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Changes in the nutrient profile and the load of mycotoxins, phytoestrogens, and pesticides in horse pastures during spring and summer in Austria

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ABSTRACT

Pastures are used for grazing and the production of conserved roughage in horses. Yet, the nutritional profile of the forage varies from spring to late summer, affecting equine nutrient supply and health. In addition, environmental factors may also favor plant contaminants such as mycotoxins. This study aimed to determine the nutritional profile and contaminant load of selected horse pastures from early spring till late summer. The nutrient composition (main macronutrients, macro elements and trace elements), as well as mycotoxins, metabolites, pesticides, and plant-derived compounds of seven horse pastures were analyzed. Each pasture was sampled three times and the samples were categorized according to the status of the pasture plants: ear emergence, early- till full bloom, and drought-damaged vegetation. Drought-damaged pastures demonstrated a rise in the acid to neutral detergent fiber ratio, calcium, iron, and magnesium but lower potassium contents. Mycotoxins and other contaminants were found in the pastures including 64 fungal compounds (ergot alkaloids (13) and metabolites from *Fusarium* (21), *Aspergillus* (2), *Penicillium* (8), *Alternaria* (8) and other fungal species (12), one bacterial metabolite (cereulide), twelve plant metabolites (including eight phytoestrogens and three cyanogenic glycosides (linamarin, lotaustralin and prunasin)), 11 nonspecific metabolites and six pesticides. *Fusarium* metabolites showed the highest concentrations among the fungal metabolites and drought-induced stress increased the contamination levels (range: 123-3873 μg/kg DM). In conclusion, there was a dominant effect of the developmental stages of the plants, botanical composition of the pastures and weather conditions on the nutritional composition and presence of contaminants on pastures.

1. Introduction

Pastures play an important role in horse nutrition, being a crucial source of structural fiber, energy, protein, minerals, and other nutrients in the equine diet. Pastures serve as a feed resource for grazing and production of conserved roughages such as hay, haylage, and grass silage [1]. Therefore, knowledge of the chemical composition and safety of the pasture plants is critical to ensure that the nutrient requirements of horses are accurately met, while avoiding both major nutrient deficiency and oversupply, which have negative consequences for the health and performance of the animals. In particular deficiencies in crude protein (CP), macro elements and trace elements are known to negatively affect reproductive performance, immunity, as well as hoof development and health [2]. On the other hand, feeding horses on pastures rich in water-soluble carbohydrates (e.g., sugars, fructans) may lead to obesity and trigger the onset of laminitis [3].

However, the pastures are also exposed to various environmental and biotic factors that can increase the load of contaminants in the

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pasture, posing an additional risk to the horse's health. Due to such stress, the endophyte and epiphyte fungi and bacteria as well as drought-resistant and toxic plants may prevail, contaminating the pasture with various harmful compounds such as mycotoxins and secondary plant compounds (phytoestrogens (PE) and plant toxins like cyanogenic glycosides). Analyses on the occurrence of such contaminants on pastures are of great interest since little is known about the contamination with these substances when compared to data in grains and conserved feeds. Moreover, most investigations have been concentrated on the regulated mycotoxins by European legislation such as aflatoxin B1, deoxynivalenol, zearalenone and fumonisins. Hence, the modified and emerging mycotoxins (metabolites from the parent forms) are left out of consideration although their presence may have detrimental effects on animal health and reproduction.

Harsh summers are increasingly evident in Europe [4]. Changing climate events could have implication on the botanical composition of pastures and thus on the nutritional profile and mycotoxin contamination. Indeed, Penagos-Tabares et al. [5] showed that temperature plays a significant role in mycotoxin and fungal metabolic occurrences in cattle pastures. Accordingly, with increasing temperatures, the number of metabolites (co-contamination) increased, whereas with temperatures below 15°C *Fusarium, Alternaria* and total fungal metabolites were low [5].

The main aim of this exploratory study was to determine changes in the nutritional profile, mycotoxins, and other contaminants of horse pastures in the province of Lower Austria from early spring till late summer. We hypothesized that mycotoxins and other contaminants are always present in pastures, though in varying concentrations, depending on the growth stage of the plants and possibly weather conditions. We performed a field study collecting horse pasture samples for subsequent analyses as well as weather data recorded in the areas of study sites.

2. Materials and methods

2.1. Sampling of pasture

For this study, seven horse pastures used exclusively for hay production and grazing of horses in the province of Lower Austria were sampled for analyses of nutritional profile and contamination levels with mycotoxins, PE, and pesticides. One farmer (pasture 2) was known by one of the authors since he provides the university with hay sometimes, another (pasture 1) was part of the university farm but nothing about previous research was known. The remaining pastures (3-7) were from farmers unknown and contacted by the author via telephone or email after searching the internet for horse owners with own hay production in Lower Austria. Due to data protection, the pastures were assigned with the numbers 1 to 7. After the informed consent of the owners, three pasture samples of each pasture at three different stages of maturity during the grazing and hay making season of 2022 were collected. The first sampling was conducted between 2nd and 4th May, where the growth of the sward of all the pastures was in the stage of ear emergence (EEM). The second sampling was conducted between 16th and 23rd May, where the growth stage of the sward of all the pastures was early- till full-bloom (EFB). This sampling was taken shortly before the first cut was harvested for hay production. The third sampling was conducted between 20th July and 10th August. Because of the drought-induced stress during summer between the first and second cut, this sampling stage was denominated as drought-damaged vegetation (DDV). In one of the pastures (pasture 6), it was not possible to collect the third sample; therefore, the third sampling consisted of n = 6, whereas EEM and EFB consisted of n = 7. Changes in the vegetation are exemplarily shown for two pastures during the first and third sampling (Supplementary Fig. 1). Throughout the sampling period, pastures 1-4 and 7 were mowed (1st time end of May, 2nd time end of July/beginning of August). Pastures 5 and 6 were grazed from the end of May to the ending of the season.

Gathered information included type and time of fertilization (Supplementary Table S1), native species and, on the day of sample collection, the stage of growth, the height of plants and botanical composition. Before cutting, the height of the plants was evaluated on the day of sampling with a tape measure (Supplementary Table S2). The pastures were sampled systematically by going over the pasture in a W-shaped line. The number of locations per pasture depended on the area of the field. We adopted the method from a previous study [5]. Briefly, the locations were selected over the entire pasture area and were cut 5-7 cm above the soil level using an electric (ISIO, Bosch) and a manual grass shear (Gardena, Husqvarna Austria GmbH, Linz, Austria).

