Atypical myopathy in Père David's deer (Elaphurus davidianius) associated with ingestion of hypoglycin A

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Inaugural-Dissertation

zur Erlangung der Doktorwürde der

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Für Opa

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Vetsuisse-Fakultät Universität Zürich (2018)

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Atypical myopathy in Père David‘s deer (*Elaphurus davidianus*) associated with ingestion of hypoglycin A

21 Père David’s deer (*Elaphurus davidianus*), also known as milu, have died for unknown reason at Zoo Duisburg from 2004 until 2016. The milus have succumbed from a myopathy that occurred seasonally with clinical signs that closely resembles those of a disease called equine atypical myopathy (EAM), which is formerly known in horses. The cause for EAM was found in the ingestion of hypoglycin A, contained in samaras and seedlings of the sycamore maple tree (*Acer pseudoplatanus*). 79 sera from all zoos that have kept milus in Germany and Austria were used and selected biochemical values were tested and additionally hypoglycin A, methylenecyclopropyl acetic acid-carnitine (MCPA-carnitine) and acylcarnitines, which have been found in horses suffering from EAM, were determined. The results showed greater values of serum activities of creatine kinase and aspartate aminotransferase in diseased milus. Moreover hypoglycin A, MCPA-carnitine and acylcarnitines were found in the blood of Père David’s deer and thus, hypoglycin A intoxication was considered to be a potential cause for the myopathies by ingestion of sycamore maple samaras that were present in the enclosure of the affected animals. Compared to horses, ruminants have a different digestive tract and further investigations will be needed to find out which factors are involved to trigger an outbreak in ruminants.

Keywords

atypical myopathy, *Elaphurus davidianus*, hypoglycin A, MCPA-carnitine, sycamore maple

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Atypical myopathy in Père David‘s deer (*Elaphurus davidianus*) associated with ingestion of hypoglycin A

Im Zoo Duisburg litt der Bestand der Davidshirsche (*Elaphurus davidianus*), auch Milus genannt, von 2004 bis 2017 unter saisonalen Myopathie-Fällen ungeklärter Ursache. Insgesamt waren 25 Tiere mit einer Mortalitätsrate von 84% betroffen und das klinische Bild war durch Muskelzittern, Salivation, Dyspnoe und ein vermindertes Fluchtverhalten gekennzeichnet. In der Pathologie zeigten sich Skelettmuskeldegenerationen. Ein ähnliches Bild zeigen Pferde, die an Equiner atypischer Myopathie (EAM) erkrankt sind. Der Auslöser für EAM ist die Ingestion des Toxins Hypoglycin A, welches in Samen und Setzlingen des Bergahorns (*Acer pseudoplatanus*) vorkommt. Es wurden 79 Blutseren aus Zoos im deutschsprachigen Raum gesammelt und auf ausgesuchte Blutparameter getestet. Die Ergebnisse zeigten bei kranken Tieren signifikant erhöhte Creatinkinase- und Aspartat-Aminotransferase- Werte. Zusätzlich wurden bei erkrankten Milus Hypoglycin A, seine metabolisierte Form Methylencyclopropylacetyl-Carnitin (MCPA-Carnitin) und Acylcarnitine nachgewiesen, die beim Pferd die Diagnose bestätigen. Bergahornsamen wurden in großer Anzahl im Gehege gefunden und eine Hypoglycin A-Intoxikation ist eine wahrscheinliche Erklärung für die Morbidität der Milus. Da Davidshirsche als Wiederkäuer einen sehr unterschiedlichen Digestionstrakt zu Pferden haben, bedarf es weiterer Studien um die Verstoffwechselung des Toxins und krankheitsauslösende Mechanismen in Wiederkäuern zu erklären.

Schlüsselwörter

Davidshirsch, Bergahorn, Hypoglycin A, Myopathie, MCPA-Carnitin

MANUSKRIPT

Atypical myopathy in Père David's deer (*Elaphurus davidianus*) associated with ingestion of hypoglycin A

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Runnig Head: Hypoglycin A Intoxication in Milus

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ABSTRACT

From 2004 until 2016, 21 Père David's deer (*Elaphurus davidianus*) have died for unknown reason at Zoo Duisburg. These deer, also known as milu, have succumbed from a myopathy that occurred seasonally in autumn and in spring. The clinical signs shown by the animals, closely resembles those of a disease called equine atypical myopathy (**EAM**), which is formerly known in horses. The cause for EAM in Europe was found in the ingestion of hypoglycin A, contained in samaras and seedlings of the sycamore maple tree (*Acer pseudoplatanus*). To test the hypothesis that the mortality of milus was caused by ingestion of hypoglycin A, 79 sera from all zoos and wildlife parks that have kept milus in Germany and Austria, including 19 diseased and 60 healthy animals, were used. Selected biochemical values and additionally hypoglycin A, methylenecyclopropyl acetic acid-carnitine (**MCPA-carnitine**) and acylcarnitines, which have been found in horses suffering from EAM, were determined. The results showed greater values of serum activities of creatine kinase ($P < 0.001$) and aspartate aminotransferase ($P < 0.001$) in diseased milus comparing to healthy ones confirming a myopathy in affected animals. Moreover, hypoglycin A and MCPA-carnitine were found in the blood of Père David's deer and thus, hypoglycin A intoxication was considered to be a potential cause for the myopathies by ingestion of sycamore maple samaras that were present in the enclosure of the affected animals. Hypoglycin A values were greater in diseased animals ($P < 0.01$) as well as MCPA-carnitine levels ($P < 0.05$). Additionally, affected milus showed greater C5-OH-Carnitine ($P < 0.01$) and C6-Carnitine ($P < 0.001$) values. Until now hypoglycin A intoxication was only known in the family of *Equidae*, in humans and in laboratory rats and it has not been previously described in other zoological families. Comparing to horses, ruminants do have a different digestive tract and it will need further investigation to find out if several factors are involved to trigger an outbreak in ruminants.

