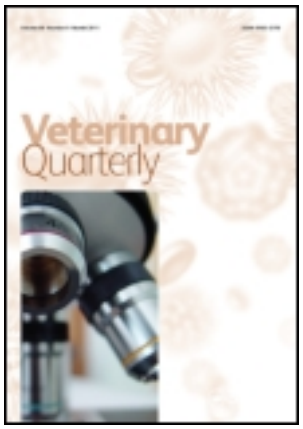

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Some notes on fatal acquired multiple acyl-CoA dehydrogenase deficiency (MADD) in a two-year-old warmblood stallion and European tar spot (Rhytisma acerinum)

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CASE REPORT

Some notes on fatal acquired multiple acyl-CoA dehydrogenase deficiency (MADD) in a two-year-old warmblood stallion and European tar spot (*Rhytisma acerinum*)

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Keywords: horse; equine; MADD; atypical myopathy; tar spot; *Rhytisma acerinum*; NMR; riboflavin; vitamin B_2; pyradizine

A two-year-old warmblood stallion was admitted to the Equine Clinic of Utrecht University on 8 April 2010 because of progressive muscle pain and reluctance to stand for two days. The horse had been pastured 11 days before admission with access to fallen maple (*Acer pseudoplatanus*) leaves covering its pasture. On arrival, the stallion had a stiff gait and extremely firm gluteal, quadriceps, longissimus, and triceps muscles. It became recumbent shortly after arrival with myoglobinuria observed. The analysis of plasma showed an increased hematocrit (0.51 L/L; reference range 0.36–0.42), leukocytosis (12.6 G/L; reference range 7–10), and increased activity of muscle enzymes like creatine kinase (CK 621779 IU/L; upper limit of reference range < 200), aspartate aminotransferase (AST 14990 IU/L; upper limit of reference range < 275), and lactate dehydrogenase (LDH 50250 IU/L; upper limit of reference range < 420). An *ante-mortem* urine sample was subjected to an analysis of organic acids, glycine conjugates, and acylcarnitines and results compared with the 95th percentile as the upper limit of the reference range based on the values obtained from 12 clinically healthy control warmblood horses. Identification-analyses of organic acids and glycine conjugates in urine were carried out by gas chromatography-mass spectrometry (Hewlett Packard 5890 series II gas chromatograph linked to a HP 5989B MS-Engine mass spectrometer). Free carnitine and acylcarnitines in urine were analyzed as their butyl-ester derivatives by electrospray tandem mass spectrometry (Micromass Quattro Ultima system equipped with an Alliance HPLC system). Urinary concentrations were expressed as analyte: creatinine ratios. The urinary metabolic screening of the stallion revealed an increased excretion (above the 95th percentile) of the organic acids ethylmalonic acid (256 mmol/mol creatinine) and 2-methylsuccinic acid (95 mmol/mol creatinine) as well as the presence of the glycine conjugates butyrylglycine, (iso)valerylglycine, and hexanoylglycine. Furthermore, the profile of acylcarnitines in urine (Table 1) showed a substantial elevation above the 95th percentile cut-off value for free carnitine and short (acylgroups less than six carbon atoms) as well as medium (acylgroups with 6–12 carbon atoms) chain acylcarnitines indicating equine acquired multiple acyl-CoA dehydrogenase deficiency (MADD) formerly known as atypical myopathy. In accord, histopathology using muscle tissue fixed in 10% buffered formalin revealed generalized myopathy with Zenker degeneration predominantly affecting Type 1 muscle fibres. At the owner's request, euthanasia was performed based on poor prognosis and financial constraints.

In addition, ^{1}H and ^{13}C nuclear magnetic resonance (NMR) measurements were performed (Bruker Avance III 750 MHz spectrometer) on *ante-mortem* urine and lateral vastus muscle tissue of the affected stallion. Muscle tissue was collected immediately after euthanasia in liquid nitrogen and stored at –80°C. Urine and lateral vastus muscle tissues of a nine-year-old warmblood mare were used as control. 1D proton, 2D–total correlation spectroscopy (TOCSY) and 2D 13C-1H heteronuclear single quantum coherence (HSQC) NMR on urine from the affected stallion revealed relatively high levels of free amino acids and myoglobin compared to the spectra from the control horse (data not shown). NMR on muscle tissue extracts in 10 volume per cent heavy water (D_2O) did not show clear differences between the control and patient spectra (data not shown). In addition, NMR measurements (Bruker Avance II 500 MHz spectrometer) were performed on maple (*A. pseudoplatanus*) leaves with and without the presence of European tar spot (*Rhytisma acerinum*) (Figure 1). An extraction of mortered fresh leaves in either heavy water or chloroform-d^{3} overnight at 20°C was used to globally compare NMR spectra of infected and uninfected leaves. The results showed a higher sucrose and glucose content in the water-soluble fraction of infected leaves compared to uninfected leaves

