Gold column (150 mm × 2.1 mm; 1.9 µm particle size) with a guard column (Thermo Fisher Scientific) at a flow rate of 0.3 ml/min and a column temperature of 40 °C. The binary mobile phase was composed of water as mobile phase A and methanol as mobile phase B, both containing 0.1% formic acid and 5 mmol ammonium formate. The gradient conditions were as follows: 0–0.5 min A: 95% / B: 5%; 7.0 min A: 50% / B: 50%; 7.5 min A: 20% / B: 80%; 7.6 min A: 0% / B: 100%; 10.1–15 min A: 95% / B: 5%.

Tandem mass spectrometry

Electrospray ionisation tandem mass spectrometry data were acquired in the positive ionisation mode on a QTRAP 6500 System (Sciex). The settings of the electrospray ionisation source were as follows: source temperature 500 °C, curtain gas 35 psi, ion source gas 1 (sheath gas) 60 psi, ion source gas 2 (drying gas) 60 psi, ion-spray voltage +5500 V and collision gas (nitrogen) medium.

Three multiple reaction monitoring transitions were measured using the first transition as a quantifier. The collision energy in eV is given in brackets: colchicine (400→383 [17], 400→358 [31]) and 400→341 [27]).

Sample preparation

For colchicine extraction, 10.0 ± 0.1 g of comminuted dried plant material was weighed into a centrifuge tube. A duplicate extraction was performed using 100 ml of aqueous extraction solution containing 0.05 M H_2SO_4. The extraction was performed using an ultrasonic bath for 15 min, followed by shaking in an overhead shaker for 20 min. The samples were centrifuged (20°C, 3800 g, 10 min). The resulting supernatant was passed through a 0.20 μm nylon membrane filter (VWR), diluted 100/200/500 fold, and subsequently analysed by an external calibration, applying a ten-point calibration curve in the range of 0.05–150 ng/ml.

2.9 | Statistics

SPSS 27® (IBM) and Microsoft Excel 2016® (Microsoft Corporation) were used to evaluate the data of the study. Data (blood parameters, contents of crude nutrients and fibre fraction in MS and hay) were tested for normal distribution using the Shapiro–Wilk Test. A paired *t*-test of normal distributed data was performed to identify significant differences between blood parameters before ($N = 6$) and after ($N = 6$) the trial. A Wilcoxon test was carried out for unnormal distributed data (WBC).

An unpaired t-test of normal distributed data was used to analyse significant differences between crude nutrients and fibre fraction in MS ($n = 4$) and hay ($n = 4$).

$P < 0.05$ was considered statistically significant.

All data were presented as mean values ±standard deviation (SD) as limited data because only few horses were available.

3 | RESULTS

3.1 | Medical monitoring and blood parameters

No anomalies in the clinical examination (e.g. heart and respiratory rate and intestinal motility) of the horses were detected during the whole trial. The blood parameters before and after the experiment were within the physiological ranges (Table 2).

3.2 | Rejection behaviour of horses

As shown in Figure 2, five of six horses ingested MS during the first two observation periods and were excluded from the trial. Four of six horses preferred MS in the first 5 min of the observation period. One horse rejected MS at the beginning of the study. After initial testing bites on day seven, an increased intake of MS was observed on the following two days. Both leaves and capsules were ingested by the horses. MS was preferentially consumed during the first 15 min within the 1-h observation period (Figure 3), although hay was available.

3.3 | Dry matter, crude nutrients and fibre fractions in MS and hay

Numeric differences were found between MS ($n = 4$) and hay ($n = 4$) in CP, CFAT, NFE and for fibre fractions (Table 3).

The CP content of MS was approximately 1.5 times higher than in hay. The CFAT content of MS was up to five times higher than that of hay. The NFE content of MS was significantly higher than that of hay. The CF content in MS was two times lower than that of hay. The CF content of hay was significantly higher than that of MS. The NDF content of hay was twofold higher than that of MS. The ADL content was higher in MS than in hay.

3.4 | Colchicine concentration in MS

The colchicine content of the capsules containing the seeds was three times higher than that of the leaves (Figure 4). The colchicine levels were numerically higher in the whole plant than in the leaves. The total colchicine content in MS plants was approximately the average of the colchicine content values of individual plant parts.