Key words atypical myopathy, *Elaphurus davidianus*, hypoglycin A, MCPA-carnitine, milu, sycamore maple

INTRODUCTION

The Père David's deer (*Elaphurus davidianus*), also known as milu, is a wild ruminant that is classified as "Extinct in the Wild" on *The IUCN Red List* (Wilson and Mittermeier, 2011). This species, which naturally occurred throughout eastern Asia, became extinct in the 19th century as a result of a flood disaster and turmoil of war during the boxer rebellion (Bannikow et al., 2000). After the extermination, the viability of the species was secured by a captive breeding program. All animals capable of breeding were gathered in England by the Duke of Bedford (Grzimek, 1988). From 1985 to 2012, the reintroduction into China took place, starting with an initial population of five breeding individuals. Today more than 5,000 milus form the world's population (Schürer and Stadler, 2016).

Zoo Duisburg, in Germany, has kept and successfully bred Père David's deer since 1966 with a total number of 77 individuals. From 2004 until 2016 the population has suffered from a disease containing high-grade myopathy. The outbreaks occurred seasonally from September until March and never happened during the summer. To this date, the majority of the milus have died, with only 4 individuals surviving out of 25 affected individuals.

The milus suffered from clinical signs that share similarities with a disease called Equine atypical myopathy (**EAM**) which is formerly known in horses. EAM can be described as a degeneration of particular muscle groups, clinically characterized by stiffness, weakness, recumbency, myoglobinuria and a high mortality rate. The disease is seasonal, and the outbreaks mostly occurred in autumn and spring (Votion, 2016). Recent studies showed that EAM in horses in Europe is associated with the ingestion of hypoglycin A contained in samaras and seedlings of the sycamore maple (*Acer pseudoplatanus*) (Votion et al., 2014; Baise et al., 2015).

We hypothesized that the morbidity and mortality of the milus at Zoo Duisburg was caused by ingestion of hypoglycin A.

MATERIALS AND METHODS

Animals and Sampling Sites

In this study, all eleven zoos and wildlife parks in different regions of Germany and Austria that have kept milus were included. Animal husbandry characteristics such as type of enclosure, vegetation, ground coverage, nutrition and medical history regarding similar symptoms to the diseased animals from Zoo Duisburg were noted in a standardized questionnaire for each holding. The diseased animals included in this study are those presenting signs compatible with a myopathy. The healthy animals are represented by not myopathic animals either from zoos other than Duisburg or milus from Duisburg that were kept in the same enclosure as the diseased deer.

Pathology

The deceased milus were brought to the CVUA (Chemical and Veterinary Investigation Office) in Krefeld, Germany. Necropsies were performed and muscle samples for histopathology were taken from many different localizations of the body, including thigh, intercostal, masseter, glossal, back, diaphragmatic, shoulder and lumbar muscles.

Blood Samples

Preserved serum samples from a total of 79 animals of different age and sex from 5 zoos were gathered. 19 samples of diseased milus from Zoo Duisburg and 60 samples of clinically healthy animals from other zoos as well as Duisburg were part of the study. The sampling years of the diseased animals were as following: 2004 (3 samples), 2005 (2 samples), 2007 (1 sample), 2010 (2 samples), 2012 (1 sample), 2013 (2 samples), 2014 (4 samples), 2015 (2 samples) and 2016 (2 samples).

28 animals under the age of 1 were classified as suckling calves. 21 animals between 1 and 3 years are representing the subadults by being sexually immature. With over 3 years of age 26 milus were considered as adults (Kern, 2008). In 4 cases the age could not be traced.

Biochemical Analysis

The sera were frozen at -18°C in Eppendorf vials and 77 samples were sent to a certified laboratory (IDEXX Laboratories, 71636 Ludwigsburg, Germany). The biochemical analysis included Cu, Se, vitamin E, Mg, urea, triglycerides, FFA and enzyme activities of creatine kinase (**CK**), alkaline phosphatase (**ALP**), aspartate aminotransferase (**AST**), glutamate dehydrogenase (**GLDH**) and, γ-glutamyl-transferase (**γ-GT**). For analyzing Se and Cu the inductively-coupled-plasma mass-spectrometry (**ICP-MS**) method was used. For Se, the isotopes Se77, 78 and 82 and for Cu, the isotopes Cu63 and 65, were analyzed at the same time by ICP-MS (Varian 820 MS). Ultrafast high performance liquid chromatography (**U-HPLC**) was taken to determine vitamin E. After protein precipitation, the extracted vitamin E was analyzed on a U-HPLC System (RS-LC 3000, Dionex/Themofisher Scientific, Dreieich, Germay). For the detection of the vitamin E a Diodenarry Detector with wavelength 295nm was used. FFA were measured photometric and the other values (Mg, Urea, CK, ALP, AST, ALT, GLDH, γ-GT) were tested by using a chemical analyzer. FFA as well as Mg, Urea, CK, AP, AST, ALT, GLDH and γ-GT have been analyzed on a Beckmann AU 5800 (Beckman Coulter, Europark Fichtenhain B13, 47807 Krefeld, Germany).