From each pasture, an overall quantity of 5 kg was collected and dry stored in cardboard boxes until further preparation back at the laboratory. It must be noted that the samples were not flash frozen and freeze-dried which could affect the preservation of fermentable/metabolizable components in the herbage. Therefore, the analyses represent the amount of ingested hay but may not be accurate for freshly grazed forage. Furthermore, the primary plant specimens (family, genus, and species) were determined by their morphological characteristics (stems, leaves, blossoms) with the help of an expert. Visually, some pastures were dominated by two or three species, but no exact proportions were determined. The collected samples were weighed into labelled aluminum grilling trays with a precisions scale (ME 4002, Mettler Toledo, Vienna, Austria). Then, the sample containing trays were put into a dry cabinet (Memmert GmbH & Co, Schwabach, Germany) for 48 hours at a temperature of 65°C. After a cooling time of approximately two hours, the samples were weighed out (ME 4002, Mettler Toledo, Vienna, Austria) and stored in sealable plastic bags. Next, the hay was pulverized to a particle size of 1 mm by a cutting mill (SM 300, Retch GmbH, Haan, Germany). The milling was conducted in 3 steps by using different sizes of sieves: firstly 6 mm, secondly 2 mm, and thirdly 1 mm. The resulting homogenate was subsequently used for further analyses.

2.2. Weather data

The climatic data were collected from the website of the Austrian Agency of Meteorology and Geodynamics (Zentralanstalt für Meteorologie und Geodynamik-ZAMG, https://data.hub.zamg.ac.at/dataset/). These included monthly data as well as data from the day before sample taking. Accordingly, the first and second samplings took place in May and therefore the same month weather data were assigned to both samplings. For the third sampling, recorded ZAMG data from July (pastures 1 and 2) and August (pastures 3 to 7) were considered. The climatic data of the pasture locations was obtained from the nearest weather station of the ZAMG. The weather stations are located in a radius of approximately 4-15 km from the sampled pastures.

2.3. Nutritional analyses

The chemical proximate (nutrient) analysis of the samples was conducted according to the protocols of the Association of German Agricultural Analytic and Research Institutes (VDLUFA) [6]. The dry matter (DM) content was determined by oven-drying the samples at 103°C for at least 4 hours (method 3.1). Ash was analyzed by combustion in a muffle furnace at 550°C overnight (method 8.1). CP was determined using the Kjeldahl method (method 4.1.1) and ether extract (EE) using the Soxhlet extraction system (method 5.1.2). Analyses of NDF (neutral detergent fiber) and ADF (acid detergent fiber) were performed following the methods described by Van Soest et al. [7]. The amount of non-fiber carbohydrates (NFC) was calculated by subtracting Ash, CP, EE and NDF from 100% : NFC = 100 – (Ash + CP + EE + NDF).

The water-soluble carbohydrates (WSC), ethanol-soluble carbohydrates (ESC), and fructans in hays were analyzed using the Anthrone method and calculated according to the method published earlier [8] and adapted later [9].

The contents of macro elements such as calcium (Ca), phosphorus (P), magnesium (Mg), potassium (K), sodium (Na), and the trace

elements like iron (Fe), manganese (Mn), zinc (Zn), and copper (Cu) were determined by inductively coupled plasma emission spectroscopy following the methods of VDLUFA (method 10.8.2) [6].

2.4. Analyses of contaminants and secondary metabolites

Various toxins and pesticides in the pasture samples were analyzed using liquid chromatography-tandem mass spectrometry (LC–MS/MS), a system enabling the accurate quantification of > 1200 biotoxins, pesticides and veterinary drugs in complex feed. A detailed description of the method is given in Steiner et al. [10]. For the analysis, the same fine-grained homogenate (dried with Memmert GmbH & Co, Schwabach, Germany; milled with SM 300, Retch GmbH, Haan, Germany) as for the previous mentioned analyses was used. The milled samples were homogeneously mixed into one representative sample per pasture. Aliquots containing 5 g of each sample were then used for the analysis which was carried out at the Department of Agrobiotechnology (IFA--Tulln) at the University of Natural Resources and Life Sciences Vienna (BOKU) in Tulln, Austria. The accuracy of the method is verified for mycotoxins addressed by regulatory limits, ergot alkaloids (EA) and tropane alkaloids by participation in a proficiency testing scheme organized by BIPEA (Genevilliers France).

Each group of metabolites is characterized by their main producers including *Alternaria, Aspergillus, Fusarium, Penicillium*, other (non-identified) fungi and unspecific metabolites (metabolites produced by fungi, bacteria and/or plants), plant metabolites (EA, PE, and cyanogenic glycosides) and anthropogenic contaminants (pesticides and veterinary drugs) (see Supplementary Table S3 [Targeted substances]).

2.5. Statistical analyses

Descriptive statistics (occurrences and concentration values of mycotoxins, PE, and pesticides: average, median, minimum, and maximum) were calculated using only the positive values (x ≥ limit of detection (LOD)). Data below LOD were considered not detectable. Concentrations lower than the respective limit of quantification (LOQ) were computed as LOQ/2. For correlations and climatic factors, the statistical analyses were performed using SAS (version 9.4; SAS Institute Inc., Cary, NC, USA). A two-tailed Pearson correlation was used (data not shown) to screen possible relationships between the concentrations of nutrients as well as different groups of metabolites and climatic data. Subsequently, targeted pairs were evaluated in detail to quantify their responses. Linear regressions of the climate day and month temperature (average, minimum and maximum), humidity and hours of sunshine duration and the nutrients and number of fungal metabolites per sample were performed using the Mixed procedure of SAS. The random effect of the pasture fields was considered in the model. The effect of sampling time on the concentrations of nutrients and grouped metabolites was evaluated using the MIXED procedure of SAS. The sampling time was grouped as mentioned before in EEM, EFB, and DDV. The statistical model of each geo-climatic factor included a fixed effect of the stage of maturity and a random effect of the pasture fields. Measurements taken on the same pasture but at different times were considered as repeated measures with an autoregressive variance-covariance matrix. The resulting data reported are the least-squares means and standard error of the least-squares mean (SEM).

