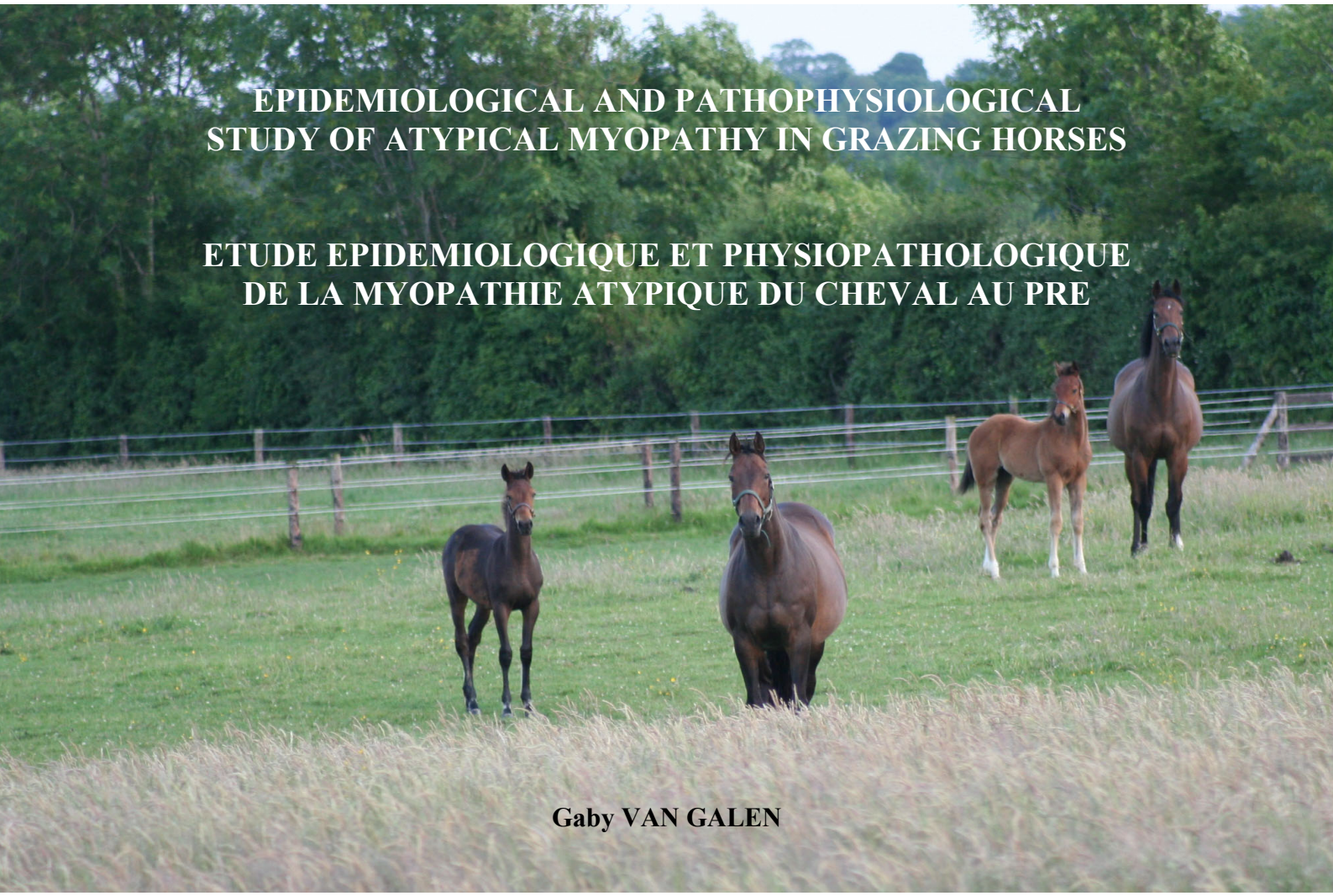
UNIVERSITY OF LIEGE
FACULTY OF VETERINARY MEDICINE
DEPARTEMENT OF CLINICAL SCIENCE
EQUINE INTERNAL MEDICINE



EPIDEMIOLOGICAL AND PATHOPHYSIOLOGICAL STUDY OF ATYPICAL MYOPATHY IN GRAZING HORSES

ETUDE EPIDEMIOLOGIQUE ET PHYSIOPATHOLOGIQUE DE LA MYOPATHIE ATYPIQUE DU CHEVAL AU PRE

Gaby VAN GALEN

WORK PRESENTED IN VUE OF OBTAINING A
« DIPLOME D'ETUDE APPROFONDIE ORIENTATION MEDECINE VETERINAIRE »

ACADEMIC YEAR 2007-2008

AKNOWLEDGEMENTS

First of all, I would like to thank my promoter, Dominique. Dominique, without you I would not have been able to be produce this DEA on such a short time. With your precision, enthusiasm and energy you pushed me to go deeper and further into the subject and you spend a lot of time yourself to do this together with me. Thanks a million. We make a good team!

Several other persons remain to be thanked for this DEA. Helene, my co-promotor, thank you are for encouraging us to proceed in this research. Prof Serteyn, you were kind of a second co-promotor. Thanks for your confidence and stimulating Dominique and me to work together. Madame Deby, thank you so much for the immense work you have done on the analysis of the fatty acids with your team and for the kindness which you explained me “Le Chinois de Chemie”. Claude Saegerman, for the statistical analysis. Dominique Cassart, for all the work you have done on autopsied cases and for your help on staining questions. Thanks also to the whole team of the “Pole Equin” (assistants, interns, secretary, stalknechten and technical staff) for their enthusiasm and courage to take care of those poor patients and their owners and to give me time to work on my DEA. Geert and Wendy, thank you for printing my DEA with lots of TLC.

Last but not least, two persons that are very dear to me. Mama, because of you I have been able to go to university and to do what I wanted to do. Denis, you have been a great support. I would not have been able to get here without you. Sorry for all the moments I was too busy working on this DEA. But, never forget: “ik hou van je”. And “dich en ich” that’s all that matters.

LIST WITH USED ABBREVIATIONS

AM: atypical myopathy

AM_1 and AM_2: horses 1 and 2 affected by atypical myopathy, respectively, used for the fatty acid analysis

AMa and AMb: horse affected by atypical at day 3 and at day 10 after the onset of the clinical signs, used for the high resolution respirometry

AMAG: atypical myopathy alert group

ATP: adenosine triphosphate

BHT: butylhydroxytoluene

Bwt: body weight

CC: case of atypical myopathy confirmed by histology

C_1, C_2 and C_3: control horses n°1, 2 and 3, respectively

$CHCl_3$: chloroform

CK: creatine kinase

D: doubtful case of atypical myopathy

FID: flame ionisation detection

ETF: electron-transferring flavoprotein

ETF:QO: ETF:ubiquinone oxidoreductase

ETS: electron transport system

FAD: flavin adenine dinucleotide

$FADH_2$: Reduced form of flavin adenine dinucleotide

FFA: free fatty acids

FMN: flavin mononucleotide

GBED: glycogen branching enzyme deficiency

GLC: gas liquid chromatography

GTP: guanosine triphosphate

HAD: 3-hydroxyacyl-CoA dehydrogenase or β-hydroxyacyl-CoA dehydrogenase

HP: case with a high probability diagnosis of atypical myopathy

HRR: high resolution respirometry

I: case infirmed for the diagnosis of atypical myopathy

IV: intravenous

LP: case with a low probability diagnosis of atypical myopathy

MADD: Multiple Acetyl-CoA Dehydrogenase Deficiency

NAD^+: oxidized form of nicotinamide adenine dinucleotide

$NADH+H^+$: reduced form of nicotinamide adenine dinucleotide

NADH-TR: NADH tetrazolium reductase

NSAID: non steroidal anti-inflammatory drugs

OXPHOS: oxidative phosphorylation

PaO_2: arterial partial pressures in O_2

PO: *per os*

Q: coenzyme Q or ubiquinone

SDH: succinate dehydrogenase

TLC: thin layer chromatography

TABLE OF CONTENTS

ETUDE EPIDEMIOLOGIQUE ET PHYSIOPATHOLOGIQUE DE LA MYOPATHIE ATYPIQUE DU CHEVAL AU PRE

RESUME EN FRANCAIS

Sur base des premiers cas belges, l'épidémiologie et la physiopathologie de la myopathie atypique ont été étudiées, mais son origine est toujours inconnue tandis que le mécanisme pathologique n'est qu'ébauché. Les objectifs de ce travail sont de préciser (1) l'épidémiologie, (2) les signes cliniques et (3) la physiopathologie de la myopathie atypique grâce à l'étude rétrospective des 165 cas européens recensés ces 2 dernières années. Pour mieux définir sa physiopathologie, la composition en acides gras et la respiration de muscles prélevés sur chevaux atteints et contrôles ont été étudiées.

Les nouveautés par rapport aux observations antérieures sont un taux de survie supérieur, l'atteinte d'ânes et de zèbres ainsi que la présence de signes cliniques peu ou/ pas rapportés antérieurement (*e.g.* hyperthermie, transit ralentis). Cette étude identifie certains facteurs pronostiques de survie (*e.g.* muqueuses normales, rester debout) et propose un traitement basé sur le dysfonctionnement observé (alimentation basée sur les sucres plutôt que les graisses). En effet, l'étude de la respiration mitochondriale et de la composition en acides gras des muscles confirmait l'hypothèse d'un problème au niveau du métabolisme énergétique et lipidique (les muscles préférentiellement atteints par la myopathie atypique contenaient plus d'acide gras dont l'acide palmitoléique).

MOTS-CLES EN FRANCAIS :

Myopathie, cheval, épidémiologie, acides gras, respirométrie

EPIDEMIOLOGICAL AND PATHOPHYSIOLOGICAL STUDY OF ATYPICAL MYOPATHY IN GRAZING HORSES

RESUME EN ANGLAIS

The epidemiological study describes European cases affected by atypical myopathy from the autumn of 2006 to the winter of 2008. Compared to previous studies, the described outbreaks were less devastating and for the first time clinically affected donkeys and zebras were reported. More horses were reported to present hyperthermia, penile prolapse and diminished digestive transit. Identified positive prognostic factors were normal mucosae and remaining standing.

In the pathophysiological study, free fatty acids analysis and high resolution respirometry were performed on muscle samples of horse(s) affected with atypical myopathy and control horses. Muscles of affected horses contained more free fatty acids (especially palmitoleic acid (C16:1)) than controls. Muscles with the majority of type I fibers contained most free fatty acids. In the affected horse the mitochondrial respiration was severely diminished in all steps of the respirometry. As well the free fatty acid analysis as the high resolution respirometry demonstrated a severe problem in muscular energy metabolism. A treatment is proposed based on the observed dysfunction (nutrition based on carbohydrates rather than lipids).

MOTS-CLES EN ANGLAIS:

Myopathy, horse, epidemiology, fatty acids, respirometry

INTRODUCTION

Several recent studies have contributed to the understanding of the pathophysiological mechanism of atypical myopathy (AM) (Cassart *et al.*, 2007; Westermann *et al.*, 2008a; Westermann *et al.*, 2008b). Nevertheless, the aetiology of AM remains unknown. Continuing epidemiological studies is of major importance to collect more information about AM in order to prevent the condition and handle affected cases in a better and more efficient way. Up to now, epidemiological studies have only been performed on Belgian cases confirmed by histology performed after post-mortem examination (Votion *et al.*, 2007b; Votion *et al.*, 2008). Differences between countries could exist since the flora, pasture characteristics, and management of horses and pastures may differ. Epidemiological data for comparison of outbreaks within countries would be of value to narrow the common factors between affected areas. This would be of help in our quest for the aetiological agent and strengthen our knowledge of identified risk and protective factors. Previously performed studies were not only geographically limited, but were also restricted to non-survivors confirmed on histology rather than including suspected cases based on clinical signs. In a first stage these studies were of great interest, excluding horses with clinical signs resembling AM but not affected by AM. However, by not including non-confirmed but highly suspected cases (including survivors) certain clinical information might have got lost.

Recently, an overview of the recent knowledge about AM has been published (van Galen *et al.*, 2008c). After a summary of this review, the pathophysiology of AM will be discussed followed by a short review of the energetic metabolism of the muscle cell, preamble to introduce the objectives of our study.

1. ATYPICAL MYOPATHY

A syndrome of a highly fatal, acute myopathy affecting grazing horses was identified in 1983 and named "atypical myoglobinuria" (Anonymous, 1985). However, since myoglobinuria is only one of the possible clinical signs of the condition, the name atypical myopathy (AM) is now preferred (Votion *et al.*, 2004). Atypical myopathy has been reported sporadically since the beginning of the 20th century in various parts of the world (Votion *et al.*, 2004), but mainly in the UK. Since the new millennium it has been recognised in an increasing number of countries (Delguste *et al.*, 2002; Gerber *et al.*, 2006; van der Kolk, 2006; Palencia and Rivero, 2007), showing the emerging character of the condition. A similar condition has also been recognised in the United States of America (Finno *et al.*, 2006). In Belgium and France, large outbreaks of AM are now encountered regularly (Puyalto-Moussu *et al.*, 2004; Votion *et al.*, 2007b).

a. Epidemiology

According to large clinical studies (Votion *et al.*, 2007b; Votion *et al.*, 2008), AM seems to occur in horses being on pasture for at least 1 week. The condition is reported to occur on bare, sloping pastures. Most of the pastures where AM declares contain humid areas and are crossed or being bordered by a

watercourse (Votion *et al.*, 2007b; Votion *et al.*, 2008). Affected horses are often young, unbroken and in poor to normal body condition, however older horses have also been affected (Brandt *et al.*, 1997; Puyalto-Moussu *et al.*, 2004; Votion *et al.*, 2004; Votion *et al.*, 2007b). The condition does not seem to be contagious, but is usually reported as outbreaks since particular environmental characteristics and specific weather conditions predispose to it. The climatic conditions favouring AM are little sunshine, cool temperature without heavy frost, heavy rainfall or humidity, and strong winds (Hosie *et al.*, 1986; Whitwell *et al.*, 1988; Votion *et al.*, 2007b). These particular meteorological conditions could be the reason of the seasonal occurrence (*i.e.* the majority of cases are observed in autumn and some in spring and winter) (Votion *et al.*, 2007b).

b. Aetiology

At this moment the aetiology of AM is still unknown, but several causes have been considered. Toxic products, like ionophores, herbicides, weed killers, nitrates and nitrites have been incriminated. However, testing for these products yielded negative results (Whitwell *et al.*, 1988; Brandt *et al.*, 1997). Phytotoxins have also been suspected, but plants known to be toxic for horses or other animals were not consistently present at the pastures of affected horses and/or were previously identified to cause other clinical signs than rhabdomyolysis (Hosie *et al.*, 1986; Brandt *et al.*, 1997; Puyalto-Moussu *et al.*, 2004; Votion *et al.*, 2007b). No evidence of viral infections was found in cases with AM (Whitwell *et al.*, 1988; Brandt *et al.*, 1997; Delguste *et al.*, 2002). Mycotoxins could be involved as they are known to have potential toxic effects in equines (Osweiler, 2001). A described human fatal myopathic condition caused by mycotoxins supports this hypothesis (Bedry *et al.*, 2001). Moreover, fungal growth and mycotoxin production depends on specific climatic conditions which are similar to those favouring AM. Several researchers have proposed clostridial toxins as a cause of AM (Delguste *et al.*, 2002; Gerber *et al.*, 2006). Unfortunately, until now no study was able to confirm neither mycotoxins, nor *Clostridium* spp. as the causative agent. Selenium or vitamin E deficiency can lead to a nutritional myopathy, resembling AM. Antioxidant status of AM cases was variable (Hosie *et al.*, 1986; Whitwell *et al.*, 1988; Brandt *et al.*, 1997), but nutritional myopathy is not thought to be the cause of AM since supplementation and treatment with selenium and vitamin E rarely improve the condition. However, these antioxidants may have a protective role against a potential oxidative stress encountered in AM.

c. Pathogenesis

The myodegenerative process of AM affects more selectively oxidative (*i.e.* the slow-twitch or type I fibers mainly found in postural and respiratory muscles) rather than glycolytic fibers (*i.e.* the fast-twitch or type II fibers predominantly found in locomotory muscles) (Brandt *et al.*, 1997; Cassart *et al.*, 2007; Palencia and Rivero, 2007). In addition, increased lipid storage is prominent in type I fibers (Cassart *et al.*, 2007). These observations alongside with no abnormal glycogen or polysaccharide accumulation

suggested an impaired oxidative metabolism and a preserved glycolytic pathway (Cassart *et al.*, 2007). Moreover, a central role of the mitochondria in the pathogenesis of AM was suggested by the observation of mitochondrial ultrastructural changes (Cassart *et al.*, 2007).