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Table 1. Concentrations of acylcarnitines (mmol/mol creatinine) in urine from a two-year-old warmblood stallion compared with the 95th percentile as the upper limit of the reference range based on the values obtained from 12 clinically healthy control warmblood horses.

Analyte	Concentration	P95
Free carnitine	739.1	12.5
C2-carnitine	382.6	1
C3-carnitine	41.0	0.1
C4-carnitine	306.9	1
C5:1-carnitine	0.22	0.01
C5-carnitine	319.9	0.1
C4:3-OH-carnitine	5.1	0
C6-carnitine	69.0	0.02
C5-OH-carnitine	5.4	0.1
C8:1-carnitine	41.8	0.01
C8-carnitine	20.5	0.03
C10:2-carnitine	31.5	0.02
C10:1-carnitine	7.5	0.01
C10-carnitine	5.8	0.02
C4DC-carnitine	0.46	0.4
C5DC-carnitine	9.1	0.5
C12:1-carnitine	0.43	0.01
C12-carnitine	0.12	0.04
C6-DC-carnitine	0.71	0.1
C14:2-carnitine	0.04	0
C14:1-carnitine	0.06	0.01
C14-carnitine	0.06	0.03
C8-DC-carnitine	0.27	0.05
C14-OH-carnitine	0.04	0.01
C16:1-carnitine	0.06	0
C16-carnitine	0.09	0.02
C10-DC-carnitine	0.12	0.02
C16:1-OH-carnitine	0.03	0
C16-OH-carnitine	0.03	0.01
C18:2-carnitine	0.00	0
C18:1-carnitine	0.05	0
C18-carnitine	0.07	0
C18:2-OH-carnitine	0.01	0
C18:1-OH-carnitine	0.02	0
C18-OH-carnitine	0.01	0
C16-DC-carnitine	0.02	0
C18:1-DC-carnitine	0.01	0

(Figure 2a). Furthermore, no large spectral changes occurred between uninfected and infected leaves, except for a scalar coupled TOCSY spin system with ^{1}H resonances at 9.22 ppm (d), 7.04 ppm, 6.06 ppm, and 2.24 ppm (m) seen only in the *R. acerinum* infected leaves (Figure 2a and 2b). The observed chemical shifts may correspond with a substituted pyradizine compound. However, the exact identity of this potential toxic compound needs to be confirmed. The chloroform (CD_3Cl) fraction of *R. acerinum* infected leaves mainly contained fatty acids in a similar abundance as seen in uninfected leaves (data not shown).

The first reports of myopathy in grazing horses concerned outbreaks that already occurred in the autumn of 1939 in the North of Wales, UK (Bowen and Craig 1942). The condition does not seem to be contagious, but it is usually reported as outbreaks since particular environmental characteristics and specific weather conditions predispose to the disease (Whitwell et al. 1988; Votion et al. 2007). The disease is associated with a lethality rate of up

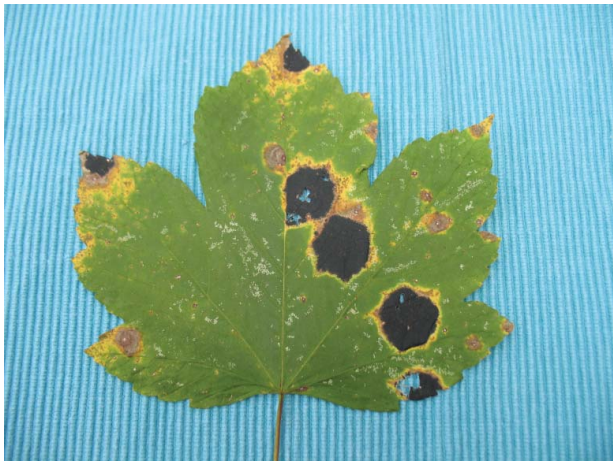

Figure 1. Maple (*Acer pseudoplatanus*) leaf covered with European tar spot (*Rhytisma acerinum*).

to 90% and death usually occurs within 72 hours preceded by severe (muscle) pain (Votion and Serteyn 2008). The latency period of the disease was estimated at up to four days (van Galen et al. 2012).