Twenty-one samples were sent to the Biochemical Genetics Laboratory of the University of Liège, Belgium for hypoglycin A and methylenecyclopropyl acetic acid-carnitine (**MCPA-carnitine**) quantification. Hypoglycin A concentration in the defrosted sera was determined by using an aTRAQ kit for amino acid analysis (Sciex, Framingham, MA, USA; Boemer et al., 2015). For MCPA-carnitine quantification, the liquid chromatography coupled with mass spectrometry (LC/MS/MS; TQ5500 mass spectrometer; Sciex, Framingham, MA, USA) was used. Serum acylcarnitines concentrations (free carnitine, C2-, C3-, C3DC-, C4-, C5-, C5-OH-, C5DC-, C6-, C8-, C8:1-, C10-, C10:1-, C10:2-, C12-, C12:1-, C14-, C14:1-, C16-, C16:1-, C18- and C18:1-Carnitine) were determined by tandem mass spectrometry (Chace et al., 2003). Briefly, serum proteins were precipitated with a methanol solution containing labelled internal standards. Supernatants were evaporated under nitrogen stream and derivatized with butanolic-HCl. Butylated samples were analyzed with a TQ5500 mass spectrometer (Sciex, Framingham, MA, USA).

Analyses of Plant Samples

Seedlings (50 g) were collected, at posteriori (spring 2017) from the enclosure where the diseased milus lived and were kept frozen (-18 °C) up to analyses. Hypoglycin A was extracted from samaras and seedlings using methanol, and high performance thin layer chromatography (**HPTLC**). Briefly, 5 g of whole maple samaras or seedlings were mechanically ground and mixed with 25 ml of pure methanol (VWR International®, Leuven, Belgium). The mixture was gently agitated for 24 h at room temperature and then centrifuged at 4,500 g for 15 min. Thereafter 12.5 ml supernatant was removed and evaporated. The residue obtained was dissolved in 3 ml of pure water. Quantification of hypoglycin A was performed by HPTLC using a CAMAG® TLC scanner 3 at 490 nm with WinCATS© 4.3 software (CAMAG, Muttenz, Switzer-land). Additional details about the procedure can be found elsewhere (Habyarimana et al., 2017).

Statistical Analysis

Data analysis was performed by using the R-program rattle (Rattle 5.0.19). This program's logistic regression function was used to identify those blood measurements which are significantly related to healthy and diseased animals respectively. Cross tabulation and Pearsons chi-square test were used to analyze whether sex and age groups were related to disease and healthiness respectively. XLSTAT (2016.06) was used to calculate the statistics for significant blood measurements including the t-test. Test results with a value of $P < 0.05$ were determined to be statistically significant.

RESULTS

A result of the questionnaire was that 25 animals from Zoo Duisburg showed the described symptoms and none of the other zoos or wildlife parks did have similar problems with their milus. The disease appeared in outbreaks in autumn-winter 2004 (11 diseased animals), 2005 (6 diseased animals), 2007 (1 diseased animal), 2010 (2 diseased animals), 2012 (1 diseased animal), 2013 (2 diseased animals), 2014 (3 diseased animals), 2015 (1 diseased animal) and 2016 (1 diseased animal) and never happened during the summer (Figure 1). One hind diseased 4 times over a period of 8 years and finally died in 2013. The diseased milus from Zoo Duisburg

showed clinical signs with a sudden onset of muscle tremor, apathy, anorexia, salivation, recumbency, labored breathing and a reduced alert response. Seven individuals additionally showed exophthalmos and 2 animals emitted dark colored urine.

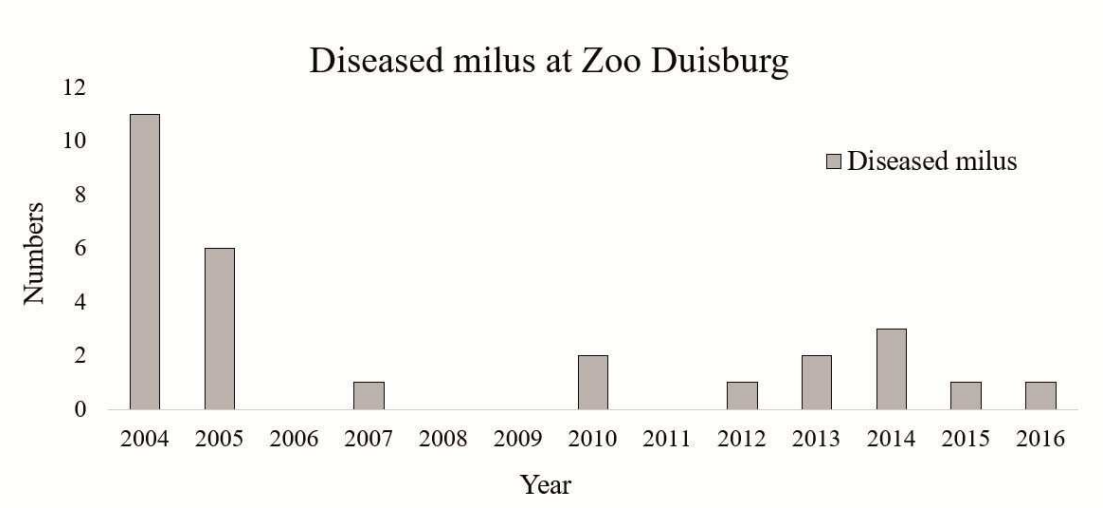

Figure 1. Numbers of diseased milus from year 2004 until 2016 at Zoo Duisburg

Over the years, the myopathic animals from Zoo Duisburg received different supportive therapies including injections of vitamin E and Se, vitamin B complex, butafosfan, menbutone, butylscopolamine and cocktail of vitamins, AA and trace elements. All of them received fluids either i.v. or s.c. during anesthesia. Different antibiotics and analgesics, including non-steroidal anti-inflammatory drugs as well as cortisone and diazepam in different combinations were given. Conversions of feeding and supplementation such as reduction of the protein amount and increase of vitamin E, Se and Cu in the ration have been made.