Recently, these observations have been supported by the description of the biochemical defect occurring in AM (Westermann *et al.*, 2007; Westermann *et al.*, 2008b). A Multiple Acyl-CoA Dehydrogenase Deficiency (MADD) was found in confirmed cases. Dehydrogenase enzymes catalyse key events in fatty acids metabolism. They catalyse oxidation-reduction reactions using a coenzyme (*i.e.* flavin adenine dinucleotide; FAD) derived from the riboflavin (vitamin B2), thus suggesting that AM might result from a riboflavin deficiency or blockage. This MADD leads to dysfunction of the most efficient way for generating energy by the mitochondria, *i.e.* the oxidative phosphorylation (OXPHOS) from lipid substrates, therefore resulting in severe rhabdomyolysis, especially in postural and respiratory muscles.

d. Clinical signs

Clinical studies (Whitwell *et al.*, 1988; Brandt *et al.*, 1997; Votion *et al.*, 2007b) report that affected horses show a sudden onset of severe general weakness and muscular stiffness and, often become recumbent within a few hours. They are sometimes even found dead on pasture without premonitory signs. Some are reluctant to move, others are so weak that they have difficulty keeping their head up (van Galen *et al.*, 2008c). The affected horses show increased heart and respiratory rate, often hypothermia and congestive mucosae. Most cases keep a good appetite, although some suffer from esophageal obstruction (Hosie *et al.*, 1986; Whitwell *et al.*, 1988; Brandt *et al.*, 1997; Votion *et al.*, 2007b). Dysuria with a distended bladder at rectal examination is frequently encountered. A lot of horses demonstrate signs of colic probably due to the bladder distension (Hosie *et al.*, 1986; Brandt *et al.*, 1997; Votion *et al.*, 2007b), but rarely intestinal causes, as described by Sherlock and Mair (Sherlock and Mair, 2008) are found. The urine is dark-brown in the acute phase due to myoglobinuria (Hosie *et al.*, 1986; Votion *et al.*, 2004; Votion *et al.*, 2007b).

Subclinical cases have been described and can be horses grazing on the same premise as a clinically affected horse. They are often recognised when performing blood analyses on co-grazers of an affected horse, and they have a significant increase of serum activities of creatine kinase (CK) (Delguste *et al.*, 2002; Votion *et al.*, 2007b). They can evolve into clinical cases and so it is important to recognise and monitor them closely. It is thought that they are in balance with the causative agent, but a sudden imbalance may occur due to a trigger or a stress factor resulting in the clinical development of AM. These stress factors are suggested to be cold weather, exercise (Votion *et al.*, 2003), transport (van Galen *et al.*, 2008c), or anaesthesia (Sherlock and Mair, 2008).

e. Diagnosis

The clinical diagnosis of AM can be made based on history, clinical signs, blood and urine analysis, muscle biopsy and finally on post-mortem examination (Delguste *et al.*, 2002; Cassart *et al.*, 2007; Votion *et al.*, 2007b; van Galen *et al.*, 2008b). History is of major importance in the diagnosis. No cases are yet described not being out on pasture, probably because the etiologic agent is only found at pasture. Excluding other forms of rhabdomyolysis, for example exercise-induced myopathies, is paramount. However, as mentioned earlier, it is speculated that exercise as any other forms of stress can predispose for developing AM (Votion *et al.*, 2003). The clinical signs are typical for any severe rhabdomyolysis, but not specific at all for AM. It is therefore difficult to make a diagnosis just based on clinical signs (van Galen *et al.*, 2008b). At blood analysis muscle enzymes are massively increased, confirming the severe rhabdomyolysis (Hosie *et al.*, 1986; Whitwell *et al.*, 1988; Brandt *et al.*, 1997; Votion *et al.*, 2007b). Often hyperglycaemia and hyperlipaemia are present and liver enzymes are increased (Hosie *et al.*, 1986; Whitwell *et al.*, 1988; Votion *et al.*, 2007b). A hypocalcaemia is almost always found, but no other important electrolyte disturbances are consistently reported. The urine is coloured due to the myoglobinuria, and should be differentiated from haematuria or haemoglobinuria (van Galen *et al.*, 2008b).

The only way to make a definitive diagnosis of AM is by means of histology performed on a muscle samples. Biopsy is best performed on postural muscles in living horses or post-mortem on intercostal muscles (Cassart *et al.*, 2007). At necropsy, discoloured muscles and myocardium, congestive lungs, petechiae and black-reddish gastro-enteric contents may be seen (Cassart *et al.*, 2007). However, necropsy may also be disappointing with no specific observation. The most specific feature on histology is the important lipid accumulations in type I muscle fibers that undergo Zenker necrosis/degeneration (Cassart *et al.*, 2007).

f. Differential diagnosis

Other severe myopathies described in the horse are sporadic or recurrent exercise-induced myopathies, glycogen branching enzyme deficiency (GBED), white muscle disease or nutritional myodegeneration, streptococcal myopathy and post-anaesthetic myopathy (van Galen *et al.*, 2008b). Besides other severe myopathies, AM can be confounded with endotoxaemia, colic, neurological disorders (*e.g.* tetanus, botulism, rabies, meningitis, spinal cord disease), intoxications (*e.g.* ionophores, strychnine, carbamates, organophosphates, cassia occidentalis, acorn, white snakeroot), haematuria or haemoglobinuria of any cause (*e.g* cystitis, exercise-induced haematuria, urethral defects), or even lameness *(e.g.* iliac thrombosis, laminitis, polyarthritis, arthritis) (van Galen *et al.*, 2008b). Worth mentioning is the fact that horses can develop increased muscle enzyme activity due to prolonged recumbency (Sherlock and Mair, 2008; van Galen *et al.*, 2008b).

g. Treatment

Since the aetiology of AM is not known, therapy of AM affected horses is mostly symptomatic and no treatment has been demonstrated to be efficient in horses with AM. One of the existing hypotheses about the aetiology includes Clostridial infections (Delguste *et al.*, 2002; Gerber *et al.*, 2006). Hypothetically, administration of metronidazole might therefore be helpful. Administration of botulism type C and D antiserum has also been proposed (Gerber *et al.*, 2006).

It is of great importance to administer fluid therapy to horses with an acute myopathy to avoid acute tubular necrosis due to myoglobinuria. Together with fluid therapy, electrolyte and acid-base balance should be maintained. Since AM cases seem prone to the development of hypocalcaemia, special attention should be carried out for calcium imbalances and administrating slow intravenous calcium borogluconate might be helpful. A lot of affected horses have difficulties urinating and should be relieved by regular or continuous bladder catheterization. When an indwelling catheter is placed or repeated catheterization is necessary, broad spectrum antibiotics should be administrated to prevent cystitis. In general, horses fully regain their capacity to urinate once the myoglobinuria has disappeared (van Galen *et al.*, 2008c). Although not all horses seem to suffer intensively (Votion *et al*., 2007), analgesia can be provided if necessary by non steroidal anti-inflammatory drugs (NSAID's), morphine or morphine-derivatives. It should be noted that NSAID's are a cause of renal toxicity, and especially with the presence of myoglobinuria caution is justified with their administration. Due to the progressively affected respiratory muscles a severe hypoxia can develop (Delguste *et al.*, 2002; Votion *et al.*, 2007b), and respiratory support in the form of nasal oxygen could be considered. For affected foals artificial ventilation might even be envisaged. Dantrolene sodium is proposed for limiting rhabdomyolyis in other myopathies (MacLeay, 2004; Nout and Reed, 2005). This drug limits the rate of calcium release from the sarcoplasmatic reticulum. Support for muscular function and repair should be offered by vitamins and anti-oxidants, like selenium, vitamin C and vitamin E. Muscle relaxants (*i.e*. acepromazine, alpha2-agonists, methocarbamol, GGE, etc) can be used, but are rarely thought to be useful (van Galen *et al.*, 2008c).

Once a horse is recumbent, regular turnings, good thick and clean bedding, and stimulating the horse to remain in sternal recumbency rather than in lateral recumbency can prevent further muscular and pulmonary compression as well as the development of important decubital ulcers (Nout and Reed, 2005). Affected horses should not be exposed to stress (for example exercise, transport), since it might aggravate their condition (Votion *et al.*, 2003; Sherlock and Mair, 2008; van Galen *et al.*, 2008c).

h. Prognosis

The prognosis for most AM cases is not favourable. The mortality rate is estimated at 85% (Votion *et al.*, 2004) and most horses die within 3 days (Votion *et al.*, 2007b). Prognostic factors recognised in clinical studies on fatal cases are normal respiration with normal arterial partial pressure of oxygen (PaO_2) levels and the absence of permanent recumbency. Serum activities of CK have shown not to be of prognostic value (Votion *et al.*, 2007b).

It is important to inform the owner about the fact that his other horses are at risk to become affected, clinically or subclinically. If possible, horses that are on the same pasture as an affected horse should be removed from this pasture and preferably placed in a box. If not enough boxes are available for all, young horses should have priority since they are more at risk for AM (Votion *et al.*, 2007b). Their level of CK activities should be checked during several days and they should be monitored closely for any annunciating signs.

2. MUSCULAR METABOLISM

To better understand the pathophysiology of atypical myopathy, it is necessary to review the pathways of energy metabolism of the skeletal muscle cell.

a. Adenosine triphosphate

The source of energy used for muscular contraction is adenosine triphosphate (ATP). The bond attaching the phosphate is a high-energy phosphate bond. When a phosphate is removed, this energy is released to be used for the contractile process. The amount of ATP present in muscles is only sufficient to sustain contraction for a couple of seconds. Therefore it is essential that new ATP is formed. This ATP can be formed by several pathways, being in chronological order the phosphocreatine-creatine system, the anaerobic glycogen-lactic acid system and the aerobic system (Guyton and Hall, 2006c, b; Votion *et al.*, 2007c; Lehninger *et al.*, 1993g).

b. Phosphocreatine-creatine system

Phosphocreatine also contains a high-energy phosphate bond, like ATP, but a bigger amount of energy is released by the removal of the phosphate. Therefore it can easily provide enough energy to reconstitute the high-energy bond of ATP. This system also is only sufficient to sustain contraction for a couple of seconds (Lehninger *et al.*, 1993g; Guyton and Hall, 2006c).

c. The glycogen-lactic acid system

Glycogen may provide a rapid energy source for either aerobic or anaerobic metabolism. The glycogen stored in the sarcolemma can be split into glucose 6-phosphate (glycogenolysis), which can enter the glycolysis (catabolic pathway from glucose 6-phosphate). Glucose, obtained from the glycogenolysis or absorbed from the bloodstream, is split into pyruvate and energy is released to form 2 ATP molecules.

Two cytosolic reduced nicotinamide adenine dinucleotide (NADH) molecules are also produced during the glycolysis. In the presence of oxygen, pyruvate is converted into acetyl-CoA and enters the Krebs cycle in the mitochondria. In absence of oxygen, pyruvate is converted to lactate in the cytoplasm, which diffuses out of the muscle cells into the interstitial fluid and blood. The ATP-production of this anaerobic system is a rapid energy source, but not an efficient compared to the aerobic system in terms of ATP yielded per mole of glucose (Lehninger *et al.*, 1993b; Guyton and Hall, 2006b, c).

d. The aerobic system

i. General

The aerobic system is the mitochondrial oxidation of glucose (and products of glycolysis), fatty acids and amino acids to provide energy (Figure 1). These substrates are conversed into acetyl-CoA, and enter the Krebs cycle in which electrons are abstracted. These electrons are carried by NADH and reduced flavin adenine dinucleotide ($FADH_2$). Hydrogen is then oxidized by the electron transport system (ETS) of the OXPHOS (Figure 2). In the overall reaction catalysed by the mitochondrial respiratory system, electrons move from NADH, succinate (via $FADH_2$), or some other primary electron donors through Complex I, Complex II or coenzyme Q which is the junction to Complex III. Electrons move from Complex III to cytochrome *c* and then to Complex IV which completes the sequence by transferring electrons from cytochrome *c* to O_2 (thus consuming O_2). Electron flow through Complexes I, III and IV is accompanied by proton flow (H^+) from the mitochondrial matrix to the intermembrane space. A last enzyme (the ATP synthase, sometimes called Complex V) catalyses the formation of ATP from ADP owing to the energy provided by the "proton-motive" force, *i.e.* the H^+ flow back into the matrix through a pore associated to the ATP synthase (Figure 2). During the Krebs cycle only a little amount of ATP is produced through the production of the high-energy compound guanosine triphosphate (GTP), but large amounts are produced during the OXPHOS (Lehninger *et al.*, 1993a; Guyton and Hall, 2006b).

ii. Fatty acids

Since a problem in lipid metabolism is suspected in AM (Cassart *et al.*, 2007; Westermann *et al.*, 2007; Westermann *et al.*, 2008b), and due to the importance of fatty acids for the aerobic metabolism in the skeletal muscle cell (MacLeay, 2004), the metabolism of fatty acids will be discussed more in detail.

The first stage of using lipids for energy is the hydrolysis of triglycerides into free fatty acids (FFA) and glycerol, which will be released into the bloodstream to be taken to the muscle cell. When glycerol enters the cell, it is immediately changed into glycerol-3-phosphate, entering the glycolytic pathway (Lehninger *et al.*, 1993e; Guyton and Hall, 2006a). The enzymes of fatty acids oxidation are located in the mitochondrial matrix. Therefore, they must be transported into the mitochondria by the carnitine shuttle, which is a rate-limiting step (Lehninger *et al.*, 1993e). The FFA with a chain length shorter than 12 carbons can however enter the mitochondria freely (Lehninger *et al.*, 1993e). In the

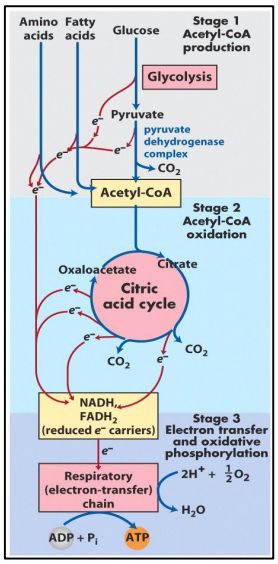
mitochondria, the fatty acids undergo β oxidation: oxidative removal of 2-carbon segments in the form of acetyl-CoA (Lehninger *et al.*, 1993e; Guyton and Hall, 2006a). This oxidation requires removal of 4 hydrogen atoms (two pairs of electrons and four H^+) per acetyl-CoA. These electrons carried by FAD and NAD (*i.e.* $FADH_2$ or $NADH_2$) enter immediately the ETS of the OXPHOS. The acetyl-CoA molecules enter the Krebs cycle and later the OXPHOS as described above (Lehninger *et al.*, 1993e) (Figure 3).

The β oxidation of FFA occurs in four steps (Lehninger *et al.*, 1993e; Guyton and Hall, 2006a). The first process is the dehydrogenation of fatty acyl-CoA with the production of a trans-fatty acid (a double bond), catalysed by acyl-CoA deshydrogenases. The acyl-CoA deshydrogenases are flavoproteins, which use the coenzyme FAD derived from riboflavin (vitamin B2) (Lehninger *et al.*, 1993e). The electrons removed from the fatty acyl-CoA are transferred to FAD. The produced $FADH_2$ immediately donates its electron to an electron carrier of the ETS, the electron-transferring flavoprotein (ETF), from which they pass to coenzyme Q via ETF:ubiquinone oxidoreductase (ETF:QO) (Figure 4). In the second step, water is added to the double bound to form β-hydroxyacyl-CoA. In the third step, β-hydroxyacyl-CoA is dehydrogenated to form β-ketoacyl-CoA by the β-hydroxyacyl-CoA dehydrogenase (or 3-hydroxyacyl-CoA dehydrogenase; HAD). The liberated electrons are transferred to the oxidized form of nicotinamide adenine dinucleotide (NAD^+) and are transported to Complex I of the ETS (Figure 4). In the fourth step, a thiolase catalyses the splitting of the β-ketoacyl-CoA into acetyl-CoA and a thioester. The four steps are repeated to yield acetyl-CoA and ATP. The ATP production from fatty acids is much more important than that from glucose (131 ATP molecules per molecule of palmitoyl-CoA *vs.* 38 ATP molecules per glucose molecule) (Lehninger *et al.*, 1993f).

3. AIM OF RESEARCH

Most of the knowledge about AM has been established on Belgian confirmed cases (Delguste *et al.*, 2002; Votion *et al.*, 2007b). Since the clinical expression of AM might be time and/or geographic dependent, *the first part of this study* aims at describing the clinical evolution and/or at better defining the clinical signs. This will be done through an epidemiological retrospective study of European cases recorded between the autumn of 2006 and the winter of 2008 and for the first time survivors of the condition will be desribed. The results of this first study might be of assistance for equine veterinarians concerning diagnosis, prognosis, treatment and prevention of AM. Due to the suspicion of a defect in mitochondrial lipid metabolism, the aim of *the second part of this study* is a search for the metabolic defect(s) associated to AM by interpreting preliminary results of a study with high resolution respirometry (HRR) applied on muscle micro-biopsy samples and by analysis of the FFA profile in affected muscles.