The biochemical defect has been identified as a deficiency of several mitochondrial dehydrogenases that utilize flavin adenine dinucleotide as a cofactor. Riboflavin or vitamin B_2 is a precursor in the synthesis of flavin adenine dinucleotide. These mitochondrial dehydrogenases include the acyl-CoA dehydrogenases of mitochondrial fatty acid β-oxidation and the dehydrogenases that degrade the CoA-esters of glutaric acid, isovaleric acid, 2-methylbutyric acid, isobutyric acid, and sarcosine. The equine disease reflects a disease similar to human multiple acyl-CoA dehydrogenase deficiency (MADD) also known as glutaric aciduria Type II (Przyrembel et al. 1976). Equine acquired MADD has been reported in Europe (Westermann et al. 2008) as well as in North America (Sponseller et al. 2012).

To date, equine acquired MADD is of hitherto unknown etiology given the fact that Koch's postulates have not been fulfilled yet although *Clostridium sordellii* toxin (Unger-Torroledo et al. 2010), maple leaves (*A. pseudoplatanus*) covered with the fungus European tar spot (*R. acerinum*) (van der Kolk et al. 2010; Sas et al. 2012), and (branched amino acid) hypoglycin A content in seeds from box elder trees (*A. negundo*) (Valberg et al. 2012) have been suggested as a causative factor. Of note, it has been shown that accumulated fallen leaves may increase the risk of equine acquired MADD associated with an odds ratio of 10.7 (Votion et al. 2007) with *A. pseudoplatanus* present in all pastures horses suffering from equine acquired MADD visited (Votion et al. 2009).

Tar spot of maple is caused by species of the ascomycete genus *Rhytisma*, and has a worldwide distribution wherever maples are found. Tar spot on Norway maple (*A. platanoides*) in North America is caused by *R. acerinum*, the same fungus found in Europe, whereas native maple species in North America have the tar spot caused by *R. americanum* (e.g. on silver maple, *A. saccharinum* and red maple, *A. rubrum*) or the speckled tar spot caused