The enclosure of the milus at Zoo Duisburg, which hosted all of the diseased animals, was surrounded by 6 maple trees. After the last milu's death in spring 2017, seedlings of these trees were found on the ground of the enclosure in large numbers (Figure 2). During botanical inspection, the trees were identified as *Acer pseudoplatanus* and digital images of the plants and the enclosure were taken. Analyses of the samples revealed that hypoglycin A was detected in samaras and seedlings of sycamore maple collected within the enclosure at the end of March 2017 was 152.0 ± 4.5 and 202.0 ± 2.9 mg/Kg fresh weight, respectively.

Figure 2. Seedlings of *Acer pseudoplatanus* on the milu enclosure at Zoo Duisburg, March 2017

Blood samples analysis showed statistically significant greater values of CK, AST, Se, hypoglycin A and MCPA-carnitine in diseased animals (Table 1) compared to healthy individuals. The diseased milus exhibited high CK levels in serum and showed an increase in AST levels (Figure 3). All diseased animals had detectable amount of hypoglycin A and significantly greater values have been computed in diseased milus for hypoglycin A (Figure 3). Greater values have also been recorded for MCPA-carnitine in diseased animals (Figure 3). The MCPA-carnitine was only detected in 6 diseased milus out of the 10 tested animals with signs of myopathy. No healthy milus had detectable amount of MCPA-carnitine.

Table 1. Significant differences in blood values between diseased and healthy milus (t-test)

		Hypoglycin A, µmol/L	MCPA-carnitine[1], nmol/L	Se, ug/L	CK[2], U/L	AST[3], U/L
Diseased	*N*	10	10	18	18	18
	Mean	0.248	6.447	146	159,353	7,686
	Median	0.197	2.280	97	116,781	5,949
	Min	0.022	0.000	53	81	43
	Max	0.643	22.600	715	908,707	32,179
	P-value	< 0.01	< 0.05	< 0.001	< 0.001	< 0.001
Healthy	*N*	11	11	57	59	59
	Mean	0.055	0.000	54	7,964	555
	Median	0.010	0.000	49	189	58
	Min	0.000	0.000	20	1	13
	Max	0.275	0.000	123	211,967	12,534

[1] MCPA-carnitine: methylenecyclopropyl acetic acid-carnitine
[2] CK: creatine kinase
[3] AST: aspartate aminotransferase

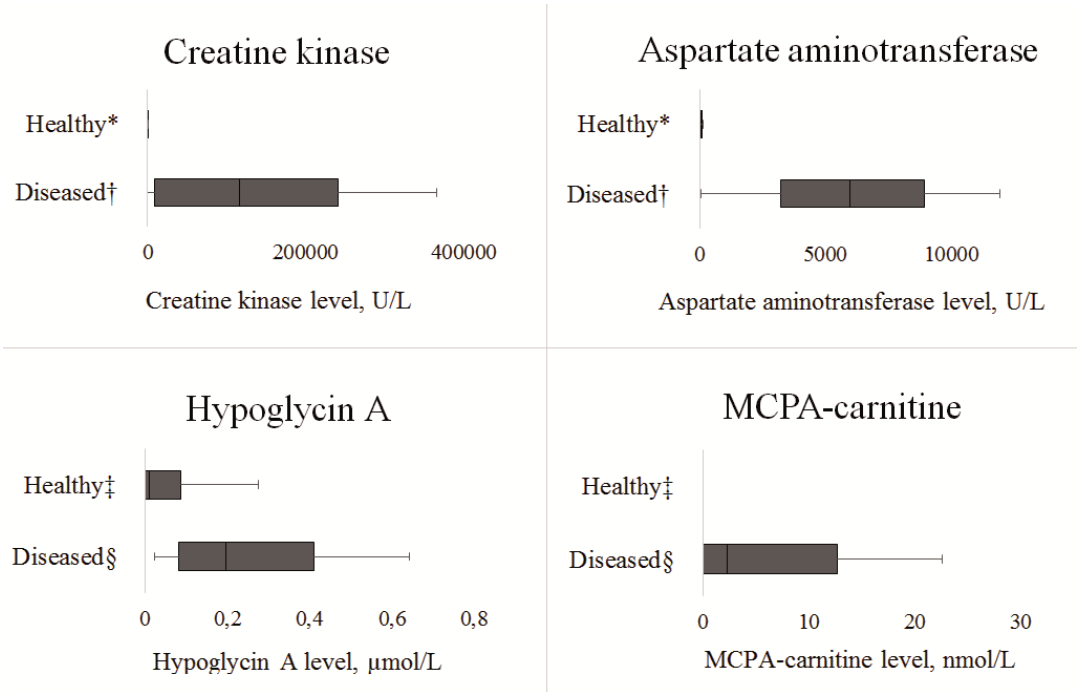

Figure 3. Creatine kinase, Aspartate aminotransferase, Hypoglycin A and MCPA-carnitine[1] values in diseased vs. healthy milus* $n = 59$, † $n = 18$, ‡ $n = 11$, § $n = 10$
[1] MCPA-carnitine: methylenecyclopropyl acetic acid-carnitine

The CK showed significant correlations with AST, MCPA-carnitine and hypoglycin A. The MCPA-carnitine showed significant correlations with hypoglycin A (Table 2).