3. Results

3.1. Botanical characterization

The botanical composition of the pastures is listed in Supplementary Table S4. At the first sample taking, identifying distinct species was impossible due to the immaturity of the plants at that stage and therefore none or little blossoms and/or seedheads were developed. However, in the second sampling (EFB), the extent of the botanical diversity could be perceived. For the current study, the main species on each pasture were registered to compare their known characteristics with the analyzed nutritional profile. At EFB stage, the plant species could be determined best. The sampled pastures contained mixtures of *Gramineae* (Family: *Poaceae*), including *Lolium perenne, Dactylis glomerata, Poa trivialis, Festuca pratensis, Alopecurus pratensis* and *Phleum pretense*) and *Leguminosae* (Family: *Fabaceae*) such as *Trifolium pratense, T. repens* and *Medicago sativa*). As identified, in Pastures 1 and 2 there was a homogenous mixture of common horse pasture plants from the families *Fabaceae-*, *Poaceae-* and *Taraxacum*. Pasture 3 was inhabited mainly by *Poaceae* with isolated spots of *Fabaceae*. Pastures 4, 5 and 7 were dominated by plants of the *Fabaceae*-family. Especially at DDV stage these pastures contained mainly *Medicago sativa* (pasture 4 and 5) and *Trifolium repens* (pasture 7) (Supplementary Table S4).

3.2. Nutritional composition

Data of chemical proximate analysis are shown in Table 1. The average DM content in the pasture samples collected at the EEM growth stage was 20.5% and did not differ from EFB (24.3%). In both cases, the DM content was lower (P = 0.002) than when taken in full summer after the first cut or DDV, being 35.8%. Ash concentrations differed between the three stages of maturity (P = 0.0038). The concentrations of CP between the EEM and EFB stages and between the EEM and DDV stages decreased (P < 0.001). Between the EFB and DDV stages, no difference was found. The NDF concentrations in the EFB collected forage were higher than in EEM and DDV stages (P = 0.003). There was a difference in ADF concentrations between EEM and the two other stages of development, whereby the lowest values were found at the stage of EEM (P = 0.005). The WSC were higher in the EEM forage than in the samples of EFB or DDV (P = 0.005). The fructans, as a subgroup of the WSC, followed the same tendency (P = 0.008). However, the ESC concentrations differed between all three stages of development (P = 0.011).

As shown in Table 2, the concentration of macro elements and trace elements in the pastures varied between the three developmental stages. However, Ca, P, Mg, K, Na, and Fe showed differences: Forage from DDV stage showed higher contents of Ca and Mg compared to EEM and EFB (P < 0.001), while K decreased compared to EEM (P = 0.002), and Fe increased compared to EFB (P = 0.002).

Table 1
Effects of changing stage of maturity on the nutrient composition of horse pastures.

Variable	Stage of vegetation[1]			SEM	*P* value
	EEM	EFB	DDV		
Dry matter (DM) (% of fresh)	20.5^{b}	24.3^{b}	35.9^{a}	2.1	0.002
Ash (% DM)	9.3^{ab}	8.3^{b}	10.9^{a}	0.72	0.038
Crude protein (% DM)	21.5^{a}	15.4^{b}	16.4^{b}	1.51	<0.001
Ether extracts (% DM)	2.4	2.4	2.4	0.138	1.000
Neutral detergent fiber (NDF) (% DM)	46.0^{b}	54.6^{a}	49.8^{ab}	2.2	0.003
Acid detergent fiber (ADF) (% DM)	24.3^{b}	31.0^{a}	33.0^{a}	1.71	0.005
Non-fiber carbohydrates (% DM)	20.7	19.3	20.5	2.11	0.752
ADF:NDF	0.53^{b}	0.57^{b}	0.66^{a}	0.023	0.002
Water-soluble carbohydrates (g/kg DM)	155^{a}	93.4^{b}	75.8^{b}	19.62	0.005
Ethanol-soluble carbohydrates (g/kg DM)	109^{a}	79.7^{b}	63.2^{b}	10.97	0.011
Fructans (g/kg DM)	46.5^{a}	13.3^{b}	12.5^{b}	10.86	0.008

[1] EEM = ear emergence collected during the first sampling before the first harvest (2nd an 4th May); EFB = early- till full-bloom collected during the second sampling before the first harvest (16th and 23rd May); DDV = drought-damaged vegetation collected at the third sampling before the second harvest.

SEM = standard error of the mean.

Means in the same row bearing different superscripts indicate significant differences (P < 0.05) based on Tukey's method.

Table 2
Effects of changing stage of maturity of the pastures on the concentrations of macro elements and trace elements of horse pastures.

Variable	Stage of vegetation[1]			SEM	*P* value
	EEM	EFB	DDV		
Calcium (g/kg DM)	7.04^{b}	7.12^{b}	12.5^{a}	1.28	<0.001
Phosphorus (g/kg DM)	3.74^{a}	3.17^{b}	3.45^{ab}	0.345	0.03
Magnesium (g/kg DM)	1.97^{b}	1.96^{b}	3.18^{a}	0.172	<0.001
Potassium (g/kg DM)	32.36^{a}	27.89^{b}	23.58^{c}	1.59	0.002
Sodium (g/kg DM)	0.35	0.25	0.3	0.075	0.352
Iron (mg/kg DM)	93.3^{a}	49.6^{b}	109^{a}	9.39	0.002
Manganese (mg/kg DM)	47.8	37.3	44.1	5.73	0.171
Zinc (mg/kg DM)	24.1	20.6	22.1	2.13	0.167
Copper (mg/kg DM)	7.6	6.0	7.3	0.75	0.127

[1] EEM = ear emergence collected during the first sampling before the first harvest (2nd an 4th May); EFB = early- till full-bloom collected during the second sampling before the first harvest (16th and 23rd May); DDV = drought-damaged vegetation collected at the third sampling before the second harvest.

SEM = standard error of the mean.

Means in the same row bearing different superscripts indicate significant differences (P < 0.05) based on Tukey's method.