TABLE 2 Blood parameters before and after the experiment in horses (data are expressed as mean ± SD) and reference ranges

Blood parameters	Reference ranges[a]	Start of the feeding period	End of the feeding period
White blood cells[b], G/L	4.4–12	7.04 ± 1.05	7.07 ± 1.23
Total protein, g/L	57.8–78.7	73.7 ± 6.67	72.7 ± 7.25
Albumin, g/L	27.3–37.0	35.9 ± 1.65	35.5 ± 2.18
Cholesterol, mmol/L	1.72–2.95	2.41 ± 0.418	2.37 ± 0.367
Bilirubin, μmol/L	15.1–14.9	21.5 ± 7.92	23.5 ± 7.93
Bile acids, μmol/L	<12	4.88 ± 2.54	4.57 ± 1.88
Triacylglyceride, mmol/L	0.13–0.61	0.45 ± 0.074	0.34 ± 0.050
Aspartate aminotransferase, U/L	213–627	321 ± 41.02	295 ± 22.9
Gamma-glutamyl transferase, U/L	6.39–44.8	19.8 ± 8.57	17.7 ± 6.17
Glutamate dehydrogenase, U/L	1.39–11.4	1.95 ± 0.187	2.0 ± 0.268
Lactate dehydrogenase, U/L	224–536	362 ± 52.4	359 ± 49.7

[a]Köller et al. (2014).

[b]Analysed by Wilcoxon test because of unnormal distribution.

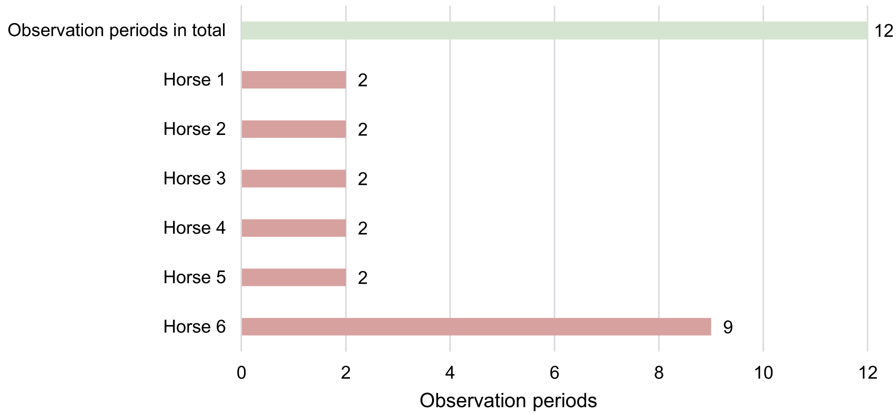

FIGURE 2 Total number of observation periods ($N = 12$) compared to the number of observation periods carried out for each horse fed with hay contaminated with meadow saffron. Number of observation periods below 12 denotes an interruption of the feeding trial due to the ingestion of meadow saffron. Data are expressed for each horse

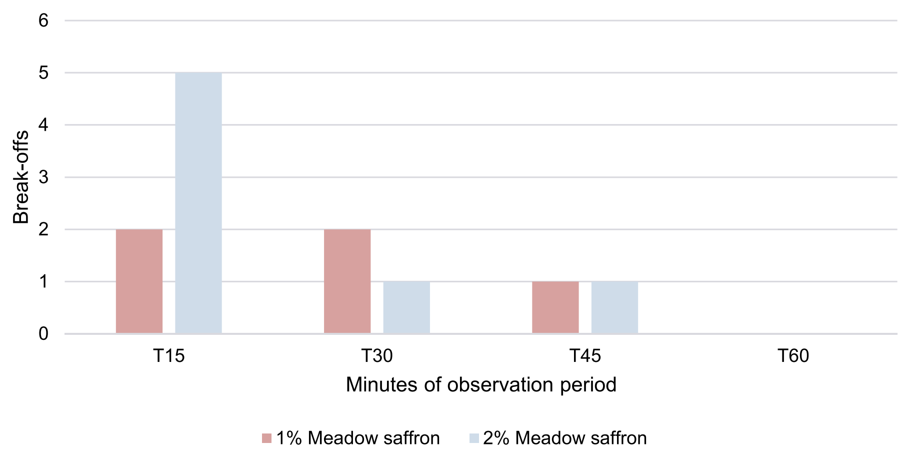

FIGURE 3 Number of break-offs ($N = 12$) in relation to the duration of observation periods (T = time in minutes)