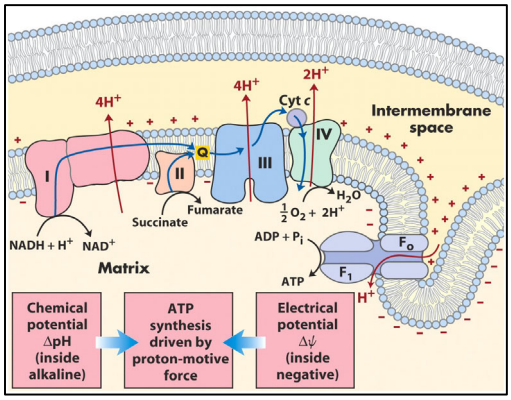
Figure 1 - Aerobic metabolism (catabolism of proteins, fats, and carbohydrates in the three stages of cellular respiration)



Legend: Stage 1: oxidation of fatty acids, glucose, and some amino acids yields acetyl-CoA. Stage 2: oxidation of acetyl groups in the Krebs (or citric acid) cycle includes four steps in which electrons are abstracted. Stages 3: electrons carried by reduced nicotinamide adenine dinucleotide (NADH) and reduced flavine adenine dinucleotide ($FADH_2$) are funnelled into a chain of mitochondrial electron carriers, providing the energy for ATP synthesis by oxidative phosphorylation.

Picture from Principles of Biochemistry, fourth edition, Ed: Lehninger, Nelson and Cox. Worth Publishers New York, 2004, pp 602.

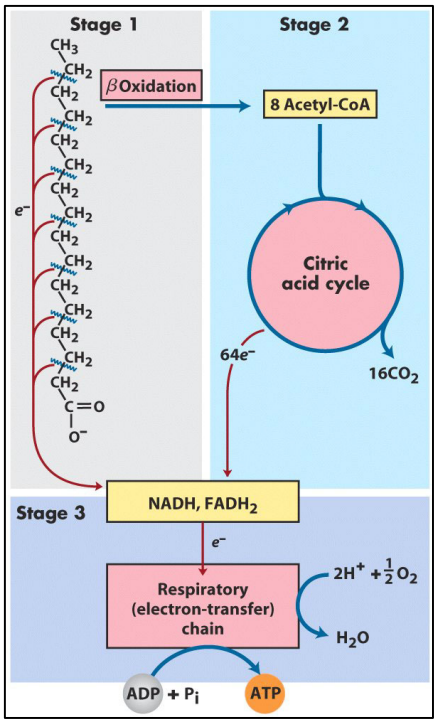
Figure 2 - The electron transport system and the oxidative phosphorylation



Legend: Electrons from reduced nicotinamide adenine dinucleotide (NADH) and other oxidizable substrates pass through a chain of carriers arranged asymmetrically in the inner membrane of the mitochondria. In this chemiosmotic model, electron flow is accompanied by proton transfer across the membrane, producing a chemical gradient (ΔpH) and an electrical gradient ($\Delta\Psi$). The inner mitochondrial membrane is impermeable to protons; protons can re-enter the matrix only through proton specific channels (F_o). The proton-motive force that drives protons back into the matrix provides the energy for ATP synthesis, catalyzed by the F_1 complex associated with F_o.

Picture from Principles of Biochemistry, fourth edition, Ed: Lehninger, Nelson and Cox. Worth Publishers New York, 2004, pp 705.

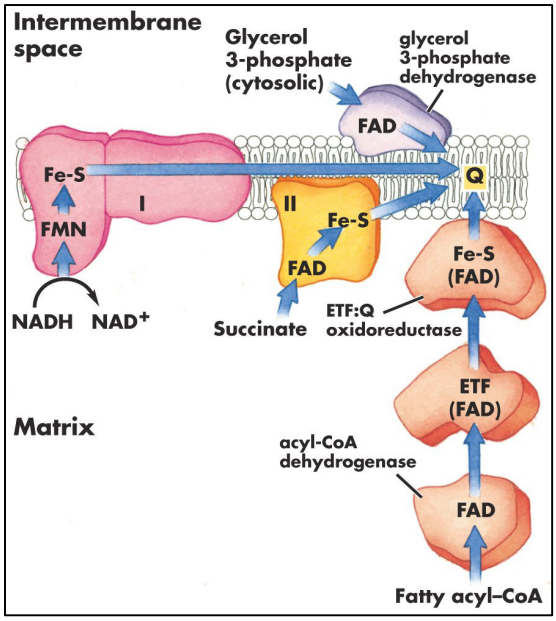
Figure 3 - Lipid metabolism (stages of fatty acid oxidation)



Legend: Stage 1 (β oxidation): a long chain fatty acid is oxidized to yield acetyl residues in the form of acetyl-CoA. Stage 2 (Krebs cycle): the acetyl groups are oxidised to CO_2 via the Krebs (or citric acid) cycle. Stage 3 (electron transfer chain): electrons derived from the oxidations of stages 1 and 2 pass to O_2 via the mitochondrial respiratory chain, providing the energy for ATP synthesis by oxidative phosphorylation.

Picture from Principles of Biochemistry, fourth edition, Ed: Lehninger, Nelson and Cox. Worth Publishers New York, 2004, pp 637.

Figure 4 - The path of electrons to ubiquinone



Legend: Electrons from nicotinamide adenine dinucleotide (NADH), glycerol 3-phosphate, succinate and fatty acyl-CoA converge to ubiquinone (coenzyme Q).

Picture from Principles of Biochemistry, fourth edition, Ed: Lehninger, Nelson and Cox. Worth Publishers New York, 2004, pp 697.

MATERIALS AND METHODS

1. EPIDEMIOLOGICAL STUDY

a. Case selection

Between the autumn of 2006 and the winter of 2008, an important amount of European cases suspected to suffer from AM were reported to the Faculty of Veterinary Medicine of Liege University in Belgium by the atypical myopathy alert group (AMAG) and by equine practitioners. These cases were allocated into 5 categories: confirmed cases of AM (CC), cases with a high probability (HP) of being AM affected, cases with a low probability (LP) of being AM affected, cases infirmed for the diagnosis of AM (I), and cases with no possibility to conclude whether they suffered from AM due to a lack of clinical information (D for doubtful).

Cases were defined as HP cases when they presented the following criteria: being out at pasture at the onset of clinical signs or at least within some days preceding, and overt signs of acute myopathy (diagnosed with an increased level of serum CK activity and/or myoglobinuria). Horses reported as being affected without any clinical information, but in the same period and at the same pasture as a previously defined HP or CC case were also considered to be HP cases. Diagnosis of AM was confirmed (*i.e.* CC cases) when histology of post-mortem samples or muscle biopsies indicated a multifocal process compatible with Zenker degeneration and necrosis in fibers of postural muscles, respiratory muscles or both (Cassart *et al.*, 2007). Cases were defined as I cases when histology of post-mortem samples or muscle biopsies did not reveal a diagnosis of AM. Cases were defined as LP cases when clinical signs typical for other conditions were present and/or when cases did not fulfil the criteria of the HP cases. Cases were defined as doubtful (D) when not enough clinical data and/or histological findings were present to confirm or infirm the suspicion of AM. Also information about co-grazers of reported cases was collected.

With the data from these reported cases and their co-grazers a retrospective study was performed to precise the clinical and epidemiological description of AM, and to identify risk and protective factors for the development of AM and prognostic factors for survival.

b. Collection of data

Geographic, seasonal and demographic data, pasture characteristics, and management of the pastures and the involved cases were obtained from interviews of the owners by E-mail, telephone or by means of questionnaires obtained via the AMAG alert website (www.myopathieatypique.be). Clinical data were obtained from medical records obtained via AMAG alert website or from interviews with veterinarians. Climatic data were obtained from national climate websites of the different countries.

c. Statistical analysis

From the defined categories, the following groups were considered for statistical analysis of the collected data: the HP/CC (that includes the data of the HP and CC cases), the LP/I (that includes the data of the LP and I cases), and the co-grazers groups. Seen the doubtful origin of the D cases they were excluded from statistical analysis. The HP/CC group was further subdivided into surviving and non-surviving cases.

The mean of the quantitative parameters in each group with unequal variance was compared with a Welch's test (Dagnelie, 1998). The risk factors calculated for different parameters were evaluated by mean of odds ratios as defined by Grenier (1990), which aimed at comparing the odds of exposure among groups. If less than 50% of the questions of a certain parameter in a group was answered, this parameter was estimated not to be interpretable for statistical analysis. The limit of statistical significance of the conducted tests was defined as $P < 0.05$.

2. FATTY ACID ANALYSIS

a. Case selection

Five horses were included in this part of the study. Two of these horses (AM_1 and AM_2) were diagnosed with AM based on history, clinical signs and presence of a multifocal process compatible with Zenker degeneration and necrosis in fibers of postural muscles, respiratory muscles or both on histology. Three other horses served as control horses (C_1, C_2 and C_3), euthanized for other reasons and without any clinical evidence of a myopathy. From all horses, muscle biopsies of different muscles were taken during general anaesthesia prior to euthanasia. The sampled muscles were immediately frozen in liquid nitrogen and then kept at – 80°C until analysis. Due to delicate circumstances with assisting owners and because some horses dying before the end of sampling, not all muscles were sampled on every horse. The sampled muscles are shown in Table I.

b. Lipid analysis

In several steps, the amount of lipids, the amount of FFA and the profile of the FFA in the muscle samples were determined, as previously described in literature (Gorski *et al.*, 1998; Nawrocki *et al.*, 1999; Nawrocki and Gorski, 2004). The first step was the extraction of the lipids from the muscle sample with chloroform. The second step consisted of an analytical thin layer chromatography (TLC) to determine the total amount of FFA present in the samples. A second TLC (preparative TLC) was performed for isolation of those FFA, which were then methylated and used in a gas liquid chromatography (GLC) for determination of the profile of the FFA.

i. Extraction of the lipids

The muscle biopsies were thawed, lyophilised and weighted. They were then crushed in a phosphate buffer (0.15 M) in a fixed volume of 10 ml at a pH of 7.4. The crushed samples were added to 8 ml of chloroform ($CHCl_3$) (in a hermetic closed tube) while being stirred during 30 minutes. The tube was then centrifuged at 2500 rotations per minute (power of centrifuge 1000 *g*) separating the aqueous phase of the lipid phase (lipids dissolved in chloroform). The aqueous phase was aspirated and 6 ml of the chloroform phase (75%) were collected in a pre-weighted tube. The solvent was evaporated under nitrogen flow and the possible traces of water were eliminated by lyophilisation. Finally, the tube with its content was weighted, giving the weight of the extracted lipids. This weight was used to calculate the weight of the total extracted lipids and finally to calculate the weight of lipids in mg per gram muscle.

ii. Analytical thin layer chromatography

The extracted lipids were replaced in solution by mixing them with 1 ml chloroform. From this solution of total lipids, 10 µl were used to perform a TLC on a plate covered with silica gel (Merck© KGaA, Darmstadt, Germany) in a solvent mixture of petroleum ether, diethylic ether, acetic acid (70; 30; 2). Two control samples (20 µl) were used on every chromatography plate: one control sample contained a mix of triolein and cholesterol (S1), and the other control sample contained oleic acid and cholesteryl oleate (S2). This TLC permitted a separation of cholesteryl esters, triglycerides, FFA, cholesterol, monoglycerides and phospholipids (in migrating order). After migration, the plate was taken out of the chromatography tank and dried by air. By vaporisation of a copper acetate solution on the plate followed by a heating of the plate at 180°C, the migrated lipids were visualised under the form of brown-black spots. The percentage of every lipid class and their value in µg in the muscle sample and per gram of muscle was calculated by the use of densitometry.

iii. Preparative thin layer chromatography and isolation of free fatty acids

A second TLC, identical to the previous one, was performed on 15 µl of the lipid samples with the same control solutions and in the same conditions. At the end of this TLC, only the S1 and S2 were visualised as described previously. The central part of the plate containing the samples was visualised only temporarily by vaporisation of iodine to be able to localise the FFA with precision. These locations were marked with a pencil. After evaporation of the iodine, the plate was vaporised with an antioxidant solution of butylhydroxytoluene (BHT) dissolved in $CHCl_3$ preventing peroxidation of the lipids by exposure to oxygen. The zone corresponding with the localisation of the FFA was removed with chloroform by the Eluchrom (Camag©, Switzerland) at slow velocity (1 ml per 10 minutes). The obtained solution of FFA was placed in a glass tube of 1 ml.

iv. Methylation and gas liquid chromatography

The obtained solution of FFA was evaporated under a nitrogen flow and then methylated by diazomethane. After methylation, the solution was evaporated and the sample was replaced in solution with 10 µl of dichloromethane of which 0.5 µl were injected in the chromatograph.

The following conditions of the GLC were used: Chromatograph Varian CX 3600 star, equipped with a capillary column Supelco SP^{TM}-2380 (cyano-silicone) (30 meter; diameter 0.25 mm; thickness of the film 0.2 µm) and a flame ionisation detection (FID). A nitrogen flow was used as gas vector (30 ml/min) at 180°C during 6 minutes, followed by programmation of increase of the temperature (10°C/min) until 210°C. Once this temperature was reached, it was maintained during 40 minutes. The gasses inducing the flame are air (300 ml/min) and hydrogen (30 ml/min). The column was standardised by a mix of methylated FFA (PUFA n°2 from Supelco; Bellefonte, PA, USA) and by individual methylated FFA (purchased from Sigma; methylated by diazomethane). The registration of the chromatogram was performed by an integrator. The column separates the FFA based on the number of the carbons and based on the number of the double bonds in the FFA.

The peaks were identified by comparing their retention time with the retention time of the FFA used as reference (PUFA n°2 or the individual FFA). After localizing and identifying the peaks, the total surface of all peaks was calculated being equal to 100%. Thereafter the surface of each individual peak was calculated in percentage and converted into the amount of that specific FFA in µg/g muscle.

c. Comparison

A comparison concerning the lipid analysis was made between the horses affected with AM and the control horses. The different muscles were also compared to determine a specific localisation of the FFA, related to the type of muscle cells. Due to the limited number of cases, no statistic analysis of these data was performed.

3. HIGH RESOLUTION RESPIROMETRY

a. Case selection

One horse affected with AM was available for micro-biopsy. This horse was sampled at 3 days (AMa) and at 10 days (AMb) after the first clinical signs occurred. As control horses, three healthy resting horses were sampled. None of the three had a history or clinical signs suggestive for myopathy.

b. Samples

Biopsies were performed *in vivo* under local anaesthesia in the *M. triceps brachii* in order to sample cells with functioning mitochondria enabling to perform HRR. The biopsy site was shaved and aseptically prepared using polyvidone iode soap (iso-Betadine® Savon Germicide, Meda Pharma, Belgium), alcohol and polyvidone iode solution (iso-Betadine® Dermique, Meda Pharma, Belgium). Mepivacaïne hydrochloride 2% (Scandicaïne® 2%, AstraZeneca, Belgium) was locally injected at the site of biopsy. The skin was incised using a number 11 blade. A 14G biopsy needle (PRO-MAG™ Ultra, Inter.V®, US) was entered 5 cm deep into the muscle and with the use of a biopsy pistol (PRO-MAG™ Ultra, Inter.V®, US) a muscle micro-biopsy was sampled. This procedure was repeated 4-5 times in the same muscle using the same skin incision. The samples were immediately placed in a specific relaxing and biopsy preservation solution named "BIOPS" (Letellier *et al.*, 1992) and were then used for HRR as previously described in literature (Wenchich *et al.*, 2003; Votion *et al.*, 2007a).

c. High resolution respirometry

The oxygraph (Oxygraph-2k®, Oroboros, Austria) measures the oxygen (O_2) use of living mitochondria (the mitochondrial respiration) of samples added in two glass chambers by means of two polarographic O_2 sensors. A scientific software for HRR (DatLab 4, Oroboros, Austria) displays on-line the O_2 flux (in pmol/(s*mg)) and the O_2 concentration (in nmol/ml) over time. Muscle fibers obtained by micro-biopsy were prepared according to the procedure of Lemieux and collaborators (2007) (Lemieux *et al.*, 2007). Mitochondrial respiration of the muscle micro-biopsies was followed at 37°C in a medium called "Miro5" (Gnaiger *et al.*, 2000) according to the titration protocol described by Votion and collaborators (2007) and summarised in Table II and Figure 5-7.

d. Comparison

Considering the fact that in the AM affected category only one horse was sampled, no statistical analysis was performed on this part of the study.