3.3. Content of contaminants and other metabolites

The occurrence and concentrations (average, standard deviation, median, minimum, and maximum, expressed in μg/kg on a DM basis) of individual and grouped natural and anthropogenic substances are shown in Tables 3 and 4. In total, 94 out of over 1500 natural and anthropogenic targeted compounds were detected in the analyzed pasture samples. These include 64 fungal compounds (such as EA (13), metabolites from *Fusarium* (21), *Aspergillus* (2), *Penicillium* (8), *Alternaria* (8) and other fungal species (12), one bacterial metabolite (cereulide), twelve plant metabolites (including eight PE) and three cyanogenic glycosides (linamarin, lotaustralin and prunasin), eleven unspecific metabolites and six pesticides. The positive samples contained on average 11.3 (range: 2-48) fungal metabolites per sample, 5.2 (range: 1-8) PE per sample, and 2.3 pesticides (range:1-5). Supplementary Table S5 shows the number of detected metabolites and co-occurrences (detected analytes/sample) of the groups of compounds detected in horse pastures in Austria.

As shown in Fig. 1, there was a notable difference in the concentrations of several groups of mycotoxins and metabolites between the three stages of development. Accordingly, samples collected at the stage of DDV presented higher concentrations of fungal metabolites (P = 0.012) including total *Fusarium* (P = 0.015) and emerging *Fusarium* metabolites (P = 0.004) compared to those of either EEM or EFB. A similar trend occurred with the number of total fungal metabolites (P = 0.003), *Fusarium* (P < 0.001) and *Alternaria* (P = 0.013), which resulted in higher concentrations in the pastures during DDV compared to EEM and EFB (Fig. 2).

The contamination with *Fusarium* metabolites was highest during the stage of DDV (Fig. 1). More precisely, in the first sampling three pastures (pasture 4: 26.45 μg/kg sample DM; pasture 3: 97.57 μg/kg sample DM; pasture 1: 328.36 μg/kg sample DM) whereas in the third sampling all pastures showed a contamination with *Fusarium* metabolites (123.08-3873.82 μg/kg sample DM). In two pastures EA were detected, in which ergovalin was the most occurrent EA. In total, three *Aspergillus* metabolites were detected, of which phenopyrrozin occurred most frequently. *Alternaria* metabolites were present in all pastures in DDV with concentrations ranging from 27.72 to 3602.58 μg/kg sample DM. Abscisic acid was found in all pastures and reached the highest concentrations of the group of other fungal metabolites in each pasture. Cereulide, an emetic toxin produced by virulent *Bacillus cereus*, was detected in one pasture during the stage of EEM. Moreover, various plant metabolites/toxins (including eight PE), three cyanogenic glycosides and some unspecific metabolites (analytes produced by different and unrelated species of fungi, bacteria and/or plants) were detected in every pasture. Regarding pesticides, a cocktail of five different pesticides was detected in DDV.

3.4. Weather conditions

Short-term weather data prior to sampling dates, including one day and 30-day averages, were recorded and the results are provided in Table 5.

Regarding the day prior to sampling, all considered parameters differed between the three times of sampling. The daily average (P < 0.001) and minimum (P < 0.001) temperature increased from EEM to EFB and further to DDV. The daily maximum temperature was lower during EEM than during EFB and DDV (P < 0.001) with no significant change during the latter ones. The average relative humidity (P = 0.003) showed a decline, whereas the 24-hour sum of sunshine duration was rising (P = 0.018). Similar patterns were observed for the 30-day data. Higher temperature variables (mean, min and max) and longer sunshine hours were observed for DDV compared to EFB and EEM (P < 0.05). On the contrary, the lowest relative humidity was observed during DDV (P < 0.001). Precipitation for 30 days of EFM was higher than EEM and DDV (P < 0.05). The number of day with mean temperature over 15°C increased from 1, to 13 and 30 days for EEM, EFB and DDV, respectively (P < 0.001).

4. Discussion

The present study explored the nutritional status as well as the concentrations of fungal contaminant and other contaminants in pastures intended for horse feeding considering the dynamic changes in these variations across botanical stages. We also examined short-term weather data to describe the weather conditions that the growing vegetation had been exposed to and to offer some information related to a rise of mycotoxins in pastures with increasing temperature [5]. Altogether, the present study was among the first studies investigating both nutrition and feed safety aspects of horse pastures.

4.1. Changes in nutrient composition

The results regarding nutrient composition are in support of previous findings. The CP concentrations, in general, were high and were higher in the first sampling than in the second. Not all pastures showed a decline in CP values until the third sampling, with pastures 5 and 7 being the exception. The explanation for pasture 5 could be the high ratio of *Fabaceae* in the third sampling. Furthermore, after the second sampling, horses were grazing on this pasture which may lead to nitrogen supply through urination and droppings. On pasture 7, a higher ratio of *Trifolium repens* (also from the family of *Fabaceae*) was found compared to the first two samples, which can explain the higher CP concentration of this particular pasture. It is also important to note that a higher variety of different species is not only more palatable for the horse, but also ensures both digestibility, and fiber supply [1,2,11]. The presence of legume species could also be beneficial in terms of yield and CP contents of pasture. We observed that rising temperatures during the succession of the seasons caused a lower yield of grasses. However, during these hot summer weeks investigated in the present study, *Fabaceae* grew lush compared to other species. That is because they are more accommodated to dry environmental conditions [2]. This suggests that maintaining drought-resistant legumes such as *Fabaceae* and nitrogen replenishment can overcome an effect of season and drought on lowering yield and the CP content of pastures. Notably, we did not have quantitative data of the botanical compositions of each pasture sampling and field management differed among pastures, for instance being mown (pastures 1-4 and 7) or grazed (pastures 5-6), which can influence the re-growing vegetation. Therefore, our assumption related to botanical composition should be taken with caution. This aspect is important to consider in future studies

Table 3
Occurrence and concentrations of mycotoxins and other fungal metabolites in the analyzed pasture samples intended for horse grazing or horse hay production.