TABLE 3 Dry matter (DM), crude nutrients and fibre fractions of meadow saffron ($n = 4$) and hay ($n = 4$) (data are presented as means ±SD and expressed in %)

Parameter	Meadow saffron (91.3 ± 0.890 DM)	Hay (91.6 ± 0.236 DM)	*p*-values
Crude fat	3.86 ± 0.254	0.80 ± 0.237	0.000
Crude protein	12.5 ± 0.717	7.84 ± 1.47	0.001
Crude fibre	17.3 ± 0.837	34.5 ± 1.85	0.000
Neutral detergent fibre	30.25 ± 3.55	66.9 ± 2.25	0.000
Acid detergent fibre	25.8 ± 1.78	39.0 ± 1.57	0.000
Acid detergent lignin	4.95 ± 1.20	4.53 ± 0.843	0.583
Nitrogen-free extractives	52.3 ± 1.24	41.4 ± 1.99	0.000

4 | DISCUSSION

In this study, the uptake of dried MS in contaminated hay by horses was observed under standardised feeding conditions. Feeding included ad libitum intake of hay to ensure satiety of the animals. Under these conditions, we hypothesised that horses would avoid the intake of dried MS.

In contrast to our hypothesis, five out of six horses ingested MS during the first two observation periods. Intake mostly occurred within the first 15 min of the observation period. One horse who was able to reject MS at the beginning of the study, started the intake of MS after seven days. In consequence, none of the horses avoided dried MS in hay under the conditions of ad libitum hay intake.

Colchicine is the main toxin in MS. According to case reports, the intake of MS led to intoxication in horses after the intake of 0.17 mg colchicine per kg BW (Wolf et al., 2009). In our study, MS contained 331 mg/kg colchicine related to DM. Considering that intoxications can occur at an intake of 0.17 mg/kg BW, symptoms would be possible after ingestion of less than 308 g of dried MS with a colchicine content of 331 mg/kg in a 600-kg horse.

It needs to be discussed why all horses ingested poisonous MS, although horses had the choice between hay or MS. Hunger as a cause of intake can be neglected, as hay was provided ad libitum.

In addition to hunger, several factors, such as oronasal sensorics, palatability and experience, influence the selection behaviour of horses.

Plants can develop antipastoral properties, such as bitter taste, to protect themselves from herbivores (Menke, 1990). In MS, colchicine and 30 other alkaloids (Jung et al., 2011) could be classified as bitter substances (Wölfle & Schempp, 2018). During the drying

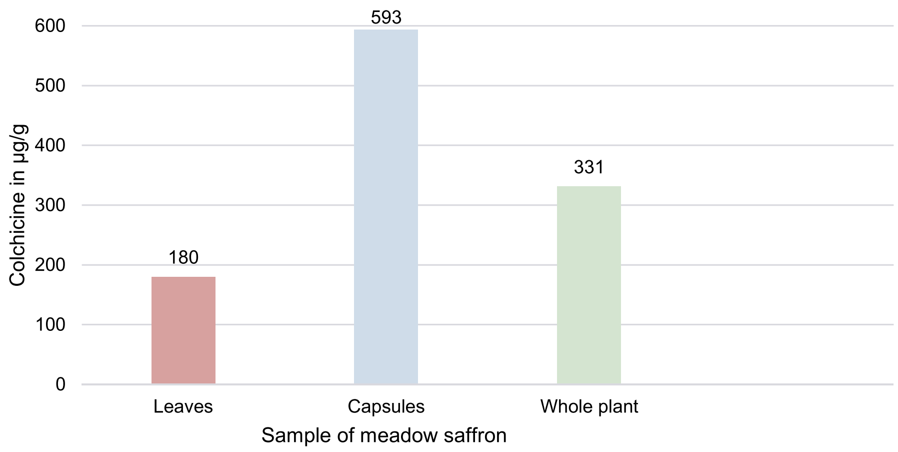

FIGURE 4 Colchicine content in mixed samples of leaves ($n = 1$), capsules ($n = 1$) and whole plant of meadow saffron ($n = 7$) related to dry matter