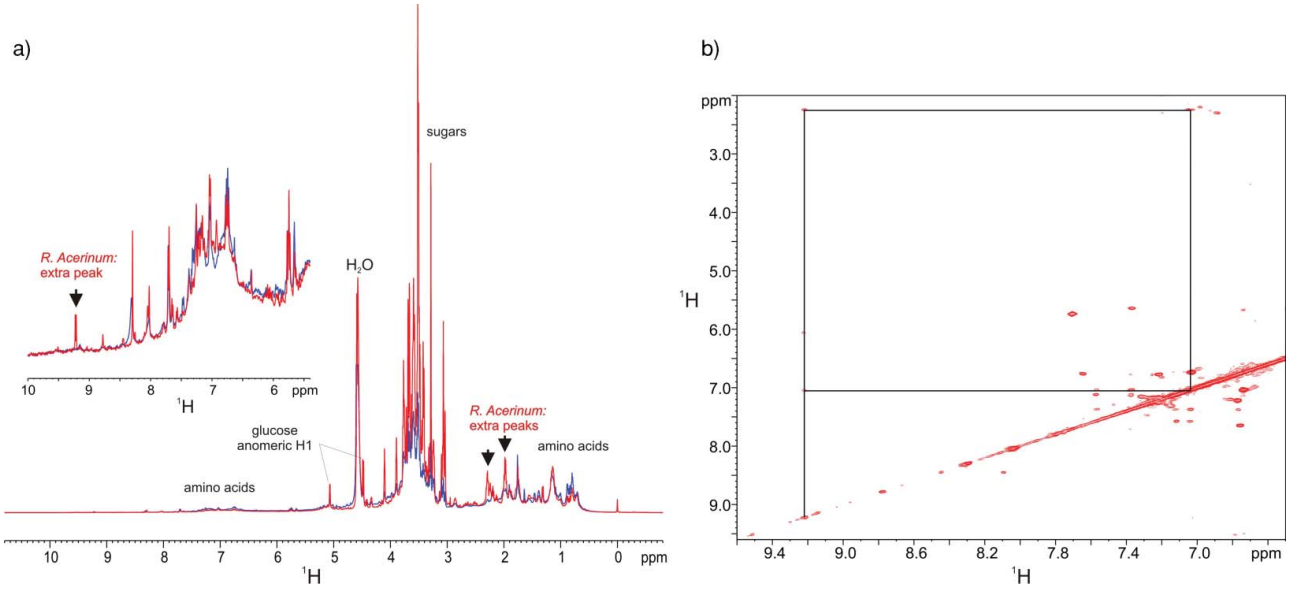

Figure 2. 500 MHz ^{1}H NMR spectra of the water-soluble fraction (D_2O solution) of maple leaves after washing with chloroform (CD_3Cl). **2a**. Comparison between the 1D proton spectrum in D_2O of control leaves (blue) and *R. acerinum* infected leaves (red). Chemical shifts are referenced to residual tetramethylsilane (TMS) (0 ppm) that was present in the deuterated chloroform used in the washing step. Arrows indicate extra peaks in the *R. acerinum* spectrum. **2b**. 2D ^{1}H-^{1}H TOCSY spectrum (100 msec mixing time) of *R. acerinum* infected leaves recorded under similar solution conditions as the red spectrum given in Figure 2a. Correlations between resonances of the coupled spin system, connected to the signal at 9.22 ppm, are indicated by black lines.

by *R. punctatum* (on big-leaf maple, *A. macrophyllum*) (Hsiang and Tian 2007). Both *R. acerinum* and Norway maple (host and pathogen) are immigrant species in North America in contrast to *R. americanum* (Hudler et al. 1998). Both *R. acerinum* and *R. americanum* can pass through an initially punctuate (speckled tar spot) stage when spots are just forming, where they might be easily confused with *R. punctatum*. *R. punctatum* is native in Western North America (Hsiang and Tian 2007).

Ascospore release from tar spots on Norway maple in Southern Ontario, Canada occurred over a three-week period, the start of which coincided with full leaf expansion. By the end of June, nearly all the asci have fully discharged their spores (Hsiang and Tian 2007). It might be hypothesized that this three-week period of ascospore release is of utmost importance in the epidemiology of equine acquired MADD with wet weather conditions in June preventing tar spot abundance. On the other hand, following heavy sporulation in June an abundant presence of infected leaves might be expected lasting about one year beyond via overwintering of *R. acerinum* on fallen leaves (Figure 3).

Of note, tar spot of maple has been increasing in incidence and severity in the Great Lakes region of eastern North America since the 1990s. Tar spot was particularly abundant in 2006 across eastern North America with most leaves of Norway Maple bearing multiple black spots (Hsiang and Tian 2007). The largest outbreaks of equine acquired MADD occurred during the autumns of 2006 and 2009 in Belgium, France, and Germany with the most recent outbreak during fall 2009 in Europe being the largest ever illustrating its emerging nature (van Galen et al. 2012). In the Netherlands, outbreaks were noticed during the autumns of 2004, 2006, and 2009 with the outbreak during autumn 2009 also being the largest.

Over the years 2000–2012, the quantity of rain fall (mm) per month, the rain duration (hours) per month, and the number of rain days per month for May, June, and both months together were obtained from meteorological data as provided by the Royal Netherlands Meteorological Institute (Figure 4). Based on this small data set, an outbreak was always preceded by a maximum of 10 rain days in June the year before combined with less than 20 rain days in June in the year of the outbreak. No doubt a dry June might maximally facilitate ascospore release and a

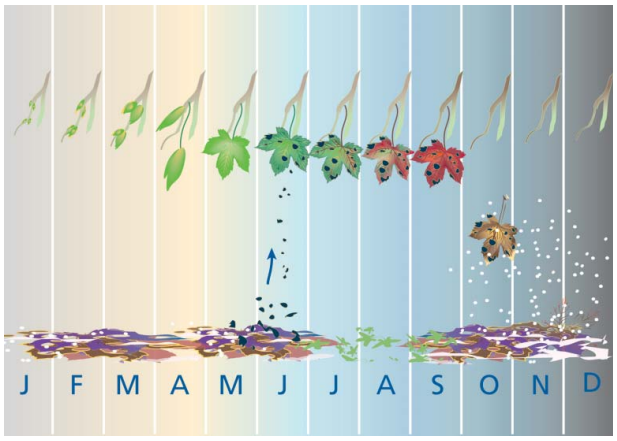

Figure 3. Seasonal pattern of *R. acerinum* (based on Hsiang and Tian 2007). Overwintering leaves might have stroma, paraphyses, and asci of *R. acerinum*. In early May, filiform ascospores might be formed averaging 55 by 2 μm. By late May, maples might be abundantly producing and shedding pollen and small samaras. By the end of June, nearly all the asci have fully discharged their spores. (Samaras not shown).