Table I - Sampled muscles on the selected horses

Horse	M. masseter	M. brachiocephalicus	M. triceps brachii	M. longissimus dorsi	M. quadriceps femoris	M. semitendinosis	M. intercostales	M. sacrocaudalis dorsalis	Cardiac muscle (interventricular septum)	Cardiac muscle (ventricular free wall)
AM_1		x		x	x		x		x	x
AM_2	x	x	x	x	x	x	x			
C_1		x		x	x	x				
C_2		x			x	x				
C_3	x	x				x		x		

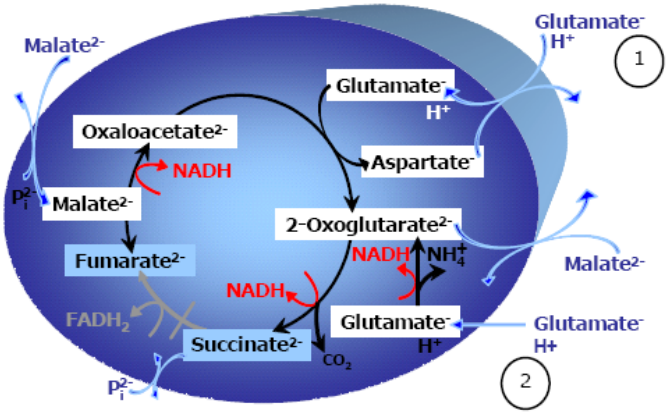
Legend: AM = atypical myopathy affected horses; C = control horses.

Table II - Multiple substrate-uncoupler-inhibitor titration protocol used for respiratory studies of equine permeabilized muscle fibers

Step	Added substrate	Respiratory state	Comments
GM	Glutamate + Malate	Coupled leak state	Respiratory resting state with substrates for Complex I, but no ADP
GM-D	ADP	Coupled OXPHOS capacity with Complex I substrates	Respiratory capacity in the active coupled state
GM-Dc	Cytochrome *c*	---	Further addition of cytochrome *c* yields a test for integrity of the outer mitochondrial membrane (loss of cytochrome *c* would induce a stimulation of respiration)
GMS-Dc	Succinate	Coupled OXPHOS capacity with Complex I+II substrates	Respiratory stimulation by convergent electron flow from Complexes I+II at the Q-junction, in the coupled state
GMS-UC	FCCP	ETS capacity with Complex I+II substrates	Uncoupling by FCCP titration as a test for limitation of OXPHOS relative to ETS capacity
S/Rot	Rotenone	ETS capacity with Complex II substrate	ETS capacity with succinate, after blocking Complex I with rotenone
AA	Antimycin A	Non-mitochondrial respiration of the permeabilized tissue	Addition of Antimycin A (inhibitor of Complex III)

Legend: ADP: adenosine diphosphate; OXPHOS: oxidative phosphorylation; FCCP: Carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone; ETS: electron transport system.

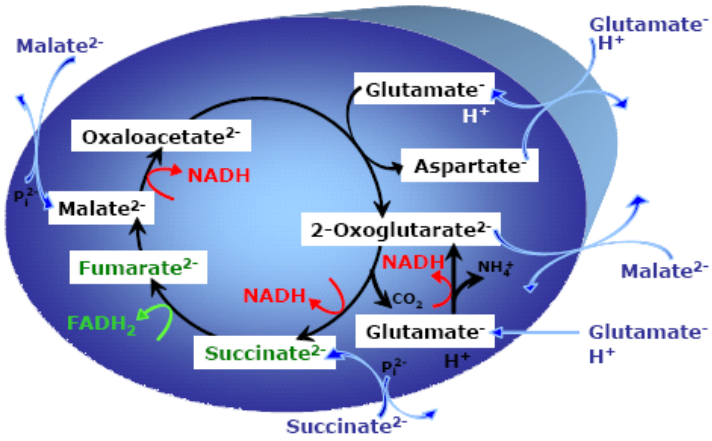
Figure 5 - Glutamate + Malate as substrates for the high resolution respirometry



Legend: Glutamate and malate activate dehydrogenase with reduction of nicotinamide adenine dinucleotide (NADH) in the Krebs cycle then feeding electrons into Complex I of the electron transport system (ETS). However, no succinate is produced (because of a malate antiport which depletes metabolites for succinate production) and thus there is no reduced flavine adenine dinucleotide ($FADH_2$) to feed Complex II of the ETS.

Picture from: www.oroboros.at

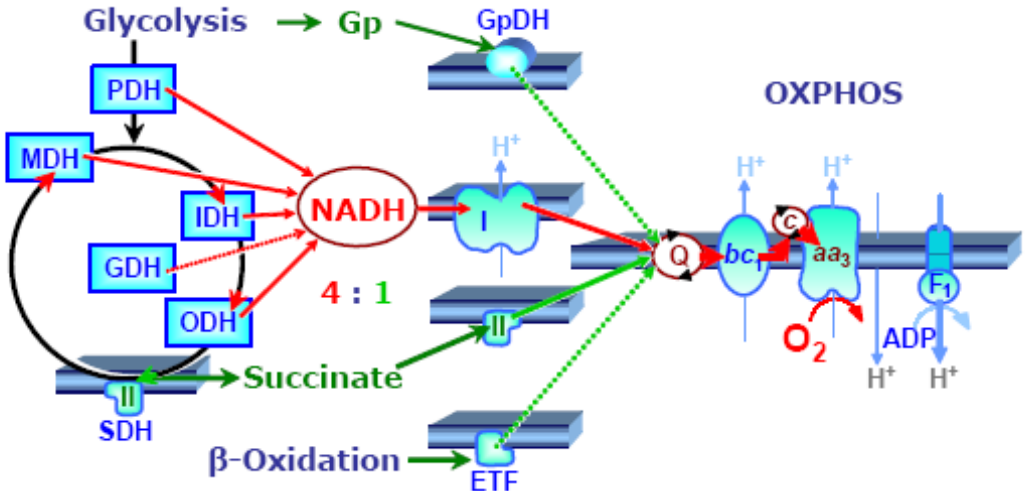
Figure 6 - Glutamate + Malate + Succinate as substrates for the high resolution respirometry



Legend: Convergent electron flow to the Q-junction (see also Figure 7) with glutamate + malate + succinate combination. The reduced nicotinamide adenine dinucleotide (NADH) and reduced flavine adenine dinucleotide ($FADH_2$) produced in the Krebs cycle feed the electron transport system with electrons at the level of Complex I and II, respectively.

Picture from: www.oroboros.at

Figure 7 - The electron transport system



Legend: Convergent electron transport at the levels of the Q-junction [Complexes I+II, glycerol 3-phosphate dehydrogenase (GpDH) and electron-transferring flavoprotein (ETF)] and at the level of the reduced nicotinamide adenine dinucleotide (NADH) pool. PDH: pyruvate dehydrogenase (DH); MDH: malate DH; IDH: isocitrate DH; ODH: oxoglutarate DH; SDH, succinate DH; GDH: glutamate DH.
Picture from: www.oroboros.at

RESULTS

1. EPIDEMIOLOGICAL STUDY

a. Division of the cases in the different categories

Between the autumn of 2006 and the winter of 2008, a total of 165 European cases were reported, and information about 65 co-grazers was collected. Figure 8a and 8b show the division of the reported cases in the different categories. Ninety-three cases (56%) were confirmed AM cases or cases highly suspected to suffer from AM (the HP/CC group).

b. Geographic and seasonal distribution

The geographic and seasonal distribution of the cases are shown in Figure 9, 10a and 10b. Most reported cases originated from Belgium and France. Most of the Belgian reported cases were from the southern part of the country (provinces of Hainaut, Liege and Luxembourg). The province Walloon Brabant was considered as protective factor for the development of AM. No difference in provinces was noted between survivors and non-survivors. Most cases occurred during the autumn of 2006.

c. Outcome and survival rate

The outcome and the survival rates of the different groups are shown in Figure 11a-11b and Table III. None of the horses of the HP/CC group were found dead before showing any clinical signs. During the autumn of 2006, a higher survival rate was noted in France (57%) compared to the rest of the study period and other countries. The HP/CC cases were significantly more often euthanized and died less often of a natural death or were less often found dead compared to the LP/I cases.

d. The horses

i. Demographic data

The age, sex, race and body condition of the reported cases are shown in Table IV and Figure 12. The HP/CC cases were often young ponies or saddle horses with a normal body condition, however older horses were also reported to be affected. Four donkeys (2 D and 2 HP cases), three zebra's (1 CC and 2 HP cases) and a deer (LP case) were present in the study population. No predisposition concerning age, race or body condition was present for the development of AM, however stallions were more and mares less at risk. Sex, age and race had no influence on the outcome, but obese horses were less at risk to die from the condition.

ii. Activity of the cases: work and pasturing

The activity of the reported cases is shown in Table V. Only a minority of the reported cases was worked and none of the HP/CC cases was worked more than twice a week. All horses were affected while at pasture, except for 2 horses of the HP/CC group that had been stabled 2 days before the onset of their clinical signs and 3 horses of the LP/I group that had been stabled 10 days before. When the condition occurred, the majority of the horses spent 24 hours per day at pasture. The period that the reported horses

grazed on the specific pasture ranged from 10 days to several years. Not being at pasture and being worked were protective factors, whilst being 24H/24H at pasture during winter was a risk factor for the development of AM. Being worked, being at pasture 24H/24H when AM occurred and the time that the horses had spent on the specific pasture had no influence on the outcome.

iii. Medical history

The medical history of the reported cases is shown in Table VI. Four reported cases had been diagnosed with a myopathy previous to the diagnosis or suspicion of AM; one D case and one LP/I case had suffered from an exercise-induced myopathy, and one LP/I case and one HP/CC case had suffered from a myopathy of unknown origin. Only a minority of the reported cases had travelled to other countries. The majority of the reported cases had been regularly dewormed and vaccinated. No significant differences regarding these parameters were noted between the groups.

iv. Nutrition

The nutritional status (complemented or not) of the reported cases is shown in Table VII. None of the reported cases had received food destined for other species. At the time of AM occurring, only about half of the HP/CC horses and their co-grazers were complemented, mostly with hay and concentrates. Complementing horses during the period in which AM occurred was a protective factor for the development of AM.

e. The pastures

A description of the pasture characteristics and management is given in Table VIII. Pastures of HP/CC cases were often sloping, non-treated, natural pastures containing trees, dead leaves, dead wood, humid zones and a water stream. Often, water from the distribution network was used and was drunk from a tank. The occurrence of previous deaths in horses (regardless the cause of death) on the pasture, and the presence of trees, dead leaves, and humid zones on the pastures were demonstrated to be risk factors for the development of AM. Dead wood was more often present on the pastures of survivors than of non-survivors.

f. Climatic conditions

The climatic conditions during the period that AM occurred are described in Table IX. Often a change in temperature, wind and rain was marked by the owners when their horses became affected by AM. No heavy frost occurred in the 10 days before the first clinical signs were noted. Less rainfall in the 10 days preceding AM was considered a risk factor for the development of AM. No statistical differences were noted between survivors and non-survivors.

g. Clinical data

i. Duration of the condition

For the non-survivors, the duration of the clinical signs ranged from 0 to 10 days (mean ± SD: 1.2 ± 1.6 days). The survivors fully recovered after 2 to 30 days (mean ± SD: 11.1 ± 6.7 days) and showed none to very few muscle atrophy.

ii. Clinical signs

Information about the clinical signs in the reported cases is given in Table X. Some owners noticed an abnormal behaviour before the onset of the clinical signs, described as depression and abnormal quietness. One horse refused to eat and another one fell over. The presence of an abnormal behaviour was not different between the studied groups.

Affected horses were often presented with congestive mucosae, tachycardia, tachypnoea, dyspnoea and moderate suffering. As well normal rectal temperature as hypo- and hyperthermia have been encountered in HP/CC cases. Myoglobinuria was almost always noted in HP/CC cases and depression, weakness, stiffness, recumbency, and trembling were frequently reported clinical signs. In HP/CC cases, quite regularly other abnormalities than a distended bladder were encountered at rectal examination indicating a diminished digestive transit: dry and/or mucus covered faeces, impaction of the large colon. Some males showed a paraphymosis. The HP/CC cases showed significantly more frequently stiffness than the LP/I cases. The other clinical signs were not significantly different between those two groups or not interpretable. Survivors showed more often normal mucosae, less recumbency and remained more standing compared to non-survivors.

iii. Creatine kinase activity

The serum activity of CK in the reported horses is described in Table XI. The values in horses of the HP/CC group were always greater than 1000 IU/L. When the CK activity was measured repeatedly, the first value was always the lowest. In some cases, the presence of a myopathy could even be questioned based on these first samples, whereas a second sample 1 or 2 days later always demonstrated clearly a severe myopathic process. The probability of AM was significantly higher in cases with a serum activity of CK higher than 15000 IU/L and significantly lower in the cases with a serum activity of CK lower than 1000 IU/L. No significant difference was noted between serum activity of CK of survivors and non-survivors, although a tendency for a higher CK activity in survivors was revealed.

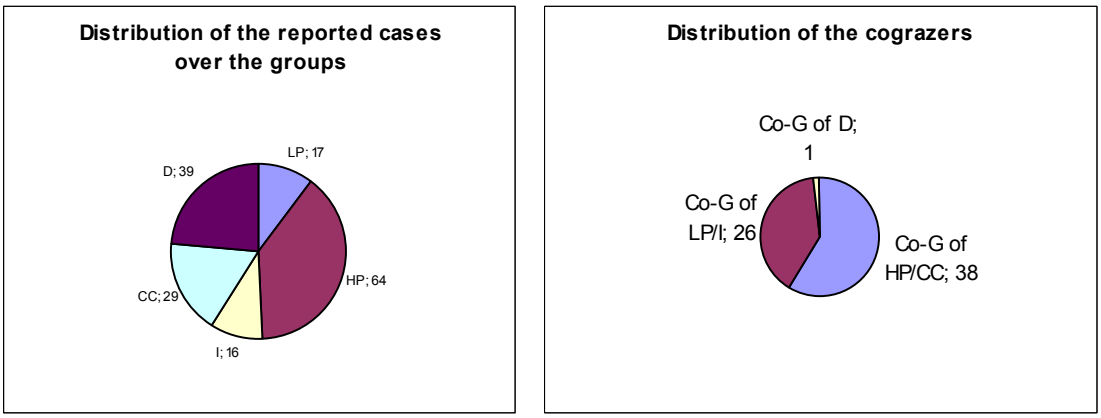
iv. Therapy

The treatment, administered to the reported cases, is shown in Table XII. The majority of the reported cases had received treatment, consisting mostly of fluid therapy, non-steroidal anti-inflammatory drugs and vitamins. The HP/CC cases were more often treated than the LP/I cases. All survivors had been treated, but no significant difference between survivors and non-survivors was noted. No significant differences in the used medication were noted between the studied groups.

v. Diagnosis

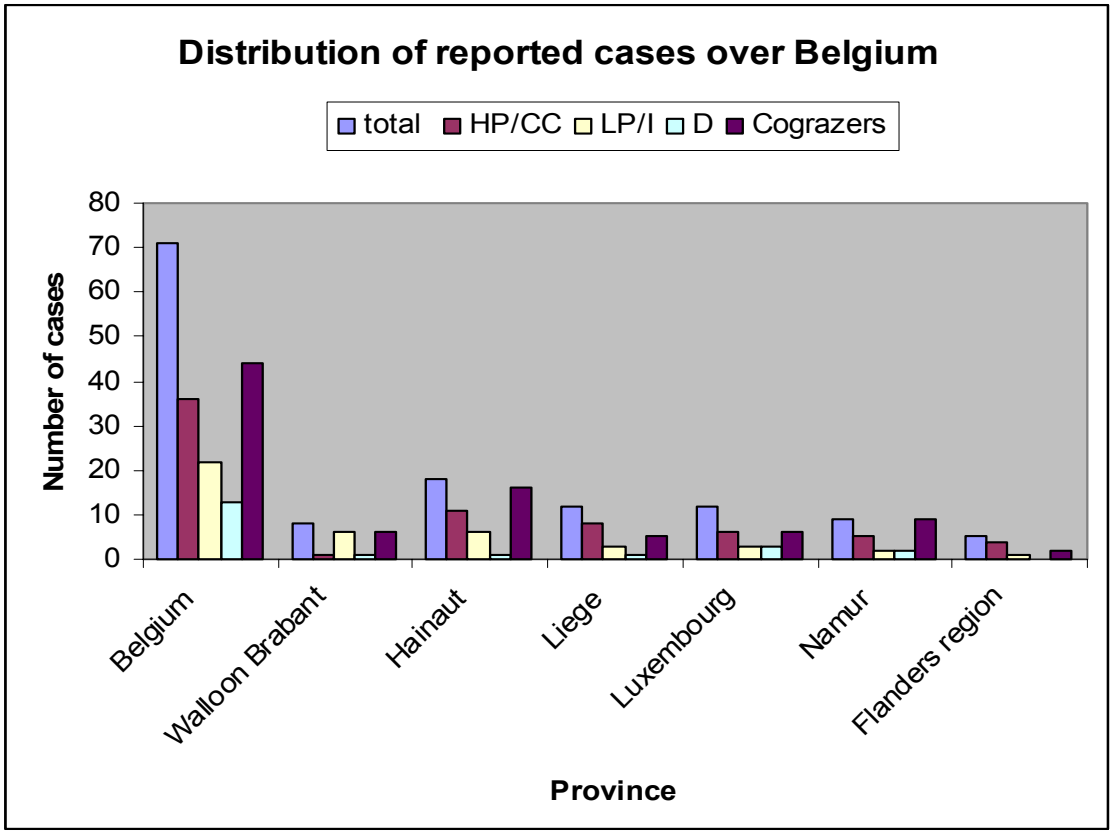
About one third of the reported cases had been autopsied to confirm or infirm the suspicion of AM (52/119) or had been diagnosed by muscle biopsy (12/165) (Table III).