process, at least some of the bitter-tasting alkaloids might be degraded, although still-high levels of colchicine have been found in our study. However, it cannot be excluded that even a bitter taste due to colchicine or other bitter-tasting substances have no impact on the selection behaviour of horses.

In holistic veterinary medicine, bitter substances are used to stimulate the appetite, support digestion or calm the gastrointestinal tract (Wölfle & Schempp, 2018). In addition, Goodwin et al. (2005) showed that horses, when given a choice, prefer bitter tastes such as fenugreek (Brendieck-Worm, 2015) or slightly bitter-tasting rosemary in a similar way than the taste of sweet banana (Goodwin et al., 2005). Therefore, it is even possible that a moderately bitter taste may have stimulated the intake of MS. As no other bitter substances were investigated, the considerations on plants taste cannot be fully discussed solely on basis of colchicine.

Not only the presence of secondary metabolites, such as bitter substances, may influence the taste of forages but also various studies have shown that horses prefer feeds rich in carbohydrates, such as sugars (Redgate et al., 2014), or high in protein (Rodiek & Jones, 2012; Van den Berg, Giagos, Lee, Brown, Cawdell-Smith, et al., 2016; van den Berg et al., 2016; van den Berg et al., 2016). In the present study, MS had higher content of CP, NFE, such as sugars or starch, and CFAT compared to hay, which may relate to a high palatability of MS. The intake of MS within the first 15 min after provision supports the assumption of high palatability as well as the observation that four out of six horses showed a preferred intake rather than an accidental intake of MS.

In contrast to the higher amounts of CP, NFE and CFAT in MS compared to hay, lower fibre fractions were found in MS. Only the lignin content was higher in MS than in hay, which is likely related to the presence of capsules and seeds.

Several studies have shown that a higher feed intake of grass and hay correlated with a lower content of NDF in the feedstuff (Allen et al., 2013; Rodiek & Jones, 2012; Staniar et al., 2010). As MS had a lower NDF content compared to hay, horses might have preferably ingested MS.

Additionally, Van den Berg, Giagos, Lee, Brown, Cawdell-Smith, et al. (2016) investigated the influence of odour on the intake of feeds with different protein content. Sweet odour (banana, coconut) increased the forage intake in comparison with the same feedstuff without sweet odour (Van den Berg, Giagos, Lee, Brown, Cawdell-Smith, et al., 2016). In another study, a familiar odour (fresh lucerne) has been shown to have a positive effect on the intake of a novel food in horses (Van den Berg, Giagos, Lee, Brown, & Hinch, 2016). Whether MS has such a sweet or familiar odour is unknown. Some horses showed searching movements with nostril across the hay followed by the intake of MS, which led to the assumption of a stimulating odour.

Beside palatability or stimulating odour, adaptation periods to feedstuff seemed to be important in selection behaviour of horses. Several studies showed that horses preferred feeds with high content of proteins, carbohydrates (starch and sugar) or energy levels if they were adapted to these diets for at least three to five days or 10 feeding sessions (Cairns et al., 2002; Redgate et al., 2014; Van den Berg, Giagos, Lee, Brown, Cawdell-Smith, et al., 2016). However, in our study, the intake of MS was mainly observed at the beginning of the experiment within the first two observation periods. From our data, the adaptation to MS was of less importance. Only one horse started to ingest MS at day seven. After initial testing bites, an increased intake of MS at the following two days could be observed. Therefore, this adaptation period as well as speculated palatability of MS might stimulate its intake.