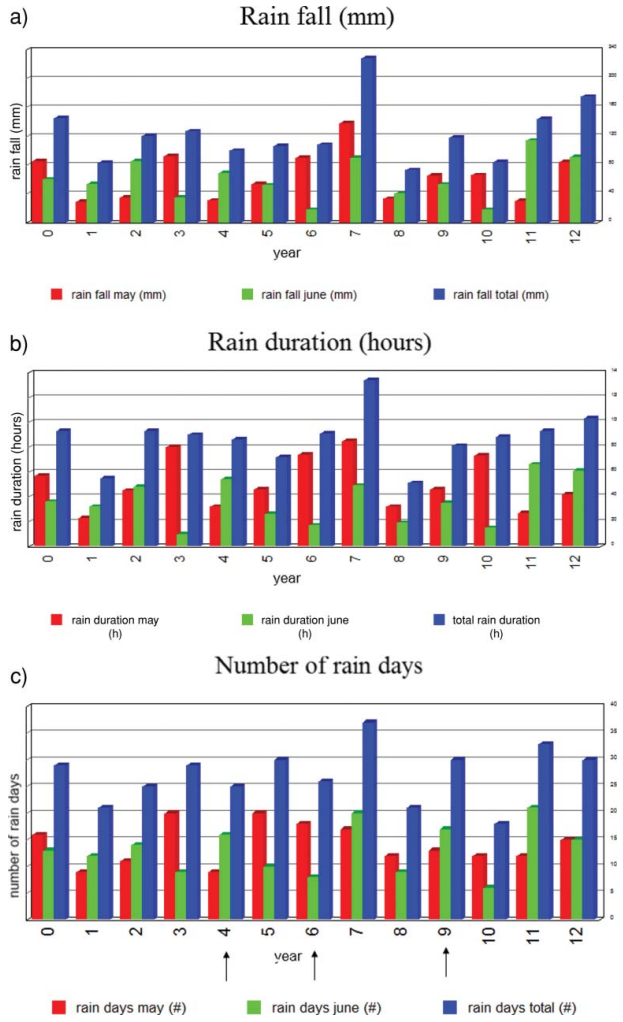

Figure 4. The quantity of rain fall (mm) per month (a), the rain duration (hours) per month (b) and the number of rain days per month (c) for May, June, and both months together over 2000–2012 obtained from meteorological data as provided by the Royal Netherlands Meteorological Institute, De Bilt, the Netherlands. Arrows indicate outbreaks of equine acquired multiple acyl-CoA dehydrogenase deficiency (MADD) in the Netherlands.

subsequent dry June the next year might even enhance it, thereby generating large tar spot abundance. On the other hand, two subsequent wet months of June might prevent tar spot abundance. The months of June 2011 and 2012 taken together generated the maximum number of rain days (36) during the period studied. In accord, tar spot is almost absent in the Netherlands in autumn 2012. Based on the previous, it might be predicted that an outbreak of equine acquired MADD in the autumn of 2012 (and spring 2013) in the Netherlands is very unlikely. In comparison, the large outbreak during autumn 2009 was preceded by a total of 26 rain days in June 2008 (9 days) and June 2009 (17 days).

Although the maple (*A. pseudoplatanus*) originally is not native in the Netherlands, it to date is very common in the Netherlands with almost total coverage in contrast to box elder trees (*A. negundo*). As shown in Figure 5, *R. acerinum* has been present in the Netherlands at least since 1830.

Figure 5. Year of collection of eldest specimen of European tar spot (*R. acerinum*) present per province in the Netherlands as stored in the National Herbarium of the Netherlands, Leiden, the Netherlands.

NMR spectra showed a higher carbohydrate (sucrose and glucose) content in the water-soluble fraction of infected leaves compared to uninfected leaves, thereby possibly facilitating ingestion by horses. This may in practice lead in horses to an eating preference for the sweeter tasting *R. acerinum* infected leaves over the uninfected leaves. Furthermore, the observed chemical shifts of the additional proton peaks in the NMR spectrum of *R. acerinum* infected maple leaves may correspond with the presence of a substituted pyradizine compound potentially leading to covalent binding to mitochondrial dehydrogenases. However, not only needs the exact identity of this potential toxic compound to be confirmed, but it should also be realized that the contamination of the leaves by pesticides based on pyradizine chemistry cannot be excluded.