Figure 8a & 8b - Distribution of the reported cases of atypical myopathy reported in the present study (n = 165) and their co-grazers (n = 65) as a function of the probability of the diagnosis



Legend: CC = AM confirmed cases; HP = cases with a high probability of AM; LP = cases with a low probability of AM; I = infirmed cases of AM; D = doubtful cases of AM; Co-G = co-grazer.

Figure 9 - The geographic distribution of the reported cases over Belgium



Legend: CC = AM confirmed cases; HP = cases with a high probability of AM; LP = cases with a low probability of AM; I = infirmed cases of AM; D = doubtful cases of AM.

Figure 10a – Geographic and seasonal distribution of the reported cases over Europe

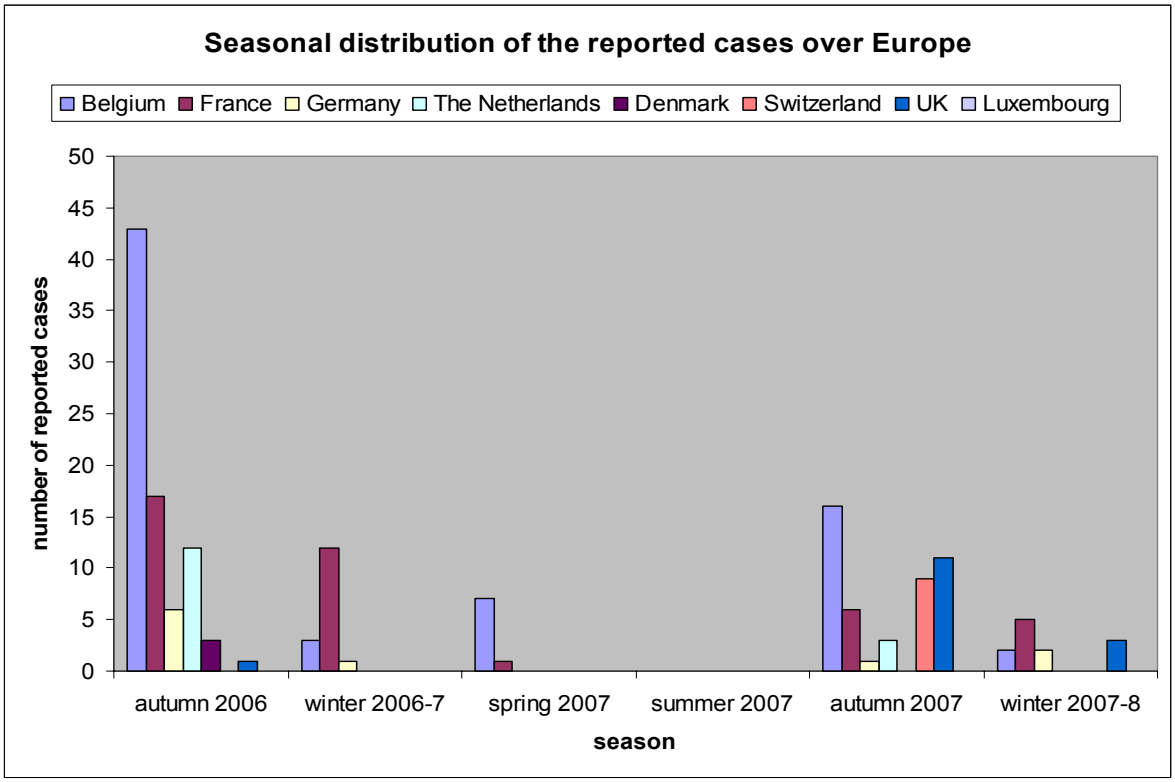


Figure 10b – Geographic and seasonal distribution of the confirmed cases or the cases with a high probability of being affected with atypical myopathy over Europe

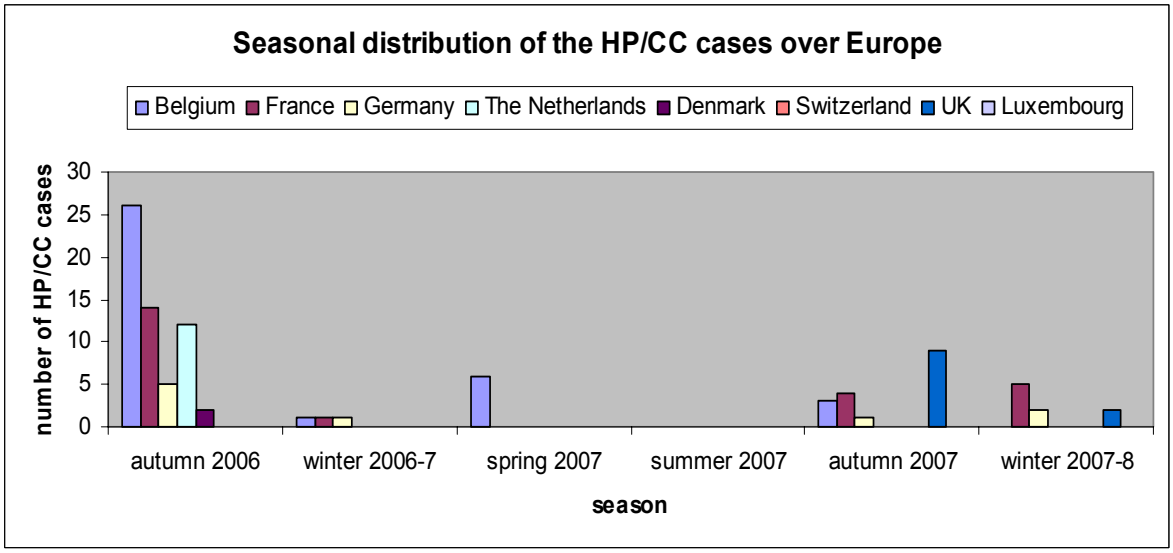
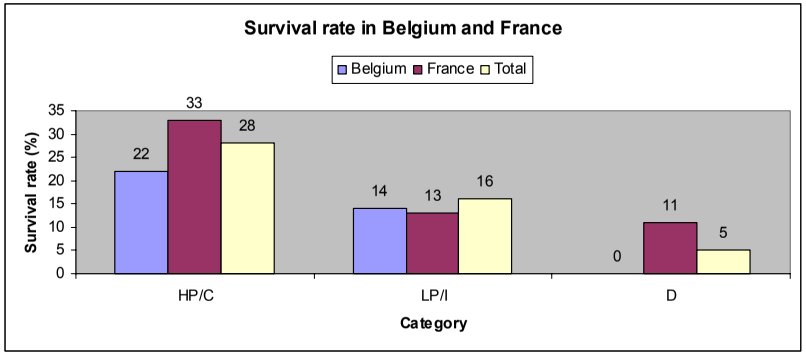
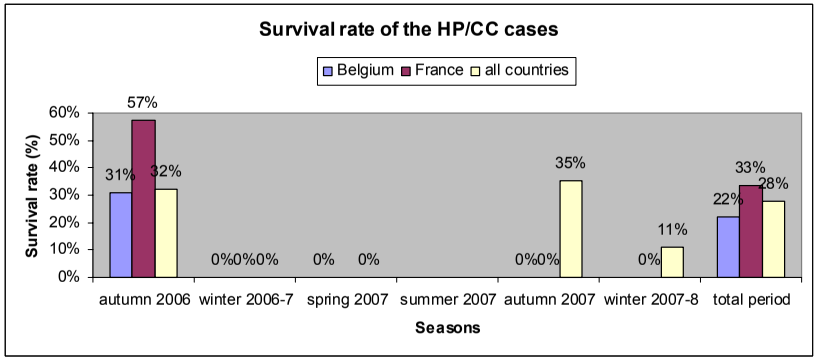


Figure 11a and 11b- The survival rate of all the cases, of the Belgian cases and of the French cases reported in the present study as a function of the probability of the diagnosis (Figure 11a) or as a function of the moment of apparition of the clinical signs (Figure 11b).





Legend: CC = AM confirmed cases; HP = cases with a high probability of AM; LP = cases with a low probability of AM; I = infirmed cases of AM; D = doubtful cases of AM.

Table III – Comparison of the outcome and way of diagnosis in the reported cases

	HP/CC			LP/I			HP/CC *vs* LP/I		
	n	**N**	**%**	**n**	**N**	**%**	**OR**	**95% CI**	
Outcome									
Euthanasia	37	81	46%	6	28	21%	3.08	1.13 – 8.41	+
Dead ("natural")	17	81	21%	14	28	50%	0.27	0.11 – 0.66	-
Dead (No precision)	11	91	12%	5	33	15%	0.77	0.25 – 2.41	ns
Alive	26	91	28%	5	33	16%	2.24	0.78 – 6.43	ns
Found dead at pasture	0	89	0%	7	32	22%	0.02	0.00 – 0.34	-
Diagnosis									
Autopsy	31	80	39%	19	33	58%	0.47	0.20 – 1.06	ns
Samples of muscles	41	79	52%	22	32	69%	0.49	0.21 – 1.17	ns

Legend: CC = AM confirmed cases; HP = cases with a high probability of AM; LP = cases with a low probability of AM; I = infirmed cases of AM; OR = odds ratio; CI = confidence interval; n = number of positive responses; N = number of total responses; % = percentage of positive responses on total responses (n/N); ns = not significant; + = risk factor; - = protective factor.

Significance is set at $P < 0.05$.

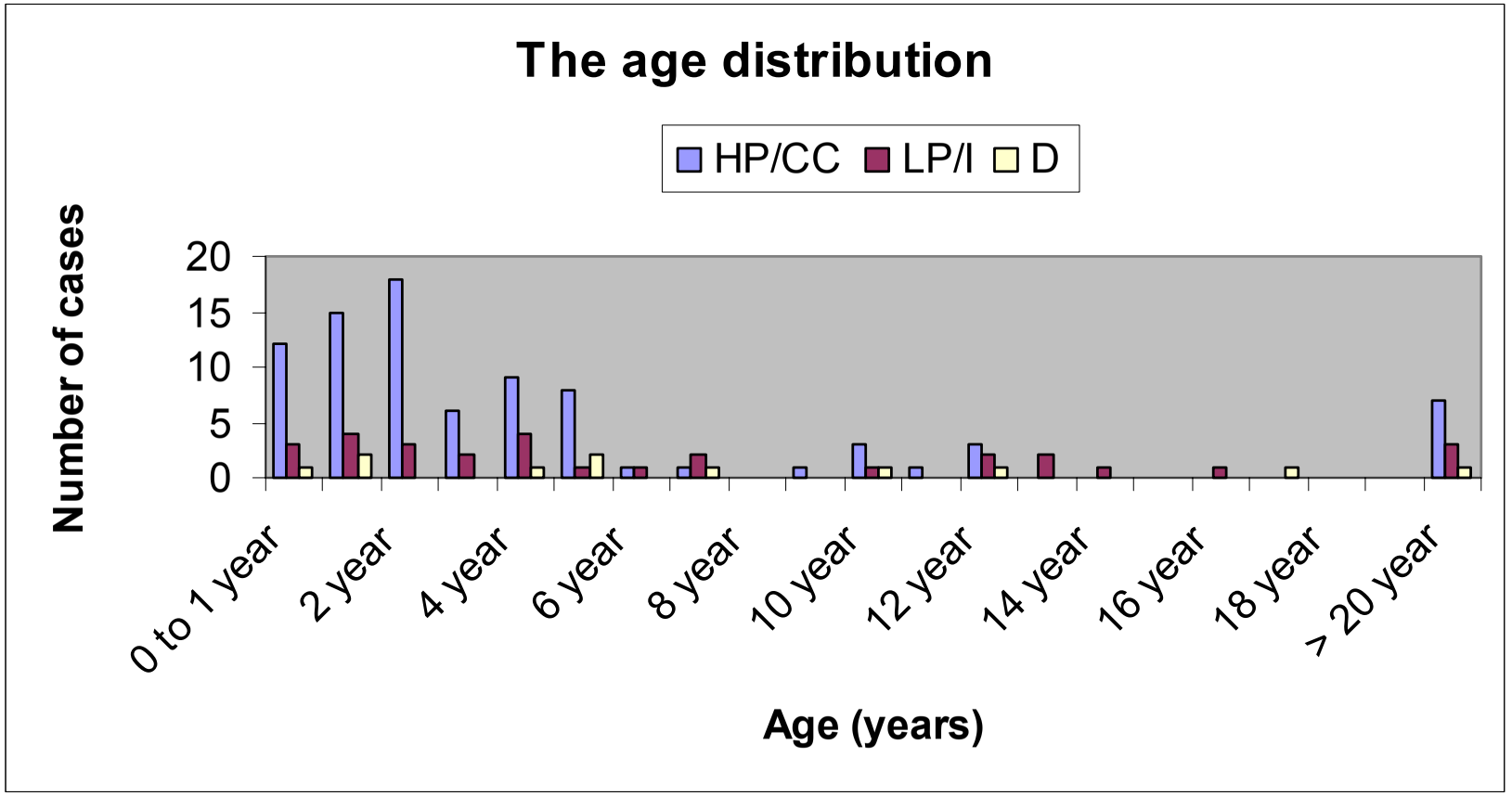
Table IV – Comparison of the demographic data of the reported cases

	HP/CC			LP/I			CoG			S			NS			HP/CC vs LP/I			HP/CC vs CoG			S vs NS		
	n	**N**	**%**	**n**	**N**	**%**	**n**	**N**	**%**	**n**	**N**	**%**	**n**	**N**	**%**	**OR**	**95% CI**		**OR**	**95% CI**		**OR**	**95% CI**	
Sex																								
Mare	39	87	45%	15	29	52%	25	36	69%	10	25	40%	30	61	49%	0.8	0.3 – 1.8	ns	0.4	0.2 – 0.8	-	0.7	0.3 – 1.8	ns
Stallion	24	87	28%	2	29	7%	4	36	11%	7	25	28%	16	61	26%	5.1	1.1 – 23.3	+	3.1	0.9 – 9.5	ns	1.1	0.4 – 3.1	ns
Gelding	24	87	28%	10	29	34%	7	36	19%	8	25	32%	15	61	25%	0.7	0.3 – 1.8	ns	1.6	0.6 – 4.1	ns	1.4	0.5 – 4.0	ns
Breed																								
Pony	27	87	31%	9	30	30%	12	38	32%	7	24	29%	20	61	33%	1.1	0.4 – 2.6	ns	1.0	0.4 – 2.2	ns	0.8	0.3 – 2.4	ns
Draft horse	12	87	14%	3	30	10%	1	38	3%	5	24	21%	7	61	11%	1.4	0.4 – 5.5	ns	5.9	0.7 – 47.3	ns	2.0	0.6 – 7.2	ns
Saddle horse	43	87	49%	17	30	57%	24	38	63%	11	24	46%	31	61	51%	0.8	0.3 – 1.7	ns	0.6	0.3 – 1.3	ns	0.8	0.3 – 2.1	ns
Donkey	2	87	2%	0	30	0%	1	38	3%	1	24	4%	1	61	2%	1.8	0.1 – 38.2	ns	0.9	0.1 – 9.9	ns	2.6	0.2 – 43.5	ns
Other	3	87	3%	1	30	3%	0	38	0%	0	24	0%	2	61	3%	1.0	0.1 – 10.4	ns	3.2	0.2– 63.3	ns	0.5	0.02– 0.5	ns
Body condition																								
Thin	4	67	6%	2	19	11%	1	36	3%	1	19	5%	4	48	8%	0.5	0.1 – 3.2	ns	2.2	0.2– 20.7	ns	0.6	0.1 – 5.9	ns
Normal	57	67	85%	14	19	74%	30	36	83%	14	19	74%	42	48	88%	2.0	0.6 – 6.9	ns	1.1	0.4 – 3.4	ns	0.4	0.1 – 1.5	ns
Overweight	6	67	9%	3	19	16%	5	36	14%	4	19	21%	2	48	4%	0.5	0.1 – 2.3	ns	0.6	0.2 – 2.2	ns	6.1	1.0 – 36.9	+
Extremely muscular	9	52	17%	2	13	15%	3	32	9%	3	18	17%	5	37	14%	1.2	0.2 – 6.1	ns	2.0	0.5 – 8.1	ns	1.3	0.3 – 6.1	ns
Age (years)	**R**	**M ± SD**		**R**	**M ± SD**		**R**	**M ± SD**		**R**	**M ± SD**		**R**	**M ± SD**		**P value**			**P value**			**P value**		
	0.33-38	5.3 ± 7.2		0.5-30	7.5 ± 7.4		0.5-24	6.7 ± 5.8		0.5 - 21	4.7± 5.4		0.4 - 38	5.6 ± 7.9		0.0797		ns	0.0956		ns	0.6207		ns

Legend: see legend Table III; S = survivors of the HP/CC group; NS = non-survivors of HP/CC group; CoG = cograzer; R = range; M = mean; SD = standard deviation.