Group	Analytes	Occurrence[1] (%)	Concentration[2] (µg/kg DM)					
			Average ± SD			Range		
Fusarium metabolites								
	15-Hydroxyculmorin	5	198			198		
	3-Acetyl-T-2 Toxin	5	6.1			6.1		
	Antibiotic Y	40	407	±	669	41.5	-	2033
	Apicidin	25	25	±	10.7	10	-	39
	Aurofusarin	30	106	±	121	7.7	-	300
	Beauvericin	45	19.4	±	45.2	1.1	-	139
	Culmorin	10	173	±	90.3	109	-	237
	Enniatin A	20	3.0	±	2.7	0.8	-	6.7
	Enniatin A1	30	10.5	±	14.4	0.8	-	37
	Enniatin B	35	37	±	54.3	2.9	-	158
	Enniatin B1	40	36.2	±	58.5	3.0	-	172
	Enniatin B2	10	3.2	±	3.0	1.1	-	5.3
	Epiequisetin	5	4.4			4.4		
	Equisetin	45	137	±	298	5.7	-	922
	Fumonisin B1	5	113			113		
	Fungerin	5	18			18		
	Moniliformin	10	21	±	2.8	19.1	-	23
	Siccanol	25	379	±	251	81.8	-	765
	T-2 toxin	5	28.5			28.5		
	W493	15	22	±	12	10.2	-	34
	Zearalenone	20	64.4	±	46.9	21.2	-	111
	Total	60	753	±	1170	1.2	-	3873
Ergot alkaloids								
	Ergocornine	5	14.7			14.7		
	Ergocorninine	5	3.6			3.6		
	Ergocristine	5	33.2			33.2		
	Ergocristinine	5	4.1			4.1		
	Ergocryptine	5	13.1			13.1		
	Ergocryptinine	5	4.1			4.1		
	Ergometrine	5	8.9			8.9		
	Ergometrinine	5	3.2			3.2		
	Ergosin	5	33.3			33.3		
	Ergosinin	5	29.8			29.8		
	Ergotamine	5	27.8			27.8		
	Ergotaminine	5	18.7			18.7		
	Ergovalin	10	7.50	±	2.1	6.0	-	9.0
	Total	10	105	±	135	9.0	-	200
Aspergillus metabolites								
	Kojic acid	5	1010			1010		
	Phenopyrrozin	35	20.7	±	11.6	9.6	-	37.4
	Total	40	144	±	349	9.6	-	1010
Penicillium metabolites								
	Barceloneic acid	15	306	±	237	159	-	580
	Bilaid A	45	11.5	±	6.2	4.0	-	22.7
	Chanoclavin	25	16.6	±	17.3	2.6	-	45.1
	Curvularin	15	8.5	±	1.5	6.8	-	9.8
	Deoxygerfelin	10	2.0	±	0.1	2.0	-	2.1
	Pyrenocin A	5	151			151		
	Questiomycin	50	100	±	192	5.0	-	636
	Secalonic acid D	5	35.2			35.2		
	Total	95	122	±	188	5.0	-	636
Alternaria metabolites								
	Alternariol	20	31.6	±	17.7	16.2	-	57.0
	Alternariolmethylether	20	28.5	±	18.4	14.5	-	54.1
	Altersetin	75	57.4	±	76.3	3.4	-	244
	Infectopyron	10	2140	±	1370	1174	-	3112
	Pyrenophorol	20	20.1	±	10.4	11.4	-	34.6
	Radicinin	30	69.6	±	146	4.5	-	367
	Tentoxin	10	23.1	±	1.73	21.9	-	24.3
	Tenuazonic acid	15	86.6	±	39.1	50.6	-	128
	Total	80	387	±	979	3.3	-	3600
Metabolites from other fungal species								
	Ascochin	5	8.1			8.1		
	Cercosporamide	5	15.8			15.8		
	Cytochalasin B	5	123			123		
	Cytochalasin D	5	18.9			18.9		
	Epoxycytochalasin C	20	52.9	±	48.9	8.6	-	121
	Ilicicolin B	20	52.5	±	33.9	19.3	-	95.6
	Ilicicolin H	5	11.1			11.1		

(*continued on next page*)

Table 3 (*continued*)

Group	Analytes	Occurrence[1] (%)	Concentration[2] (µg/kg DM)					
			Average ± SD			Range		
	Monocerin	25	31.2	±	29.0	4.5	-	75.8
	Rubellin D	15	19.9	±	16.8	7.3	-	39.0
	Sporidesmolide II	50	117	±	230	2.8	-	751
	Sporidesmolide III	20	25.8	±	34.1	3.7	-	75.7
	Sydowinin A	40	333	±	225	68.2	-	725
	Total	70	340	±	375	8.1	-	1070
Total fungal metabolites		100	1180	±	2130	38.7	-	8670

[1] n = 20 pastures, samples with values > limit of detection (LOD)
[2] Excluding data < LOD. In case values > LOD and <limit of quantification (LOQ), LOQ/2 was used for calculation.

Table 4
Occurrence and concentrations of phytoestrogens, cyanogenic glycosides, pesticides, and other secondary metabolites in the analyzed pasture samples intended for horse grazing or horse hay production.

Group/Analyte	Occurrence[1] (%)Analyte	Concentration[1] (µg/kg DM)AverageOccurrence[1] (%)	Concentration[2] (µg/kg DM)					
			Average ± SD			Range		
Bacterial metabolites								
	Cereulide	5	14.0			14.0		
Phytoestrogens								
	Biochanin	75	12,220	±	11,060	110	-	31,220
	Coumestrol	80	434	±	609	17.3	-	1960
	Daidzein	40	818	±	631	201	-	2060
	Daidzin	55	3680	±	5820	201	-	20,750
	Formonetin	55	17,390	±	8860	7890	-	31,210
	Genistein	55	952	±	782	123	-	2680
	Genistin	70	14,160	±	15,750	238	-	51,530
	Ononin	85	10,470	±	11,310	128	-	34,170
	Total	100	40,770	±	46,330	17.3	-	144,660
Cianogenic Glycoside								
	Linamarin	70	100,290	±	123,320	1205	-	366,630
	Lotaustralin	90	107,370		145,180	135	-	402,160
	Prunasin	5	887			887		
	Total	90	185,420	±	256,900	135	-	764,580
Other plant metabolites								
	Abscisic acid	100	3007	±	1570	675	-	5820
Total plant Metabolites		100	210,660	±	279,040	1560	-	825,650
Pesticides								
	Dichlorprop	5	4.0			4.0		
	Dimethomorph	5	15.0			15.0		
	Fluopyram	5	8.3			8.3		
	Iprovalicarb	5	9.9			9.9		
	Metrafenon	5	11.1			11.1		
	Spiroxamin	10	4.2	±	1.1	3.4	-	4.9
	Total	15	18.9	±	26.3	3.4	-	49.3
Unspecific metabolites								
	3-Nitropropionic acid	10	3580	±	3080	1400	-	5760
	Brevianamid F	55	20.9	±	12.5	8.9	-	48.2
	Chlorocitreorosein	5	5.7			5.7		
	Chrysophanol	20	258	±	160	137	-	483
	Citreorosein	30	121	±	127	14.6	-	313
	cyclo(L-Pro-L-Tyr)	45	112	±	58.8	57.4	-	235
	cyclo(L-Pro-L-Val)	100	163	±	150	16.2	-	584
	Emodin	65	262	±	256	10.5	-	771
	Iso-Rhodoptilometrin	5	11		11			
	Norlichexanthone	40	67.9	±	77.7	5.7	-	249
	Tryptophol	80	817	±	332	402	-	1470
	Total	100	1520	±	1380	63.2	-	6620