The measured concentrations of acylcarnitines in the sera turned out to be higher in diseased milus compared to healthy ones except for 2 medium chained acylcarnitines (C8:1- and C10:2-Carnitine) where the values were equal. Statistically greater values have been found for C5-OH-Carnitine and C6-Carnitine in diseased vs. healthy milus (Table 3).

Table 2. Correlation (Pearson) between significant blood values in diseased milus

	AST[1]	MCPA-carnitine[2]	CK[3]	Hypoglycin A
AST[1]	1	0.46	0.94	0.55
MCPA-carnitine[2]	0.46	1	0.88	0.75
CK[3]	0.94	0.88	1	0.87
Hypoglycin A	0.55	0.75	0.87	1

[1] AST: aspartate aminotransferase
[2] MCPA-carnitine: methylenecyclopropyl acetic acid-carnitine
[3] CK: creatine kinase

Table 3. Serum (mean ±SD) concentrations of free carnitine and acylcarnitines (µmol/L) in diseased and healthy milus and in control horses[1] and horses with EAM[2] (non-survivors)[1]

	Healthy milus	Diseased milus, Non-survivors	Control horses	Horses with EAM, Non-survivors
	(*n*=11)	(*n*=10)	(*n*=44)	(*n*=40)
Free carnitine				
Free carnitine	79.42±61.86	121.11±50.31	26.02±1.07	87.04±7.95
Short chain acylcarnitines (C2 to C5)				
C2-Carnitine	13.02±9.42	20.00±11.36	8.77±0.67	55.23±4.24
C3-Carnitine	0.50±0.44	0.65±0.40	0.56±0.03	2.92±0.27
C3DC-Carnitine	0.04±0.04	0.06±0.03	0.03±0.00	0.34±0.04
C4-Carnitine	1.42±2.70	6.23±6.44	0.36±0.02	26.62±3.67
C5-Carnitine	0.68±1.22	2.06±1.61	0.21±0.01	24.71±3.20
C5-OH-Carnitine	0.08±0.04**	0.11±0.05**	0.06±0.0	0.56±0.07
C5DC-Carnitine	0.29±0.48	0.51±0.56	0.18±0.01	2.20±0.28
Medium chain acyl-carnitines (C6 to C10)				
C6-Carnitine	0.06±0.10***	0.39±0.45***	0.04±0.00	5.40±0.81
C8-Carnitine	0.06±0.09	0.17±0.15	0.02±0.00	1.45±0.18
C8:1-Carnitine	0.04±0.05	0.04±0.02	0.09±0.01	1.26±0.15
C10-Carnitine	0.04±0.07	0.13±0.12	0.02±0.00	0.76±0.09
C10:1-Carnitine	0.02±0.02	0.03±0.02	0.05±0.01	0.67±0.07
C10:2-Carnitine	0.01±0.00	0.01±0.00	0.03±0.00	1.31±0.14
Long chain acyl-carnitines (C12 to C18)				
C12-Carnitine	0.16±0.38	0.34±0.22	0.04±0.01	0.28±0.03
C12:1-Carnitine	0.04±0.09	0.06±0.04	0.02±0.00	0.23±0.03
C14-Carnitine	0.22±0.56	0.63±0.42	0.03±0.00	0.21±0.02
C14:1-Carnitine	0.07±0.15	0.23±0.15	0.03±0.00	0.32±0.04
C16-Carnitine	0.80±2.27	2.59±1.8	0.14±0.04	0.68±0.07
C16:1-Carnitine	0.13±0.36	0.40±0.28	0.02±0.00	0.24±0.03
C18-Carnitine	0.33±0.68	2.81±1.86	0.07±0.01	0.28±0.02
C18:1-Carnitine	0.31±0.85	0.77±0.44	0.04±0.01	0.57±0.06

[1] Boemer F et al., 2017

[2] EAM: equine atypical myopathy

**$P < 0.01$; ***$P < 0.001$; Superscripts indicate statistically greater values in diseased vs. healthy milus

There were no statistically significant differences in Cu, vitamin E, Mg, urea, ALP, GLDH, triglycerides, FFA and γ-GT comparing healthy or diseased animals. Also, there were no statistically significant differences for diseased animals between the 2 sexes and the 3 age groups (Table 4 and 5).

Table 4. Cross tabulation and Chi-square test results show no significance for age group

Age group	healthy	diseased
< 1 yr	22	9
1 to 3 yrs	14	11
> 3 yrs	19	12
not applicable	4	0

X-squared = 3.7077, df = 3, *P*-value = 0.2948

Table 5. Cross tabulation and Chi-square test results show no significance for gender

Sex	healthy	diseased
Female	35	20
Male	24	12

X-squared = 0.087635, df = 1, *P*-value = 0.7672

Pathological reports of 15 deceased milus were provided. Forty percent revealed macroscopic lesions consisted of partial pale discoloration of skeletal muscle. Histologically, skeletal muscles of all milus, except for 2 individuals, demonstrated diffuse low to high grade acute to subacute hyalinous myofiber degeneration (Figure 4), depending on the localization of the muscle. Severe degenerations were especially found in the intercostal and thigh muscles. In 3 milus multifocal low to high grade acute myocardial fiber degeneration was additionally found. The kidneys of 4 individuals exposed a tubular epithelial degeneration and the renal pelvis was filled with reddish brown urine. 5 individuals had a collateral degeneration of liver cells. Other pathological findings were unremarkable.