Significance is set at $P < 0.05$.

Figure 12 - The age distribution of the reported cases



Legend: CC = AM confirmed cases; HP = cases with a high probability of AM; LP = cases with a low probability of AM; I = infirmed cases of AM; D = doubtful cases of AM.

Table V – Comparison of the activity of the reported cases: work and pasturing

	HP/CC			LP/I			CoG			S			NS			HP/CC *vs* LP/I			HP/CC *vs* CoG			S *vs* NS		
	n	**N**	**%**	**n**	**N**	**%**	**n**	**N**	**%**	**n**	**N**	**%**	**n**	**N**	**%**	**OR**	**95% CI**		**OR**	**95% CI**		**OR**	**95% CI**	
Worked	10	54	19%	9	20	45%	13	36	36%	4	19	21%	6	35	17%	0.3	0.1 – 0.8	-	0.4	0.2 – 1.1	ns	1.3	0.3 - 5.3	ns
24H/24H pasturing																								
Winter	28	48	58%	10	15	67%	10	33	30%	10	17	59%	18	31	58%	x	x	x	3.2	1.3 – 8.2	+	x	x	x
Spring	35	51	69%	12	15	80%	26	34	76%	12	18	67%	23	33	70%	x	x	x	0.7	0.3 – 1.8	ns	x	x	x
Summer	41	51	80%	12	15	80%	26	32	81%	14	18	78%	27	33	82%	x	x	x	0.9	0.3 – 2.9	ns	x	x	x
Autumn	43	54	80%	13	15	87%	29	33	88%	16	19	84%	27	35	77%	x	x	x	0.5	0.2 – 1.9	ns	1.5	0.4 – 6.8	ns
When AM occured	44	54	81%	15	22	68%	31	36	86%	17	19	89%	27	35	77%	2.1	0.7 – 6.4	ns	0.7	0.2 – 2.3	ns	2.5	0.5 - 13.3	ns
Not at pasture	0	54	0%	5	22	23%	0	36	0%							0.0	0.0 – 0.6	-	0.7	0.0 – 34.5	ns			
	R		**M ± SD**	**R**		**M ± SD**	**R**		**M ± SD**	**R**		**M ± SD**	**R**		**M ± SD**	**P value**			**P value**			**P value**		
Period at pasture (days)	-2 – 730		367.8 ± 1152.9	-10 – 4745		490.8 ± 1235.8	14 – 7300		741.56 ± 1642.72	10 – 420		92.2 ± 108.4	10 – 7300		527.0± 1428.3	0.2862		ns	0.6091		ns	0,1920		ns

Legend: see legend of Table III and IV; Grey = less than 50% answers; x = no statistical analysis possible due to lack of information in at least one of the compared groups.

Significance is set at $P < 0.05$.

Table VI – Comparison of the medical history of the reported cases

	HP/CC			LP/I			CoG			S			NS			HP/CC *vs* LP/I			HP/CC *vs* CoG			S *vs* NS		
	n	**N**	**%**	**n**	**N**	**%**	**n**	**N**	**%**	**n**	**N**	**%**	**n**	**N**	**%**	**OR**	**95% CI**		**OR**	**95% CI**		**OR**	**95% CI**	
Previous myopathy	1	50	2%	0	16	0%	0	36	0%	1	19	5%	0	31	0%	x	x	x	2.2	0.1 – 55.9	ns	x	x	x
Vaccination	43	60	72%	9	15	60%	26	36	72%	15	21	71%	28	39	72%	x	x	x	1.0	0.4 – 2.4	ns	1.0	0.3 – 3.2	ns
Deworming	50	54	93%	14	15	93%	36	36	100%	18	19	95%	32	35	91%	x	x	x	0.2	0.0 – 2.9	ns	1.7	0.2 – 17.5	ns
Travelling	4	42	10%	5	13	38%	16	36	44%	3	15	20%	1	27	4%	x	x	x	x	x	x	x	x	x
	R		**M ± SD**	**R**		**M ± SD**	**R**		**M ± SD**	**R**		**M ± SD**	**R**		**M ± SD**	**P value**			**P value**			**P value**		
Frequency deworming per year	0 – 5		2.9 ± 1.5	0 – 6.5		2.7 ± 1.6	1 – 6.5		3.2 ± 1.2	0 – 4		3.3 ± 1.2	0 – 5		2.7 ± 1.6	x		x	x		x	x		x

Legend: see legend of Table III, IV and V.

Significance is set at $P < 0.05$.

Table VII – Comparison of the complementation of food of the reported cases

	HP/CC			LP/I			CoG			S			NS			HP/CC *vs* LP/I			HP/CC *vs* CoG			S *vs* NS		
	n	**N**	**%**	**n**	**N**	**%**	**n**	**N**	**%**	**n**	**N**	**%**	**n**	**N**	**%**	**OR**	**95% CI**		**OR**	**95% CI**		**OR**	**95% CI**	
Complementation																								
Winter	41	48	85%	11	11	100%	30	33	91%	14	18	78%	27	30	90%	x	x	x	0.6	0.1 – 2.5	ns	x	x	x
Spring	23	48	48%	6	12	50%	13	33	39%	7	18	39%	16	30	53%	x	x	x	1.4	0.6 – 3.5	ns	x	x	x
Summer	21	48	44%	2	12	17%	19	33	58%	7	18	39%	14	30	47%	x	x	x	0.6	0.2 – 1.4	ns	x	x	x
Autumn	30	51	59%	7	12	58%	18	34	53%	9	19	47%	21	32	66%	x	x	x	1.3	0.5 – 3.0	ns	x	x	x
When AM occurs	29	51	57%	18	20	90%	19	34	56%	9	20	45%	20	31	65%	0.1	0.0 – 0.7	-	1.0	0.4 – 2.5	ns	x	x	x

Legend: see legend of Table III, IV and V.

Significance is set at $P < 0.05$.

Table VIII – Comparison of the pasture characteristics and management of the reported cases

	HP/CC			LP/I			CoG			S			NS			HP/CC *vs* LP/I			HP/CC *vs* CoG			S *vs* NS		
	n	**N**	**%**	**n**	**N**	**%**	**n**	**N**	**%**	**n**	**N**	**%**	**n**	**N**	**%**	**OR**	**95% CI**		**OR**	**95% CI**		**OR**	**95% CI**	
Present on pasture																								
Shelter	23	49	47%	10	20	50%	17	38	45%	8	16	50%	15	33	45%	0.9	0.3 – 2.5	ns	1.09	0.5 – 2.6	ns	1.2	0.4 – 3.9	ns
Trees	51	53	96%	15	19	79%	35	38	92%	18	18	100%	33	35	94%	6.8	1.1 – 40.8	+	2.19	0.3 – 13.8	ns	2.8	0.1 – 60.7	ns
Dead leaves	46	53	87%	6	16	38%	25	38	66%	18	18	100%	28	35	80%	x	x	x	3.42	1.2 – 9.7	+	9.7	0.5 – 181.0	ns
Dead wood	31	51	61%	7	18	39%	27	38	71%	14	17	82%	17	34	50%	2.4	0.8 – 7.3	ns	0.63	0.3 – 1.6	ns	4.7	1.1 – 19.3	+
Humid zones	31	50	62%	6	19	32%	27	38	71%	11	17	65%	20	33	61%	3.5	1.1 – 10.9	+	0.66	0.3 – 1.6	ns	1.2	0.4 – 4.0	ns
Water stream	25	50	50%	2	15	13%	25	38	66%	8	17	47%	17	33	52%	x	x	x	0.52	0.2 – 1.2	ns	0.8	0.3 – 2.7	ns
Nature pasture																								
Natural	41	50	82%	16	17	94%	34	38	89%	14	17	82%	27	33	82%	0.3	0.0 – 2.4	ns	0.5	0.2 – 1.9	ns	0.8	0.3 – 2.7	ns
Not-natural	2	50	4%	1	17	6%	0	38	0%	1	17	6%	1	33	3%	0.7	0.1 – 7.9	ns	3.9	0.2 – 85.1	ns	1037.0	0.2 – 4.8	ns
Treatment pasture																								
Fertilizers	19	49	39%	8	15	53%	22	38	58%	5	17	29%	14	32	44%	x	x	x	0.5	0.2 – 1.1	ns	x	x	x
Not-treated	27	49	55%	6	15	40%	14	38	37%	11	17	65%	16	32	50%	x	x	x	2.1	0.9 – 5.0	ns	x	x	x
Removal of manure	4	45	9%	1	14	7%	4	38	11%	1	16	6%	3	29	10%	x	x	x	x	x	x	x	x	x
Spreading of manure	14	45	31%	5	14	36%	9	38	24%	4	16	25%	10	29	34%	x	x	x	x	x	x	x	x	x
Lush when AM occurred	14	44	32%	4	14	29%	13	38	34%	3	14	21%	11	30	37%	x	x	x	x	x	x	x	x	x
Drinking tool																								
Bucket	4	44	9%	2	15	13%	1	32	3%	2	18	11%	2	26	8%	x	x	x	x	x	x	x	x	x
Tank	19	44	43%	11	15	73%	19	32	59%	7	18	39%	12	26	46%	x	x	x	x	x	x	x	x	x
Water course	6	44	14%	0	15	0%	1	32	3%	1	18	6%	5	26	19%	x	x	x	x	x	x	x	x	x
Automatic drinker	15	44	34%	4	15	27%	11	32	34%	8	18	44%	7	26	27%	x	x	x	x	x	x	x	x	x

Water source																								
Rain	9	42	21%	2	15	13%	13	33	39%	3	16	19%	6	26	23%	x	x	x	x	x	x	x	x	x
Distribution	23	42	55%	11	15	73%	19	33	58%	8	16	50%	15	26	58%	x	x	x	x	x	x	x	x	x
Springwater	13	42	31%	4	15	27%	2	33	6%	6	16	38%	7	26	27%	x	x	x	x	x	x	x	x	x
Water stream	5	42	12%	0	15	0%	1	33	3%	3	16	19%	2	26	8%	x	x	x	x	x	x	x	x	x
Slope																								
Yes	36	51	71%	7	15	47%	32	38	84%	13	17	76%	23	34	68%	x	x	x	0.5	0.2 – 1.3	ns	1.6	0.4 - 5.9	ns
100%	14	32	44%	0	6	0%	15	29	52%	5	13	38%	9	19	47%	x	x	x	x	x	x	x	x	x
>10%	12	35	34%	1	6	17%	14	30	47%	5	13	38%	7	22	32%	x	x	x	x	x	x	x	x	x
Animals																								
Other animals on pasture	26	56	46%	6	18	33%	12	36	33%	12	20	60%	14	36	39%	1.7	0.6 – 5.3	ns	1.7	0.7 – 4.1	ns	2.4	0.8 - 7.2	ns
Co-grazing	9	19	47%	2	5	40%	9	12	75%	5	9	56%	4	10	40%	x	x	x	x	x	x	x	x	x
Death of animals																								
Other horses	12	49	24%	6	15	40%	2	36	6%	5	18	28%	7	31	23%	x	x	x	5.5	1.1 – 26.4	+	x	x	x
Other animals	0	32	0%	1	10	10%	0	26	0%	0	12	0%	0	20	0%	x	x	x	0.8	0.0 – 42.5	ns	x	x	x
Intensity of use pasture	**R**	**M ± SD**		**R**	**M ± SD**		**R**	**M ± SD**		**R**	**M ± SD**		**R**	**M ± SD**		**P value**			**P value**			**P value**		
Live stock unit per hectare	1 – 13	2.4 ± 2.5		1 – 175	22.6 ± 53.9		0 – 175	12.7 ± 37.2		0.1 – 4.4	2.1± 1.4		0.1 – 13.3	2.6 ± 3.1		x		x	x		x	x		x
Horses / year	1 – 12	2.9 ± 2.4		2 – 12	5.7 ± 4.4		0 – 12	4.5 ± 3.4		0.4 - 6	2.1 ± 1.4		0.2 - 12	3.4 ± 2.7		x		x	x		x	x		x
Horses / year / hectare	1 – 13	1.8 ± 2.7		1 – 175	20.7 ± 54.3		0 – 175	8.9 ± 33.0		0.3 – 12.5	1.9 ± 3.2		0.2 – 12.5	1.8 ± 2.4		x		x	x		x	x		x

Legend: see legend of Table III, IV and V.

Significance is set at $P < 0.05$.

Table IX – Comparison of the climatic conditions when atypical myopathy occurred in the reported cases

	HP/CC			LP/I			S			NS			HP/CC *vs* LP/I			S *vs* NS		
	n	**N**	**%**	**n**	**N**	**%**	**n**	**N**	**%**	**n**	**N**	**%**	**OR**	**95% CI**		**OR**	**95% CI**	
Climatic conditions reported by the owners																		
Change in T	19	37	51%	6	13	46%	8	15	53%	11	22	50%	x	x	x	x	x	x
Fog	5	33	15%	2	13	15%	1	12	8%	4	21	19%	x	x	x	x	x	x
Dew	22	39	56%	9	12	75%	5	13	38%	17	26	65%	x	x	x	x	x	x
Frost	6	42	14%	5	15	33%	2	15	13%	4	27	15%	x	x	x	x	x	x
Sun	9	32	28%	6	12	50%	3	12	25%	6	20	30%	x	x	x	x	x	x
Freezing	4	40	10%	4	14	29%	1	15	7%	3	25	12%	x	x	x	x	x	x
Rain	34	40	85%	11	14	79%	12	15	80%	22	25	88%	x	x	x	x	x	x
Wind	19	37	51%	6	13	46%	12	14	86%	21	24	88%	x	x	x	x	x	x
National climatic conditions	**Range**	**M ± SD**		**Range**	**M ± SD**		**Range**	**M ± SD**		**Range**	**M ± SD**		**P value**			**P value**		
Min day T 10 days before (°C)	-2 – 15	7.9 ± 3.7		-1 – 12	5.8 ± 3.1		4 – 15	7.5 ± 3.5		-2 – 15	8.2 ± 3.8		0.0309	+		x		x
Max day T 10 days before (°C)	9 – 22	14.9 ± 3.5		9 – 21	13.6 ± 3.2		9 – 18	13.6 ± 3.2		9 – 22	15.5 ± 3.6		0.1209	ns		0.1019		ns
Min night T 10 days before (°C)*	-4 – 6	-0.7 ± 2.8		-2	-2.0		-4 – 0	-2.3 ± 1.9		-3 – 6	0.0 ± 2.9		x	x		x		X
Rainfall 10 days before (mm)	4 – 66	20.3 ± 14.1		9 – 50	30.9 ± 14.4		7 – 46	17.0 ± 9.7		0 – 39	20.9 ± 14.9		0.0077	-		0.3716		ns
Monthly sunshine (Hours)	44 – 284.2	97.3 ± 63.7		34.5 – 158.1	75.6 ± 30.7		40 – 108.25	77.1 ± 22.7		40 – 284.2	105.9 ± 71.7		0.3825	ns		0.1474		ns
Mean monthly atmospheric pressure (hPa)	1010 – 1023	1017.1 ± 1.7		1010 – 1023	1017.5 ± 3.2		1013 – 1023	1016.8 ± 3.1		1010 – 1023	1017.0 ± 3.5		x	x		x		x
Mean monthly windspeed (km/H)	6.7 – 12.6	10.2 ± 1.7		5.5 – 16.4	11.6 ± 2.6		10.1 – 12.6	11.1 ± 0.8		10.1 – 12.6	9.9 ± 1.7		x	x		x		x

Legend: see legend of Table III, IV and V; T = temperature; Min day T 10 days before = the minimal day temperature in the 10 days preceding the onset of AM; Max day T 10 days before = the maximal day temperature in the 10 days preceding the onset of AM; Min night T 10 days before = the minimal night temperature in the 10 days preceding the onset of AM.