[1] n = 20 pastures, samples with values > limit of detection (LOD);
[2] Excluding data < LOD. In case values > LOD and < limit of quantification (LOQ), LOQ/2 was used for calculation; DM: Dry matter; SD: Standard deviation.

to confirm our assumptions. Overall, CP-rich roughage of these pastures would provide sufficient CP to cover increased protein requirements of breeding horses and foals, though potentially containing excessive CP amounts for horses at maintenance level or sport [12]. It may be that if fed ad libitum, an excessive CP amount as found on pastures 5 and 7 may lead to gastrointestinal dysbiosis [2].

Furthermore, WSC concentrations declined throughout the sampling period. The findings of our study confirm that high WSC concentrations are related to low average temperatures and early developmental stages of the plants. However, varying WSC concentrations could also be measured when the botanical composition changed: pastures with a high percentage of *Fabaceae* showed a lower WSC concentration. This

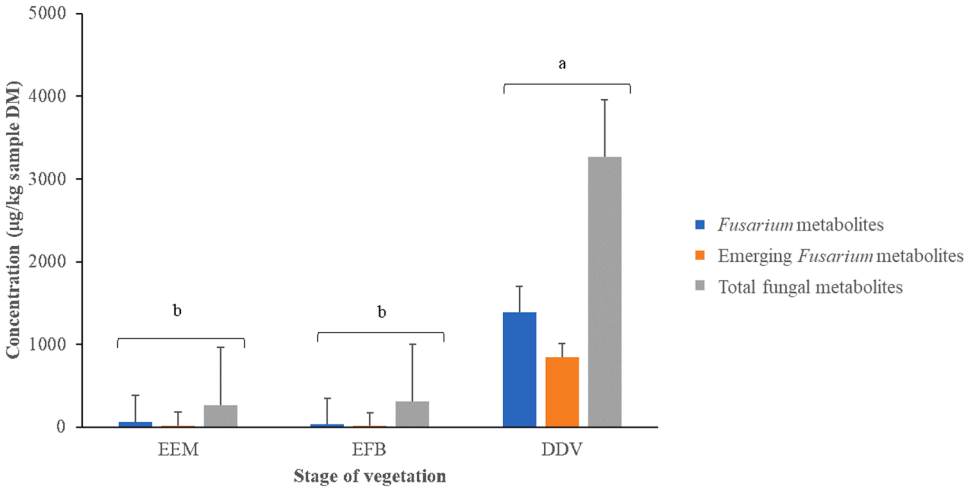

Fig. 1. Effects of vegetation and season on the concentration of various fungal metabolites in the analyzed pasture samples. Per group of metabolites, the vertical bars represent the least square means per each stage of vegetation and the arrow bars represent the standard error of the mean. Significantly different ($P \leq 0.05$) results of detected mycotoxin metabolites are indicated with differing letters. EEM: ear emergence (n = 7); EFB: early-till full-bloom (n = 7); DDV: drought-damaged vegetation (n = 6).

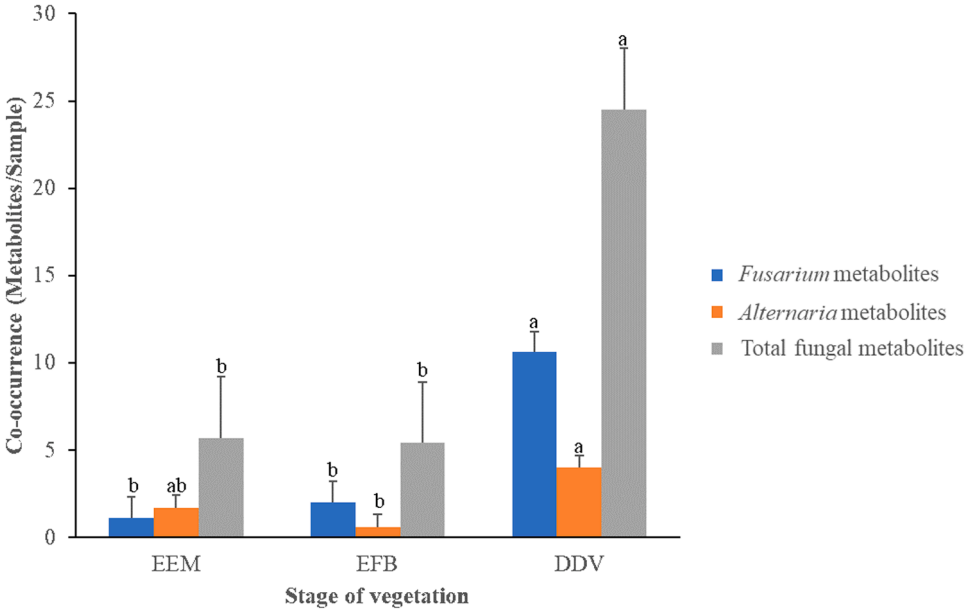

Fig. 2. Effects of vegetation and season on the number of mycotoxins/metabolites produced by *Fusarium, Alternaria*, and fungi (*Alternaria, Aspergillus*, ergot alkaloids, *Penicillium* and other fungi) in the analyzed pasture samples. Per group of metabolites, the vertical bars represent the least square means per each stage of vegetation and the arrow bars represent the standard error of the mean. Significantly different ($P \leq 0.05$) results of detected mycotoxin metabolites are indicated with differing letters. EEM: ear emergence (n = 7); EFB: early- till full-bloom (n = 7); DDV: drought-damaged vegetation (n = 6).

supports the common finding that *Fabaceae* generally have lower WSC values than other plants [13].