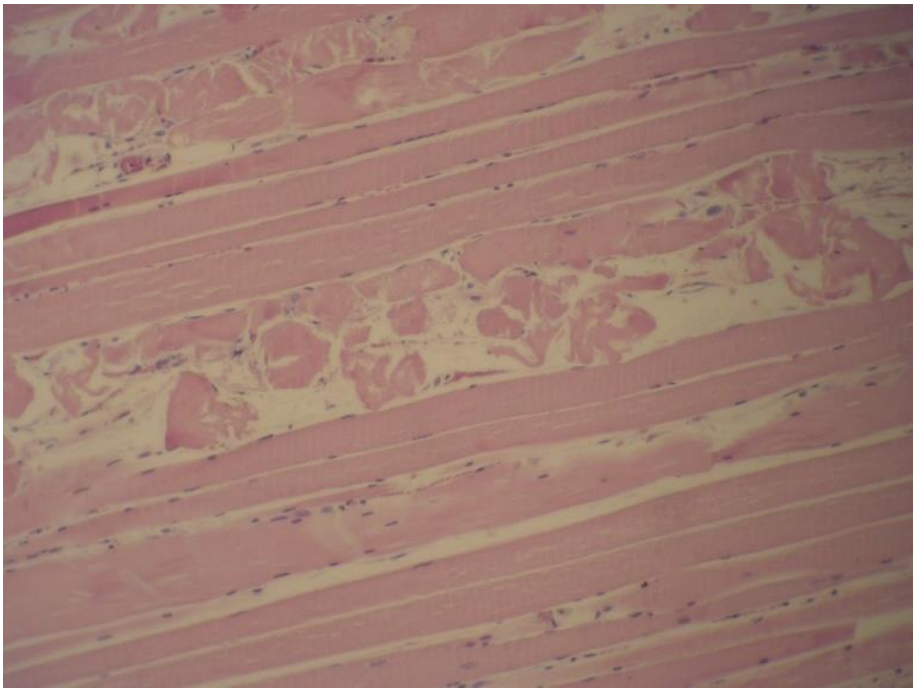

Figure 4. Skeletal muscle of a milu, hyalinous myofiber degeneration (x10 magnification) Histological specimen: CVUA, Krefeld, Germany

DISCUSSION

The results of this study show the close connection of the symptoms of the milus at Zoo Duisburg to a hypoglycin A intoxication. This shows that our hypothesis that morbidity and mortality of the milus at Zoo Duisburg was caused by ingestion of hypoglycin A was proven due to the following reasons:

Firstly, the milu enclosure was surrounded by sycamore maple trees and every autumn samaras of these trees fell onto the ground of the enclosure in large numbers. Samaras and seedlings of the sycampore maple contain the toxin hypoglycin A which is the etiological factor responsible for EAM in horses. In horses, EAM outbreaks can be divided into 2 groups. An autumnal outbreak is defined from September up to the end of February mainly caused by the ingestion of samaras. The spring outbreak from mid-March presumably originates from mostly ingestion of seedlings (Baise et al., 2015). The outbreaks in the Père David's deer at Zoo Duisburg always occurred from September until early March which suggests that the milus mainly suffered from autumnal outbreaks by ingesting samaras. After the last milu's death in 2017, seedlings of the sycamore maple were seen in large numbers on the former milu enclosure, supporting the hypothesis that the animals may have ingested the samaras in the previous autumn. The concentration of hypoglycin A from autumnal samaras cannot be extrapolated from the one found in spring. At the concentration found (about 150 mg/Kg fresh weight), almost 170 g of

samaras would be sufficient to intoxicate a 500 kg horse as hypothesized based on experiments on laboratory animals (Valberg et al. 2013). The large number of seedlings found within the enclosure (Figure 2) strengthen the hypothesis of a strong toxic pressure in autumn and thus that hypoglycin A intoxication is a possible explanation for the mortality of the deer.

Secondly, a shared temporality between outbreaks in horses and in milus could be observed. The severe outbreaks in milus happened in autumn-winter 2004 and a year later in 2005. Although there were no official recordings from the "Atypical Myopathy Alert Group" at an European level, there were outbreaks observed in horses in Belgium in both years (Votion et al., 2007). From 2006, where EAM was started to be recorded at an European level, EAM cases were reported yearly (D. Votion, personal communication). So, the outbreaks in milus in 2007, 2010, 2012, 2013, 2014, 2015 and 2016 were temporally accompanied by outbreaks in horses which corroborates our hypothesis.

In addition, the clinical signs shown by horses suffering from EAM and shown by affected milus substantiate a hypoglycin A intoxication. Both horses and milus showed clinical signs like trembling, recumbency, depression, dyspnea, weakness, normothermia, stiffness and pigmenturia, but in different severities. In EAM-horses, 96 percent showed pigmenturia during the autumnal outbreak in 2013, whereas only 2 milus out of 25 animals emitted dark colored urine. Horses additionally showed tachycardia, sweating, heart murmurs, colics, dysphagia, a distended bladder on rectal palpation and congested mucous membranes that has not occurred in milus (van Galen et al. 2012). It must be mentioned, that all clinical examinations in the milus were performed under anesthesia which might implicate a different depiction of clinical signs. Contrary to horses, the milus showed salivation and anorexia. The reduced alert response which was the first clinical sign in most cases, is not comparable with domesticated horses.