Significance is set at $P < 0.05$.

Data from: www.meteobelgique.be, www.knmi.nl, www.climate-uk.com. National climatic conditions only available for Belgium, The Netherlands and UK.

Table X – Comparison of the clinical data of the reported cases

	HP/CC			LP/I			S			NS			HP/CC *vs* LP/I			S *vs* NS		
	n	**N**	**%**	**n**	**N**	**%**	**n**	**N**	**%**	**n**	**N**	**%**	**OR**	**95% CI**		**OR**	**95% CI**	
Abnormal behavior prior to AM	13	63	21%	4	19	21%	6	19	32%	7	44	16%	1.0	0.3 – 3.4	ns	2.4	0.7 - 8.6	ns
Mucosae																		
Normal	14	48	29%	5	14	36%	8	14	57%	6	34	18%	x	x	x	6.2	1.6 – 24.7	+
Congestive	28	48	58%	2	14	14%	6	14	43%	22	34	65%	x	x	x	0.4	0.1 – 1.5	ns
Cyanotic	4	48	8%	4	14	29%	0	14	0%	4	34	12%	x	x	x	0.2	0.0 – 4.6	ns
Icteric	3	53	6%	3	15	20%	0	14	0%	3	39	8%	x	x	x	0.4	0.0 – 7.4	ns
Respiratory																		
Normal respiration	25	55	45%	7	11	64%	11	12	92%	14	43	33%	x	x	x	x	x	x
Dyspnoea	25	50	50%	5	11	45%	1	11	9%	24	39	62%	x	x	x	x	x	x
Cardiac																		
Tachycardia	45	56	80%	8	11	73%	13	15	87%	32	41	78%	x	x	x	1.8	0.3 – 9.6	ns
Murmur	4	49	8%	2	9	22%	0	13	0%	4	36	11%	x	x	x	0.3	0.0 – 5.3	ns
Arrhythmia	7	48	15%	0	5	0%	2	12	17%	5	36	14%	x	x	x	x	x	x
Temperature																		
Normal temperature	30	59	51%	6	18	33%	7	15	47%	23	44	52%	2.07	0.69 – 6.25	ns	0.8	0.2 – 2.6	ns
Hypothermia	19	61	31%	6	19	32%	5	15	33%	14	46	30%	0.98	0.32 – 2.97	ns	1.1	0.3 – 4.0	ns
Hyperthermia	10	59	17%	6	18	33%	3	15	20%	7	44	16%	0.41	0.12 – 1.35	ns	1.3	0.3 – 5.9	ns
Signs suggestive for myopathy																		
Weakness	60	68	88%	15	19	79%	14	15	93%	46	53	87%	2.0	0.5 – 7.5	ns	2.1	0.2 – 18.8	ns
Stiffness	58	65	89%	13	19	68%	15	16	94%	43	49	88%	3.8	1.1 – 13.3	+	2.1	0.2 – 18.8	ns
Recumbency	50	78	64%	16	23	70%	8	20	40%	42	57	74%	0.8	0.3 – 2.1	ns	0.2	0.1 – 0.7	-
Remains standing most of the time	34	76	45%	7	22	32%	14	19	74%	19	56	34%	1.7	0.6 – 4.7	ns	5.5	1.7 – 17.4	+
Trembling	43	66	65%	8	18	44%	11	16	69%	32	50	64%	2.3	0.8 – 6.7	ns	1.2	0.4 – 4.1	ns

Sweating	33	65	51%	12	19	63%	6	16	38%	27	49	55%	0.6	0.2 – 1.7	ns	0.5	0.2 – 1.6	ns
Myoglobinuria	68	72	94%	6	12	50%	16	17	94%	52	55	95%	x	x	x	0.9	0.1 – 9.5	ns
Pain																		
Signs of colic	19	69	28%	6	21	29%	6	18	33%	13	51	25%	0.9	0.3 – 2.8	ns	1.5	0.5 – 4.7	ns
Depressed	52	72	72%	12	21	57%	12	18	67%	40	54	74%	1.9	0.7 – 5.3	ns	0.7	0.2 – 2.2	ns
No suffering	5	42	12%	3	11	27%	4	13	31%	1	29	3%	x	x	x	x	x	x
Medium suffering	26	42	62%	4	11	36%	8	13	62%	18	29	62%	x	x	x	x	x	x
Severe suffering	11	42	26%	4	11	36%	1	13	8%	10	29	34%	x	x	x	x	x	x
Digestive																		
Anorexia	23	64	36%	5	19	26%	4	17	24%	19	47	40%	1.6	0.5 – 4.9	ns	0.5	0.1 – 1.6	ns
Craving for food	5	37	14%	4	14	29%	0	6	0%	5	31	16%	x	x	x	x	x	x
Dysphagia	15	60	25%	3	18	17%	1	17	6%	14	43	33%	1.7	0.4 – 6.6	ns	0.1	0.0 – 1.1	ns
Esophageal obstruction	5	52	10%	1	16	6%	1	17	6%	4	35	11%	x	x	x	0.5	0.0 – 4.7	ns
Defecation	26	40	65%	5	8	63%	9	12	75%	17	28	61%	x	x	x	x	x	x
Borborygmi present	23	36	64%	5	9	56%	8	12	67%	15	24	63%	x	x	x	x	x	x
Gastric reflux	0	22	0%	1	2	50%	0	7	0%	0	15	0%	x	x	x	x	x	x
Rectal examination																		
Distended bladder	20	36	56%	3	5	60%	6	12	50%	14	24	58%	x	x	x	x	x	x
Other abnormalities	16	36	44%	2	6	33%	7	12	58%	9	24	38%	x	x	x	x	x	x
General examination	**R**	**M ± SD**		**R**	**M ± SD**		**R**	**M ± SD**		**R**	**M ± SD**		**P value**			**P value**		
Heart rate	36 – 94	58.3 ± 15		36 – 96	64 ± 20.5		36 – 80	54.9 ± 12.8		35 – 94	59.6 ± 15.8		x		x	0.3504		ns
Respiratory rate	12 – 72	31 ± 17		18 and 44	X		8 – 40	21 ± 9.8		18 – 72	35 ± 18		x		x	0.0195		ns
CRT	1 – 5	2.4 ± 1		2 – 4	2 ± 0.8		2 – 3	2.3 ± 0.4		1 – 5	2.4 ± 1.1		x		x	0.7211		ns
Rectal temperature	34.5 – 40.2	38 ± 1.1		33.0 – 41.0	37.0 ± 2.3		34.5 – 39	38 ± 1.2		35.7 – 40.2	37 ± 1.1		x		x	0.8586		ns

Legend: see legend of Table III, IV and V.

Significance is set at $P < 0.05$.

Table XI – Comparison of the serum activities of creatine kinase of the reported cases

	HP/CC			LP/I			S			NS			HP/CC *vs* LP/I			S *vs* NS		
	n	**N**	**%**	**n**	**N**	**%**	**n**	**N**	**%**	**n**	**N**	**%**	**OR**	**95% CI**		**OR**	**95% CI**	
Value CK activity (IU/L)																		
< 1000	0	64	0%	4	17	24%	0	21	0%	0	42	0%	0.02	0.0 – 0.5	-	2.0	0.0 – 103.1	ns
1000 – 5000	9	64	14%	5	17	29%	3	21	14%	6	42	14%	0.4	0.1 – 1.4	ns	1.0	0.2 – 4.5	ns
5000 – 15000	7	64	11%	1	17	6%	2	21	10%	5	42	12%	1.9	0.2 – 17.2	ns	0.8	0.1 – 4.4	ns
>15000	48	64	75%	7	17	41%	16	21	76%	31	42	74%	4.3	1.4 – 13.1	+	1.1	0.3 – 3.8	ns
Value CK activity(IU/L)	**Range**	**Mean ± SD**		**Range**	**Mean ± SD**		**Range**	**Mean ± SD**		**Range**	**Mean ± SD**		**P value**			**P value**		
First measured value	1 000 – 7059880	265 628 ± 921 555		495 – 54 500	13 812 ± 18 246		1 300 – 7 059 880	480 738 ± 1 511 593		1 000 – 2 172 000	158 586 ± 368 121		x		x	0.1706		ns
Highest value	1 300 – 7059880	312 221 ± 929 130		495 – 54 500	15 079 ± 17 865		1 300 – 7 059 880	502 194 ± 1 505 380		1 300 – 2 172 000	216 907 ± 396 755		x		x	0.2501		ns

Legend: see legend of Table III, IV and V; CK = creatine kinase.

Significance is set at $P < 0.05$.

Table XII – Comparison of the treatment of the reported cases

	HP/CC			LP/I			S			NS			HP/CC *vs* LP/I			S *vs* NS		
	n	**N**	**%**	**n**	**N**	**%**	**n**	**N**	**%**	**n**	**N**		**OR**	**95% CI**		**OR**	**95% CI**	
Treatment	53	59	90%	15	24	63%	20	20	100%	33	39	85%	5.30	1.63 – 17.27	+	7.96	0.43 – 148.76	ns
Fluidtherapy	38	51	75%	8	14	57%	15	20	75%	23	31	74%	x	x	x	x	x	x
Calcium	6	50	12%	1	14	7%	3	19	16%	3	31	10%	x	x	x	x	x	x
NSAID's	38	50	76%	12	14	86%	12	19	63%	26	31	84%	x	x	x	x	x	x
Corticosteroids	12	50	24%	4	14	29%	5	19	26%	7	31	23%	x	x	x	x	x	x
DMSO	6	49	12%	1	14	7%	3	19	16%	3	30	10%	x	x	x	x	x	x
Metronidazole	3	49	6%	0	14	0%	2	19	11%	1	30	3%	x	x	x	x	x	x
Vitamins and minerals	21	49	43%	5	14	36%	11	19	58%	10	30	33%	x	x	x	x	x	x
Antibiotics	16	49	33%	7	15	47%	9	19	47%	7	30	23%	x	x	x	x	x	x
Myorelaxants	5	49	10%	1	14	7%	2	19	11%	3	30	10%	x	x	x	x	x	x
Alpha2-agonists	6	50	12%	1	14	7%	1	19	5%	5	31	16%	x	x	x	x	x	x
ACP	12	49	24%	1	14	7%	6	19	32%	6	30	20%	x	x	x	x	x	x
Diuretics	3	50	6%	0	14	0%	1	19	5%	2	31	6%	x	x	x	x	x	x

Legend: see legend of Table III, IV and V.

Significance is set at $P < 0.05$.

2. FATTY ACID ANALYSIS

a. Extraction of the lipids

No difference between AM affected horses and control horses was present concerning the weight of the total lipids in mg/g muscle (Table XIII and Figure 13).

b. Analytical thin layer chromatography

The muscle samples of the control horses often had shown none or just traces of FFA at the TLC. These FFA were then considered to be < 10 µg or < 0.5% of the measured weight of the total muscular lipids in order to be able to perform the calculations for the GLC.

In all muscles of the AM affected horses the FFA were clearly visible at the TLC, in contrast to the control horses. The muscles of the control horses that showed FFA at the TLC were the *M. masseter* (C_3) and the *M. brachiocephalicus* (C_1 and C_3; see Figure 14a and 14b). Differences in the other lipid classes were also visible (no quantitative data shown): in the affected horses less triglycerides were visible at the TLC than in the control horses and in some muscles of affected horses monoglycerides were identified. In none of the horses, cholesterylesters were present.

The mean muscular FFA in affected horses was higher than that of the control horses (Figure 15). The muscles with the highest FFA values were the *M. masseter* (in as well control horses as in AM affected horses), the *Mm. intercostales* (only sampled in AM affected horses) and the cardiac muscles (only sampled in AM_1), however compared to the control horses the *M. masseter* had the lowest increase in FFA in affected horses (Figure 16 and Table XIV).

c. Gas liquid chromatography

It was not possible to identify all peaks of the GLC. In all horses, small peaks with a very long retention time (longer than C20:5n3 or C24:2 or C22:4n6) were present, which were not identified due to their absence in the reference FFA (Figure 17).

The profiles of the chromatograms were about the same in all horses. Some FFA were only present in 1 horse: C_1 was the only horse with C24:2, while it had no C12:0 and no C14:1. The FFA C22:4n6 only was present in the *M. quadriceps* of C_2; C22:0 was only present in AM_1; C22:1 was only present in some muscles of AM_2 and C_2. Other FFA were present in all horses except one: C20:5n3 was not present in AM_1; C22:1 was not encountered in C_3. All these FFA, which were not present in all horses, were only present in small amounts.

In *percentage*, the FFA also showed a quite similar division, except for C16:1 which was clearly present in a higher percentage in both affected horses compared to the controls, and C16:0 which was present in a higher percentage in the controls compared to the AM affected horses (Figure 18). In every muscle of the affected horses, the highest value of the FFA was obtained for C16:1 (20.9% - 42.1% and 225.57 - 1435.7 µg/g muscle; mean 733.36 µg/g muscle) with the exception of the *M. triceps* of AM_2 (a

long chain unidentified FFA; the next highest was C14:0). The FFA with the highest value in the control horses was as follows: C_1 had as highest FFA in the 4 muscles C16:1, C10:0, and twice a very short chain unidentified FFA (< C10:0); C_2 in all 3 muscles C16:0 (24.1% - 43.85% and 43.37 - 90.05 µg/g muscle; mean 72.48 µg/g muscle); and C_3 in all 4 muscles C16:0 (14.77% - 40.42% and 42.12 - 1328.05 µg/g muscle; mean 479.31 µg/g muscle [but the *M. masseter* increases the mean substantially]). The peak of the C16:0 in the control horses expressed in µg/g muscle was much lower than the peak of C16:1 in the affected horses.

The amount of the FFA was almost always higher in the AM affected muscles than in the control muscles, although differences were not that clear in all FFA as in C16:0 and C16:1 (Figure 19). The exceptions consisted mostly of short and medium chains FFA (C10:0, C12:0, C14:1 and C16:0) in single muscles, and in C_1 some long FFA (C20:5n3 and C24:2). These exceptions were often not visible in the charts with mean values.

The FFA can be divided in saturated, mono-unsaturated and poly-unsaturated FFA dependant on the presence of and the number of double bonds. Control horses had a majority of saturated FFA. In general, affected horses had more of all three groups FFA compared to control horses, but they had a majority of mono-unsaturated FFA.

Table XV shows the values of the identified FFA. The identified FFA were 34.7% to 79.82% of the total FFA, with a mean of 53.8% for the AM cases and 56.9% for the controls.

Table XIII - Weight of the total lipids and of the free fatty acids (FFA) in the sampled muscles of the control horses and atypical myopathy affected horses

Horse	Muscle	Weight lyophilised muscle (g)	Weight total lipids (mg/g muscle)	Weight FFA (µg/g muscle)	Mean (±SD) FFA (µg/g muscle)
Atypical myopathy affected horses					
AM_1	M.brachiocephalicus	0.9030	21.12	2 673.79	
	M. longissimus dorsi	1.1898	10.98	848.75	
	M. quadriceps femoris	0.7950	22.60	1 627.20	3 100
	Mm. intercostales	0.7286	36.60	2 470.50	± 1 977
	Interventricular septum of the myocardium	1.0241	57.91	5 107.66	
	Ventricular free wall of the myocardium	1.2956	42.84	5 877.24	
AM_2	M. masseter	1.2783	29.83	4 671.38	
	M. brachiocephalicus	1.0900	18.72	2 515.43	
	M. triceps brachii	1.1750	22.13	2 367.70	2 806
	M. longissimus dorsi	1.0642	32.32	2 472.86	± 1 318
	M. quadriceps femoris	1.2524	61.43	1 898.16	
	M. semi-tendinosis	1.0142	43.78	1 164.49	
	Mm. intercostales	0.4613	54.92	4 552.62	
Control horses					
C_1	M. brachiocephalicus	1.2495	21.45	1 454.31	
	M. longissimus dorsi	0.8806	29.37	146.85*	474
	M. quadriceps femoris	1.2152	21.81	109.05*	± 653
	M. semi-tendinosis	0.9438	37.86	189.30*	
C_2	M. brachiocephalicus	1.1871	35.94	179.70*	159
	M. quadriceps femoris	1.2335	41.07	205.35*	± 13.6
	M. semi-tendinosis	1.0313	40.08	200.40*	
C_3	M. masseter	1.0039	26.03	3 877.79	
	M. brachiocephalicus	1.1016	45.27	1 616.59	1 529
	M. semi-tendinosis	1.0216	57.03	285.15*	± 1682
	M. sacrocaudalis dorsalis	1.1531	67.41	337.05*	

Legend: (*) FFA not present or only in traces visible on the TLC, so < 10 µg or < 0.5 % of the weight of the measured total lipids. To be able to perform the calculations for the GLC, the quantity of those FFA was arbitrary chosen to be 0.5% of the weight of the measured total lipids.