As plants develop, concentrations of macro elements and trace elements decrease during the first cut [13]. During the maturation, the amount of cell wall-components in the plant increase at the cost of the cell content, including the macro elements and trace elements. This decrease was also perceived in the present study for all measured macro elements and trace elements from the first to the second sample taken, except for Ca and Mg, which stayed at the same level. This result is likely because legumes, which prevailed in many pastures of the third sampling, usually have a higher concentration of Ca and Mg than grasses [11]. Hence, younger plants do not always contain higher concentrations of minerals and trace elements. It is expected that during the second cut, macro elements and trace elements are generally higher [13]. This was only partially confirmed in our results. The concentrations of Ca, Mg and Fe increased, but the concentrations of K were lower in the samples taken at the stage of DDV. Results from our analyses were compared to studies conducted in New Zealand [14] and Poland [15]. Looking at the concentration of macro elements in the pastures, our data suggest that these pastures met and exceeded the requirements in Ca, P, Mg, and K in all sampled, based on the estimated needs of horse at maintenance level [12]. Yet, in nearly all pastures a deficiency of Na, Zn and Cu was observed, and deficiency of Mn and Fe in a few pastures.

High concentrations of Ca were detected on pastures with a high ratio of *Fabaceae*. However, against the paradigm that Ca concentrations decrease with high precipitation rates [13], in our study the highest average Ca concentrations coincide with the lowest monthly sum of rainfall over the sampling period.

Table 5
Weather data as daily and monthly indices during the experiment.

Category	Variable	Stage of vegetation[1]			SEM	*P* value
		EEM	EFB	DDV		
Day data[2]	Mean Temp. (°C)	13.2^{c}	16.6^{b}	20.7^{a}	0.50	<0.001
	Max. Temp. (°C)	21.6^{b}	23.7^{a}	29.5^{a}	1.00	<0.001
	Min. Temp. (°C)	4.77^{c}	9.43^{b}	12.0^{a}	0.70	<0.001
	Relative humidity (%)	59.4^{a}	52.0^{ab}	41.8^{b}	2.86	0.003
	Sunshine duration (h/d)	7.37^{b}	10.9^{ab}	12.2^{a}	1.20	0.018
30-day data[2]	Mean Temp. (°C)	9.6^{c}	14.3^{b}	21.9^{a}	0.46	<0.001
	Max. Temp. (°C)	15.5^{c}	20.8^{b}	29.3^{a}	0.33	<0.001
	Min. Temp. (°C)	3.7^{c}	7.8^{b}	14.6^{a}	0.55	<0.001
	Days with mean Temp. > 15°C (d)	1.0^{c}	13.4^{b}	30.0^{a}	0.96	<0.001
	Relative humidity (%)	61.4^{b}	63.0^{a}	51.6^{c}	1.47	<0.001
	Precipitation (mm/30 d)	24.7^{b}	36. 4^{a}	15.8^{b}	2.88	<0.001
	Total sunshine duration (h/30 d)	179^{c}	221^{b}	279^{a}	9.74	<0.001

[1] EEM = ear emergence collected during the first sampling before the first harvest (2nd an 4th May); EFB = early- till full-bloom collected during the second sampling before the first harvest (16th and 23rd May); DDV = drought-damaged vegetation collected at the third sampling before the second harvest.

[2] Weather data were obtained from the website of the Austrian Agency of Meteorology and Geodynamics (ZAMG) of the nearby weather stations of the pastures. Day data were the day prior to sampling date and 30-day data were average values of 30 days prior to sampling date.

SEM = standard error of the mean.

Means in the same row bearing different superscripts indicate significant differences (P < 0.05) based on Tukey's method.

It is generally known that forages are rich in K but very low in Na [13]. This was also expected and found in our study, so that an oversupply in K but a deficiency of Na is expected in terms of meeting the requirements of horses. Therefore, free access to salt lick stones containing sodium chloride (NaCl) for all horses is recommended. For horses with physical activity especially during summer and high-performance horses with an increased sweat loss through exercise, the specific supplementary feeding of NaCl is recommended, based on the level of exercise and sweating.

Average Cu concentrations were low throughout the sampling periods and our values were comparable to the results of studies in New Zealand [14] and Poland [15]. With the concentrations measured, it is conceivable that the Cu requirements of horses are not fully met. Considering the key role of Cu for equine health, our data indicate that Cu supplementation of hay or pastures should be considered.

Surprisingly, Mn concentrations in our study were very low compared with data from New Zealand [14] and Poland [15]. Low concentrations of Mn can be caused by factors like chalky soils, Ca rich fertilization, or longer periods of drought [13]. However, the last two can be excluded as the values showed no correlation with the raised temperature data and none of the used fertilizers suggested being rich in Ca. This leaves the chalky soil as the most likely cause of low Mn concentrations in our study. To evaluate this factor, analyses of soil samples would be necessary. Since the minimal required concentrations for Mn are not met, horses fed with forage from the pastures as in our study, may require supplementation of Mn through mineral supplement.

As none of the pastures met the recommended minimum Zn concentrations, and as Zn is influenced by the same environmental factors as Mn, the assumption that a chalky soil is the cause of low Mn and Zn values, seems to be confirmed. Therefore, deficiencies in Zn, Cu, and likely Mn of the pastures should be taken into account in the mineral supplementation of the equine diets [2].

4.2. Changes in mycotoxins and contaminants

To the best of our knowledge, the present study is the first that documents the occurrences of a broad spectrum of mycotoxins, but also of relevant plant-derived compounds (PE and cyanogenic glycosides) as well as unspecific (multi-kingdom) secondary metabolites in pastures used for horse grazing and hay production. However, there are two related surveys on mycotoxins and other naturally occurring metabolites on Austrian pastures and complete rations intended for feeding dairy cattle [5,16], and one study from Germany on mycotoxins in commercial horse feed preparations (complementary feeds) [17]. Since mycotoxins, their metabolites, and plant-derived compounds can have negative effects on equine health, this lack of data is quite surprising. The only mycotoxin that has been widely studied in equine health is fumonisin B1 (FB1), which can cause equine leukoencephalomalacia (ELEM) [17–19]. Case evaluations in the USA associated concentrations > 10 mg FB1/kg feed with an increased risk for horses to develop ELEM, whereby with concentrations < 6 mg FB1/kg, no effect was assumed. Furthermore, FB1 is the only regulated mycotoxin with guidance values for horses by the European Food Safety Authority (EFSA) [18].