Hypoglycin A toxicity is also known in humans suffering from the Jamaican vomiting sickness and in laboratory rats that were exposed to a hypoglycin A diet. In humans, the affected show gastrointestinal distress, hypoglycaemia, central nervous system depression, vomiting, hypotonia and seizures (Barceloux, 2009), which totally differs from clinical signs in horses. Indicators of hypoglycin A toxicity in rats were abnormal motor movements, arched backs, raised hairs, blackening of faecal matters and a loss in body weight (Blake et al., 2006). Compared to the response of humans and laboratory rats to hypoglycin A, the clinical signs of horses and milus are much more alike. But even the differences in clinical signs are obviously usual between different kind of species.

Furthermore, the pathological findings in the milus are analogical to the findings in horses. Macroscopic discolorations of certain muscle groups have been found in horses as well as in

milus during necropsy. In horses suffering from EAM, a degeneration of particular muscle groups is very distinctive. Preferentially postural, respiratory skeletal muscles and the myocardium are affected. In the deceased milus the most severe and frequent lesions were found in the intercostal and thigh muscles which resembles EAM cases. Three individuals showed additionally hyalinous myofiber degeneration of the myocardium. The filling of the renal pelvis with reddish brown urine in 4 milu cases is matchable with myoglobin casts that could be consistently identified in the kidneys of horses with EAM. In horses, microscopic examination of the liver did not reveal significant changes in contrast to five deer where degenerations of liver cells were observed (Cassart et al. 2007).

The pathological findings in the milus were the first indicators that the animals might suffer from a disease similar to EAM, because no other potential cause of death has been found over the years.

The results of the statistical analysis of the blood samples are equally supportive of this topic. Creatine kinase is a blood parameter which showed highly increased levels in diseased animals. It is the most muscle-specific enzyme with occurrence in the skeletal and cardiac muscle and is therefore a very sensitive indicator for damage of striated muscles. A high increase of CK may have many reasons such as muscle trauma, exercise myopathy, diet-related deficiency states or inflammatory processes (myositis) (Moritz, 2013). In wild animals, capture myopathy is a common non-infectious, metabolic disease that is associated with pursuit, capture, restraint or transportation of animals and goes along with elevations of AST and CK in serum. Clinical signs include ataxia, muscle stiffness and prostration and histological findings are characterized by necrotic muscles (West et al., 2014). Thus, the increased activities of CK in combination with AST could be related to a capture myopathy with necrotizing muscle injury. However, at Zoo Duisburg the milus developed the symptoms without a trigger like being captured or transported. Moreover, the measured values of the CK are similar to the levels described in horses with EAM which is accompanied by increased serum activity of CK with values from 10,000 U/L to 100,000 U/L (Zuraw et al., 2015).

Diseased milus also showed a significant increase in AST levels. The most abundant causes of AST elevations are hepatopathy, muscle damage (necrosis or inflammation) or hemolysis. In the studied samples, hepatopathy and hemolysis can be excluded by other blood values (Willard and Tvedten, 2006). Glutamate dehydrogenase, ALP and γ-GT are liver-specific enzymes (Moritz, 2013) and were not significantly greater in the diseased deer. A hemolysis can be clarified by measuring the hematocrit (**hct**) and evaluating the color of serum or plasma (Willard and Tvedten, 2006). Hematocrit was not measured in this study but was always part

of measurements taken when examining the diseased milus at Zoo Duisburg. The hct was always within normal reference values for most animals. Most likely the AST increase in combination with the high CK levels is associated with a myopathy. Aspartate aminotransferase is also elevated in horses with EAM (Votion et al., 2007).

Selenium levels were also significantly increased in diseased milus compared to healthy individuals. Selenium is an essential trace element with antioxidant activity and it occurs in the enzyme glutathione peroxidase. Exposure to excessive amounts of Se results in intoxication by severe oxidative tissue damage (Forth et al., 2001). In 2011, Al-Dissi et al. (2011) described Se toxicosis in white-tailed deer (*Odocoileus virginianus*) concomitant with exhibited signs of anorexia and unsteadiness on their feet. In necropsy, myocardial necrosis and mineralization were found. However, it is unlikely that the milus in this present study suffered from a Se intoxication. The high values of Se are much more likely due to the treatment of the diseased deer with vitamin E and Se supplements either oral or via injection. This treatment was chosen, because of the elevated CK and AST serum levels in blood and the degenerative muscle damages that were found during necropsy of other deer. Due to these findings, a deficiency of Se was presumed (McGavin and Zachary, 2009). Thus, a Se intoxication can be excluded.

Moreover, the toxin hypoglycin A has been demonstrated in all diseased milus. Hypoglycin A is a nonproteinogenic amino acid that is found in the samaras and seedlings of the sycamore maple (*Acer pseudoplatanus*) (Baise et al., 2015). After ingestion, this toxin is metabolized into MCPA-CoA, an inhibitor of acyl-CoA dehydrogenase (Osmundsen and Sherratt, 1975; Ikeda and Tanaka, 1990). This enzyme catalyzes each cycle of fatty acid β-oxidation in the mitochondria. A reduced activity of the dehydrogenase engenders an accumulation of acyl-CoAs in the mitochondria. In the next step the acyl-CoAs may change into acylcarnitines that leave the mitochondria and circulate in the blood, which leads to a characteristic blood acylcarnitine profile in horses (Boemer et al., 2017). All muscles, including respiratory muscles and myocardium, utilize fatty acids as a primary energy source and the inhibition of fatty acid oxidation results in muscle damage and often leads to death. The toxic metabolite MCPA may form an ester with carnitine to MCPA-carnitine, which has been found in horses suffering from EAM (Votion et al., 2014).