Further research is necessary to assess hypoglycin A content of seeds and leaves of *Acer pseudoplatanus* and *R. acerinum*. In addition, it would be of interest to study seasonal occurrence of maple samaras as the equine disease is usually reported as outbreaks. Last but not the least, Koch's postulates should be fulfilled.

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References

Bowen JN, Craig JF. 1942. Myoglobinuria in horses. *Vet Rec*. 35:354–355.

Hsiang T, Tian XL. 2007. Sporulation and identification of tar spot of Maple in Canada. *Acta Silv Lign Hung Spec Edition*. 71–74.

Hudler GW, Jensen-Tracy S, Banik MT. 1998. *Rhytisma americanum* sp. nov: a previously undescribed species of *Rhytisma* on maples (*Acer* spp.). *Mycotaxon*. 68:405–416.

Przyrembel H, Wendel U, Becker K, Bremer HJ, Bruinvis L, Ketting D, Wadman SK. 1976. Glutaric aciduria type II: report on a previously undescribed metabolic disorder. *Clin Chim Acta*. 66:227–239.

Sas AMC, van der Kolk JH, Dank M, Westermann CM. 2012. Atypical myopathy: a review and description of the outbreak in the Netherlands during autumn 2009 and spring 2010. *Tijdschr Diergeneeskd*. 137(8):514–521.

Sponseller BT, Valberg SJ, Schultz NE, Bedford H, Wong DM, Kersh K, Shelton GD. 2012. Equine multiple acyl-CoA dehydrogenase deficiency (MADD) associated with seasonal pasture myopathy in the midwestern United States. *J Vet Intern Med*. 26(4):1012–1018.

Unger-Torroledo L, Straub R, Lehmann AD, Graber F, Stahl C, Frey J, Gerber V, Hoppeler H, Baum O. 2010. Lethal toxin of *Clostridium sordellii* is associated with fatal equine atypical myopathy. *Vet Microbiol*. 144:487–492.

Valberg SJ, Sponseller BT, Hegeman AD, Earing J, Bender JB, Martinson KL, Patterson SE, Sweetman L. Forthcoming 2012. Seasonal pasture myopathy/atypical myopathy in North America associated with ingestion of hypoglycin A within seeds of the box elder tree. *Equine Vet J*.

Van der Kolk JH, Wijnberg ID, Westermann CM, de Sain-van der Velden MG, Kranenburg LC, Duran M, Dijkstra JA, van der Lugt JJ, Wanders RJ, Gruys E. 2010. Equine acquired multiple acyl-CoA dehydrogenase deficiency (MADD) in 14 horses associated with ingestion of Maple leaves (*Acer pseudoplatanus*) covered with European tar spot (*Rhytisma acerinum*). *Mol Genet Metab*. 101: 289–291.

van Galen G, Marcillaud Pitel C, Saegerman C, Patarin F, Amory H, Baily JD, Cassart D, Gerber V, Hahn C, Harris P, et al. 2012. European outbreaks of atypical myopathy in grazing equids (2006–2009): spatiotemporal distribution, history and clinical features. *Equine Vet J*. 44(5): 614–620.

Votion DM, Linden A, Delguste C, Amory H, Thiry E, Engels P, van Galen G, Navet R, Sluse F, Serteyn D, Saegerman C. 2009. Atypical myopathy in grazing horses: a first exploratory data analysis. *Vet J*. 180:77–87.

Votion DM, Linden A, Saegerman C, Engels P, Erpicum M, Thiry E, Delguste C, Rouxhet S, Demoulin V, Navet R, et al. 2007. History and clinical features of atypical myopathy in horses in Belgium (2000–2005). *J Vet Int Med*. 21:1380–1391.

Votion DM, Serteyn D. 2008. Equine atypical myopathy: a review. *Vet J*. 178:185–190.

Westermann CM, Dorland L, Votion DM, de Sain-van der Velden MG, Wijnberg ID, Wanders RJ, Spliet WG, Testerink N, Berger R, Ruiter JP, van der Kolk JH. 2008. Acquired Multiple AcylCoA Dehydrogenase Deficiency in 10 horses with atypical myopathy. *Neuromuscul Disord*. 18:355–364.

Whitwell KE, Harris P, Farrington PG. 1988. Atypical myoglobinuria: an acute myopathy in grazing horses. *Equine Vet J*. 20:357–363.