Our study clearly showed that the concentrations of *Fusarium* and total fungal metabolites were the highest at the stage of DDV, indicating a correlation to dry weather conditions and the load of mycotoxins. However, according to temperature changes, the concentrations of *Fusarium* and total fungal metabolites remained comparably low when the 30-day average temperature was below 16°C (EEM, EFB) and rose when the average temperature was 21.9°C (DDV). A similar trend occurred with the fungal metabolite contamination in the study from Penagos-Tabares et al. [5], where over the critical temperature of 15°C concentrations of *Fusarium, Alternaria* and total fungal metabolites rapidly rose in pastures used for dairy production. They also observed a linear correlation between temperatures with the number of fungal and plant metabolites. Consistently, we also observed that the samples collected late in the season had higher concentrations of co-contamination of fungal metabolites.

Both regulated and emerging *Fusarium* metabolites were detected in the samples. It is worth mentioning the toxicity risk of these fungal metabolites in the pastures for horses. Among the EU-regulated mycotoxins, FB1, the mycestrogen zearalenone, and the cytotoxic type A trichothecene T-2 were present in some samples. For horses, there are only specific guidance values for FB1, with maximum allowed concentrations of 5000 μg/kg in corn and corn-byproducts [20]. In experimental studies, adverse effects of FB1 (neurological abnormalities) were observed at a minimum intravenous dose of 0.01-0.05 mg/kg body weight pure FB1. With the assumption of a bioavailability of 5% in horses, this would mean an oral dose of 0.2-1.0 mg/kg body weight per day. In the current study, only pasture 3 had once a FB1-positive sample with a measured value of 0.1 mg/kg sample. Assuming a 500-kg horse with a daily intake of 1.5 kg hay/kg body weight and a consistent incidence of FB1 over the whole pasture, the oral dose would reach 0.002 mg/kg body weight. Therefore, health consequences are very unlikely [18]. Although the adverse effects after the intake of regulated and emerging mycotoxins are not properly characterized, these compounds can interact with other well-recognized fungal toxins, increasing their toxicological activity [16].

It is interesting to note, that in the samples of pasture 7 at DDV, a total of 13 EA were detected. Individual EA presented concentrations of 3-33 μg/kg per sample; however, the accumulated concentration was detected up to concentrations of 200 μg/kg. Such concentrations of total EA could represent a risk if we consider that mares are sensitive to even lower values, starting from 50-100 μg/kg. The alkaloids of ergot are known to cause delayed parturition, dystocia, and agalactia, among other health impairments including circulatory and neurological disorders in mares and other animals [21]. EA are mainly produced by the fungi *Claviceps* and *Epichloë* spp., which parasitize on a wide range of monocotyledonous plants, including the family of *Poaceae*. Since

Poaceae were present on all pastures and the weather conditions were roughly the same at all locations, it remains unclear why only pasture 7 showed such a high number of EA in the last sample. Ergovaline is associated with the presence of fescue grass (*Festuca* spp.), as its producing fungi parasitizes on these plants [5].

A notable number of PE were present in all samples. PE are plant compounds, which show a resemblance to the hormone 17β-estradiol (E2). Therefore, they can bind on estrogen receptors, which can lead to endocrine disorders (influence on the estrous cycle) [22]. In sheep, cattle and horses, the PE coumestrol is known to cause a declining reproductive efficiency [5]. The legumes alfalfa and clover are two types of estrogenic plants being rich sources of PE [5,22]. This would suggest that a high ratio of alfalfa and clover on pastures could lead to high PE concentrations in the pastures. Surprisingly, the current study showed the opposite: PE concentrations were low on pastures with a high legume-ratio (Pastures 4 and 5) compared to pastures containing a more diverse botanical variety (Pastures 1 and 2). This suggests that not only plant species but also other, yet unknown, factors determine the presence of PE in forage. Research on health effects of PE is scarce and guidance values for horse feed are missing. Therefore, more research needs to be done on this issue.

3-Nitropropionic acid (3-NPA) was detected on pasture 3 during EEM and DDV. This neurotoxin is known to be produced by bacteria, fungi, animals, and plants. In plants and insects, it has a protective function against herbivores and predators, respectively. 3-NPA, among others, is produced by fungal species such as *Aspergillus* and *Penicillium*. In plants, its derivates most frequently occur in the *Fabaceae*-family. However, in horses and other species intoxication can cause neurological damage [23,24]. The cause for the occurrence of 3-NPA only in pasture 3 at the stages of EEM and DDV could not be identified with this study, since visually no species of the plants associated with this metabolite in the USA [24] and Hungary [23] were identified. Furthermore, plants of the *Fabaceae*-family were also present on other sampled Austrian pastures.

Pesticides were only present in very low amounts in the pastures investigated. However, it was remarkable that in the third sample of pasture 3, five pesticides were detected even though this pasture is declared as a conservation area, where the use of pesticides is not allowed. Since the farmer declared that no pesticides were used, a rational explanation could be the airborne contamination from nearby potato fields. Five out of six detected pesticides were fungicides; however, the contamination of fungal metabolites was not lower.

5. Conclusions

The data of this exploratory study indicate that forage harvested at an early stage of development and/or pastures rich in *Fabaceae* contain high concentrations of CP and Ca, making it suitable for horses in growth and lactation. Furthermore, early stages of plant development were associated with high WSC concentrations making this early-cut forage inappropriate for horses with a history or a predisposition for equine laminitis. Thus, grazing should be avoided or restricted in the early grazing season and postponed to a later period for horses with a risk for laminitis. In the case of the sampled pastures in this study, this would mean using conserved forage from a late first cut (mid-May) or second cut (July-August) and allowing grazing from mid-May until August, respectively. A late first cut will have the benefit of more mature plants with lower WSC content but could mean lower yields due to dry conditions in late summer and economic losses for the farmer. The higher ADF/NDF ratio in late-cut hay may result in a lower digestibility in the equine gastrointestinal tract, making it less suitable for horses requiring a highly digestible forage such as performance horses. A varying number of mycotoxins was always present on pastures. However, forage in late summer showed a higher contamination with mycotoxins and other contaminants. Since little is known about the effects of different mycotoxins and other fungal or plant-derived compounds, the occurrence of these substances in horse as well as their toxicological interactions and negative effects on health should be further investigated.

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Ethics Statement

The work described in this article was performed in accordance with the Code of Ethics. No animals were used in this research.

Declaration of Competing Interest

I would like to highlight that none of the authors has any conflict of interest with this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jevs.2023.104958.

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