The presence of hypoglycin A in the blood of diseased milus suggests the ingestion of sycamore maple samaras or seedlings.

Compared to horses with EAM (mean 5.47 ± 1.60 µmol/l) (Baise et al., 2015) the hypoglycin A value in milus is much lower. In horses, the kinetics of hypoglycin A are unknown at present and above all they are unknown in different species like in deer. In horses, usually AA are

reabsorbed in the small intestine, whereas reabsorption in ruminants is taking place in the rumen (von Engelhardt, 2010). If this is also the case for hypoglycin A, this might lead to different concentrations of the toxin in blood despite equal input of samaras or seedlings in horses and ruminants.

Hypoglycin A has also been detected in healthy deer kept at Zoo Duisburg of this study. All *E. davidianus* were kept in the same enclosure the whole time. Certainly, every individual had access to samaras of the surrounding maple trees. In horses, hypoglycin A was also found in the blood of apparently healthy co-grazers which had no clinical signs (Baise et al., 2015). This led to the suggestion that hypoglycin A ingestion alone is not the sole trigger for EAM, but that there may be additional mechanisms that are involved in the pathogenesis of this disease or that there are different toxic levels according to individuals. A possible explanation for individuals that are staying subclinical might be a resistance to the toxic metabolite or blockage of the hypoglycin A conversion into MCPA by in- or extrinsic factors like immunity or antioxidant status (Baise et al., 2015). The demonstration of the presence of hypoglycin A in the blood of all diseased animals confirms the exposition to the toxin and thus the presumably ingestion of sycamore maple samaras.

In horses, acylcarnitines can be used to confirm a diagnosis of EAM and also some of the acylcarnitines are informative regarding prognosis of survival of the affected horses comparing values between survivors and non-survivors (Boemer et al., 2017). The determination of survivors was not possible in this study due to the death of all included milus. The acylcarnitines can be sorted into 3 groups: short, medium and long chain acylcarnitines, corresponding to their metabolic pathways. Most of the acylcarnitine mean serum concentrations of diseased milus, including all short and long chain acylcarnitines as well as free carnitine, were higher compared to healthy ones. Only the medium chain acylcarnitine C8:1 and C10:2 values were equal respectively healthy and diseased milus. C5-OH-Carnitine and C6-Carnitine were statistically significant showing greater values in diseased vs. healthy animals. This leads to the conclusion that acylcarnitines may be used not only in horses, but also in milus to confirm the diagnoses of a myopathy caused by hypoglycin A. Comparing the values of healthy deer with control horses, greater concentrations are noticeable in the milus except in four acylcarnitines (C3, C8:1, C10:1 and C10:2). A possible explanation for these greater values is that the healthy milu group consisted of co-grazers that were likewise exposed to the samaras. Diseased milus showed lower concentrations of short and medium chain acylcarnitines, but greater concentrations in free carnitine and in six out of eight longchain acylcarnitines (C12, C14, C16, C16:1, C18 and C18:1) comparing to non-survived horses with EAM.

Finally, the MCPA-carnitine, representing the toxic metabolite of hypoglycin A, was only detected in diseased milus and not in healthy ones.

The measured MCPA-carnitine levels were lower than mean concentrations in diseased horses (20.39 ± 17.24 nmol/l) (Votion et al., 2014). As the metabolism of hypoglycin A in ruminants is uncovered, this might lead to different ranges of MCPA-carnitine in the blood comparing to horses.

Unexpectedly, in 40 percent of the diseased deer MCPA-carnitine could not be detected. This matter of fact was also found in horses, where a high concentration of hypoglycin A, but a low concentration of MCPA-carnitine in serum was detected. In this particular case, it was assumed that the early euthanasia of the horse after the beginning of intoxication impede MCPA-carnitine accumulation in the blood (Bochnia et al., 2015). In the 40 percent of this study, additionally slightly lower CK values were recorded. Due to these circumstances, it leads to the assumption that there might be several interacting factors followed by the intoxication of the deer. The milus might have suffered from an unresolved disease that only in combination with ingestion of hypoglycin A led to the myopathy. This hypothesis leaves a margin for further studies.

As statistical analysis has pointed out, there is a high correlation between the four values (hypoglycin A, MCPA-carnitine, CK and AST), especially for MCPA-carnitine and CK (R= 0.88). Thus, it can be said that the greater the MCPA value in a deer, the more muscle damage will occur, which will be represented by the high CK value.

CONCLUSION

Hypoglycin A was detected in the blood of all diseased milus thus confirming toxin exposure possibly by ingestion of samaras of *Acer pseudoplatanus* at Zoo Duisburg.

Until now EAM was only known in the family of *Equidae* belonging to the order of *Perissodactyla* such as a horse (*Equus ferus caballus*) (Votion et al., 2009). Besides this, hypoglycin A intoxication has only been described in humans suffering from the Jamaican vomiting syndrome (Barceloux, 2009) and in laboratory rats (Blake et al., 2006) and it has not been published for other zoological families or orders. *Elaphurus davidianus* belongs to the family of *Cervidae* within the order of *Artiodactyla* (Gaisler and Zejda, 1997).

As ruminants have a different digestive tract comparing to horses, it will need further investigation to find out if there are several factors involved that trigger an outbreak of this disease in deer and to solidify this hypothesis.

However, there is the need for prevention of this myopathy. As *A. pseusoplatanus* is ubiquitous in the north of Europe it is difficult to control the exposure to the toxin (Baise et al., 2015). Prevention is even more complicated in zoos as the susceptible species have not been established at this stage.

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