Provenza (1995) and Villalba & Provenza (2000a, 2000b) postulated that post-ingestive effects such as nutrient release in the gut and fermentation products, for example short-chain fatty acids or ammonia are related to feed intake in ruminants. The feedback mechanisms allow the selection of forages based on their digestibility or nutrient content (Provenza, 1995; Villalba & Provenza, 2000a, 2000b). The relation between post-ingestive effects and selection behaviour in horses remains open from the present data. However, the total amount of MS ingested by the horses in this study was low and, therefore, less likely to trigger metabolic effects.

Apart from mentioned factors, experience may influence the horses' ability to select feedstuffs.

In horses, the selection of edible or inedible plants is a learned behaviour, where foals mostly learn from their mares (Bolzan et al., 2020; Marinier & Alexander, 1991, 1995). However, adult horses are still able to learn food aversion (Houpt et al., 1990). In comparison with foals, the learning process of adult horses was mainly influenced by negative experiences, such as illness when occurring less than 30 min after the feed intake (Houpt et al., 1990). The symptoms of colchicine intoxication usually occur between 4 and 12 h after the intake of MS and thus exceed the 30-min benchmark (Brvar et al., 2004; Kamphues & Meyer, 1990; Yamada et al., 2000). Therefore, a learned aversion of MS by illness seems to be unlikely and was at least not present in the current study. Additionally, an intoxication by MS could be excluded because of physiological blood parameters and unremarkable clinical examination of the horses.

One of the horses showed a good rejection behaviour at the beginning of the experiment. However, in the course of time, the horse ingested parts of MS. It can only be speculated that this horse might have learned to avoid MS from his dam. The inconsistent rejection behaviour indicated that the premature food aversion might have failed during the feeding trial for unknown reasons. In contrast, the horse might have shown feed neophobia, which has been described as a protective reaction to avoid ingestion of an unknown and potentially harmful feedstuff (Provenza & Balph, 1988; Thorhallsdottir et al., 1987; Van den Berg, Lee, et al., 2016).

Furthermore, the horses' individual preference for a certain plant structure or taste should be recognised for the assessment of rejection behaviour.

Because rejection behaviour is influenced by many factors and depends on the horses' preferences, the reasons for ingesting MS cannot be investigated within this study.

However, despite ad libitum feeding, all horses were not able to reject MS from hay. On the contrary, four horses even preferred the intake of MS compared to hay. Owing to the high content of colchicine in capsules and the accumulation of MS in nests, intoxication cannot be excluded when contaminated hay is used for feeding in horses. According to the EU Regulation (EC) 178/2002, Article 15, 'feed shall not be placed on the market or fed to any food-producing animal if it is unsafe'. Feedstuffs are 'unsafe [...], if they are considered to have an adverse effect on [...] animal health', and such feedstuffs include hay contaminated with MS. The EU Regulation (EC) 767/2009 extended the ban to non-food-producing animals. Additionally, colchicine is considered a prohibited substance for food-producing animals based on EU Regulation 37/2010, which may also be applied to slaughter horses.

5 | CONCLUSION

In this study, the intake of MS by horses could not be excluded even under feeding conditions such as the use of hay ad libitum. The reasons for the intake of MS are not fully understood in horses. Since intoxication by MS cannot be excluded from the routine feeding practice in horses, meadows containing MS are not suitable for hay production.

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CONFLICT OF INTEREST

The authors declare no competing interests.

AUTHOR CONTRIBUTION

C. Mueller contributed to study design, study execution, data analysis and preparation of the manuscript. L. Sroka contributed to study design, study execution and data analysis. L.-M. Hass contributed to study execution. S. Aboling contributed to study design. A. These contributed to colchicine measurement and manuscript preparation. I. Vervuert contributed to study design, study execution, data analysis and manuscript preparation. All authors gave their final approval of the manuscript.

ANIMAL WELFARE STATEMENT

The project was approved by the Ethics Committee for Animal Rights Protection of the Leipzig District Government (No. TVV 17/19) in accordance with German legislation for animal rights and welfare. The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. The authors confirm that they have followed EU standards for the protection of animals used for scientific purposes.

ETHICS STATEMENT

This project was approved by the ethics committee for animal rights protection of the Leipzig district government (TVV 17/2019), in accordance with German legislation for animal rights and welfare.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in at http://doi.org/[doi].

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