Figure 13 - Mean weight of the total muscular lipids for each individual

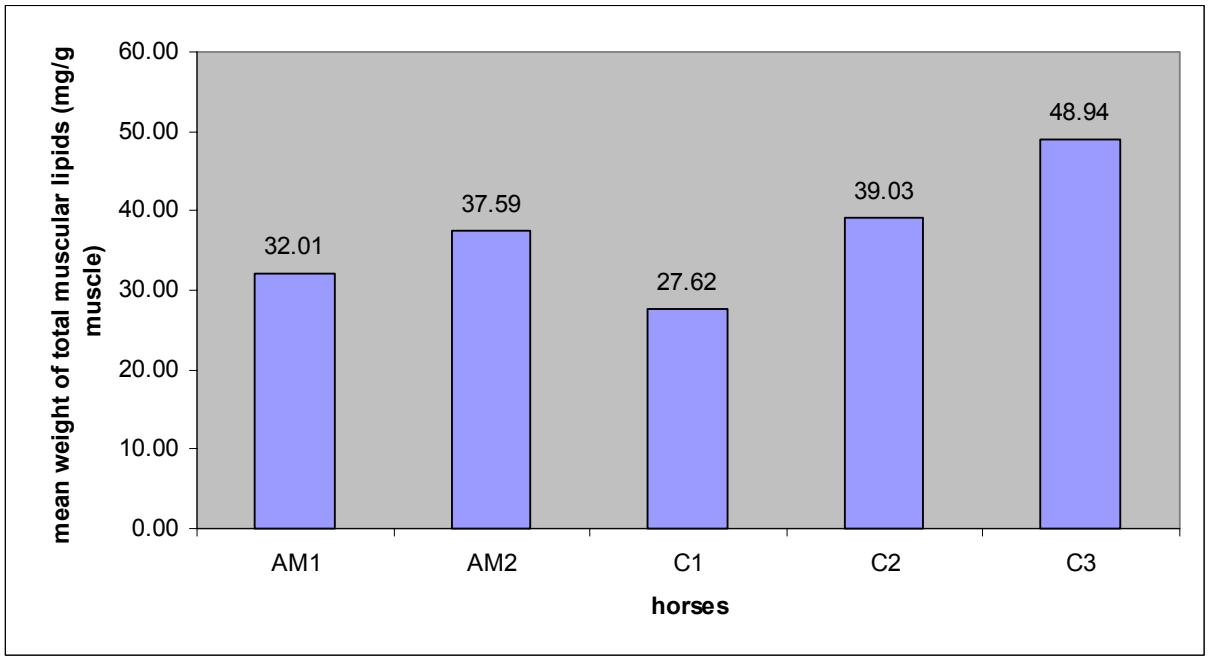


Figure 15 - Mean weight of the muscular free fatty acids

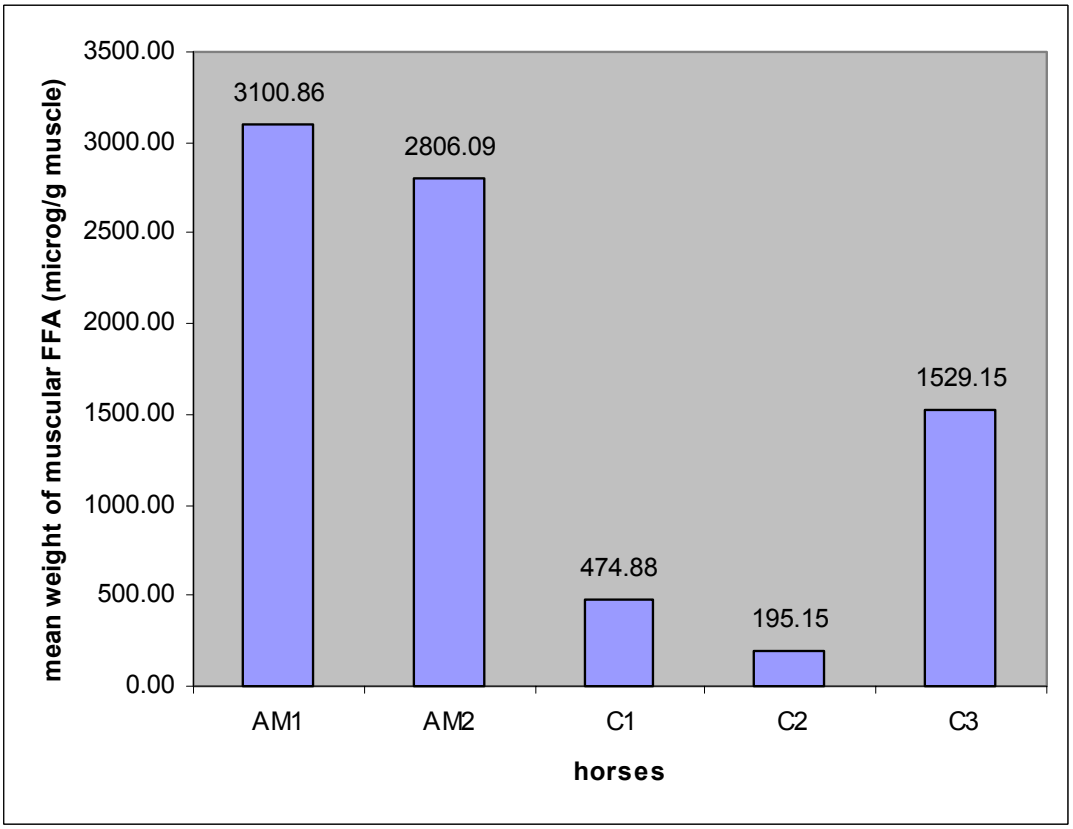
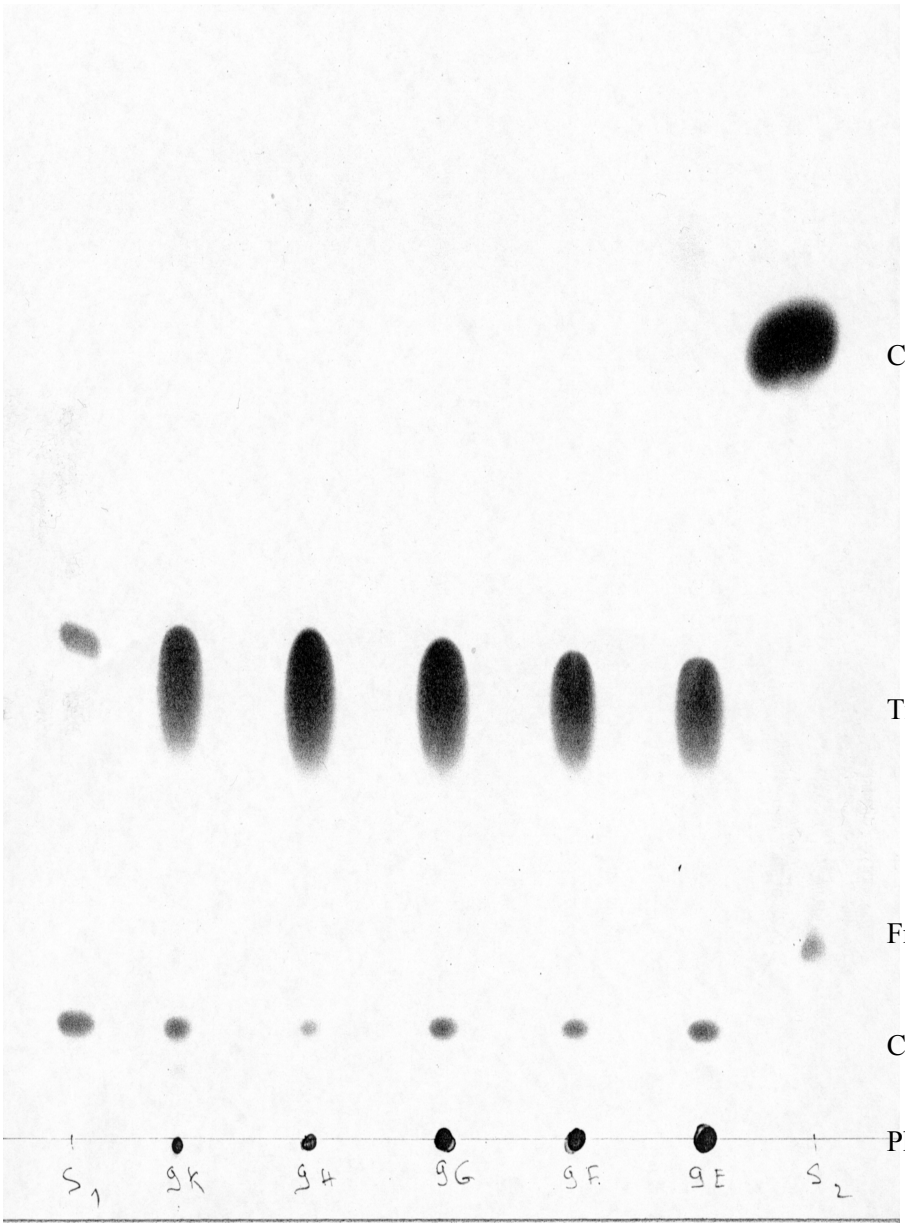
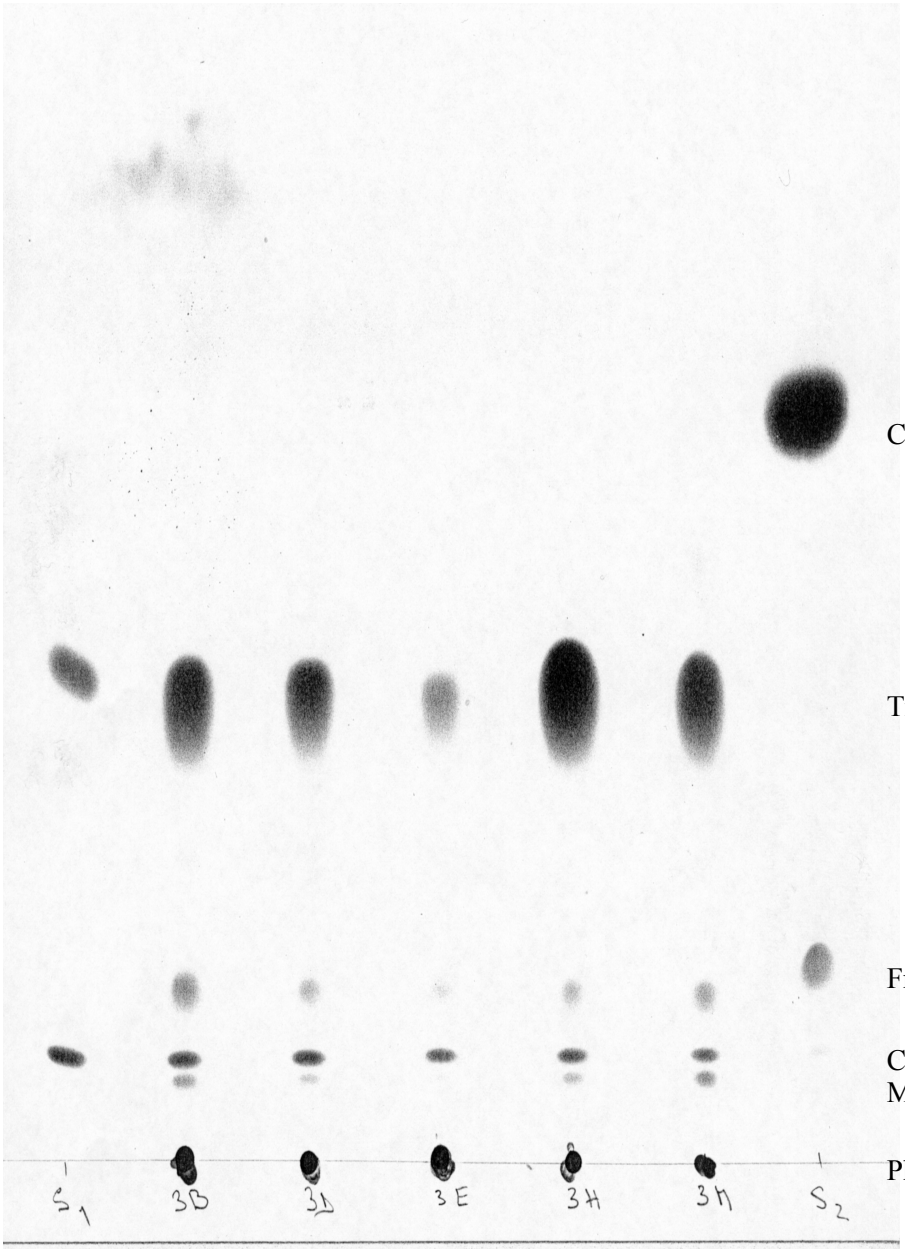


Figure 14a - Thin layer chromatography of a muscle sample of horse affceted with atypical myopathy



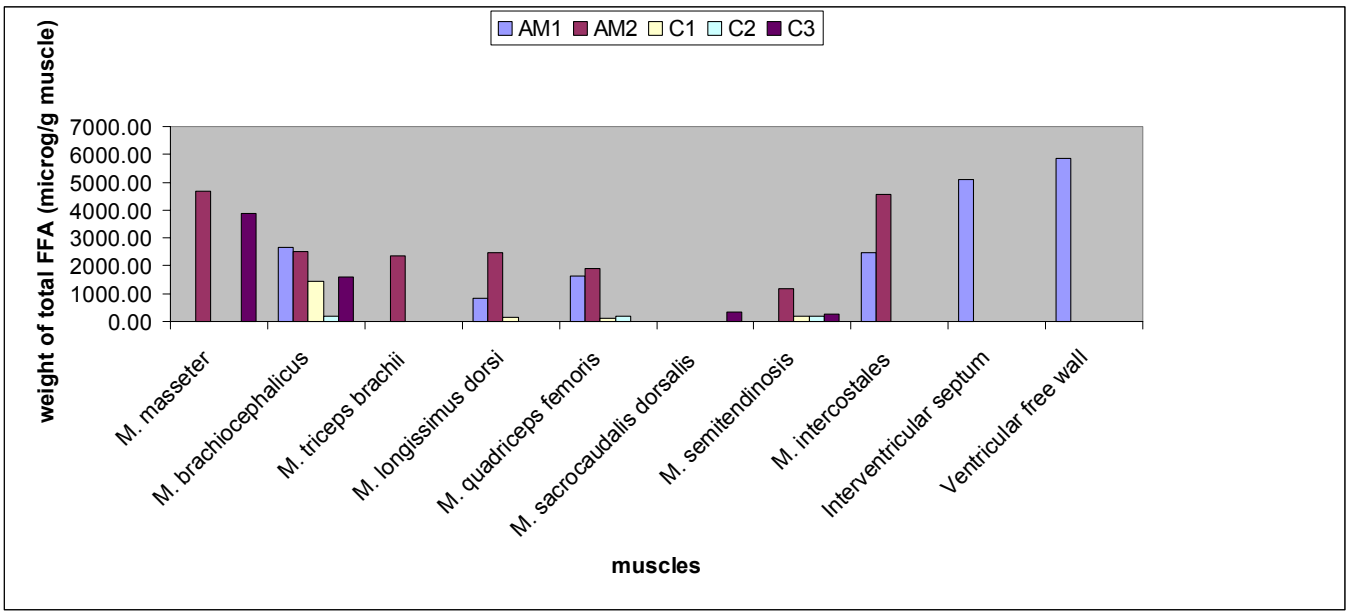
Legend: A chromatography plate with 5 samples (9E-9K) of a control horse and 2 control samples (S1 and S2). The migration of the lipids occurs from ventral to dorsal and the TLC permits a separation of cholesteryl esters, triglycerides, FFA, cholesterol, monoglycerides and phospholipids. After migration, the migrated lipids were visualised under the form of brown-black spots.

Figure 14b – Thin layer chromatography of a muscle sample of a control horse



Legend: A chromatography plate with 5 samples (3B-3H) of an affected horse and 2 control samples (S1 and S2). The migration of the lipids occurs from ventral to dorsal and the TLC permits a separation of cholesteryl esters, triglycerides, FFA, cholesterol, monoglycerides and phospholipids. After migration, the migrated lipids were visualised under the form of brown-black spots.

Figure 16 - Weight of the total free fatty acids per muscle



Legend : FFA = free fatty acids ; AM1 = case 1 affected with atypical myopathy ; AM2 = case 2 affected with atypical myopathy ; C1 = control horse 1; C2 = control horse 2; C3 = control horse 3

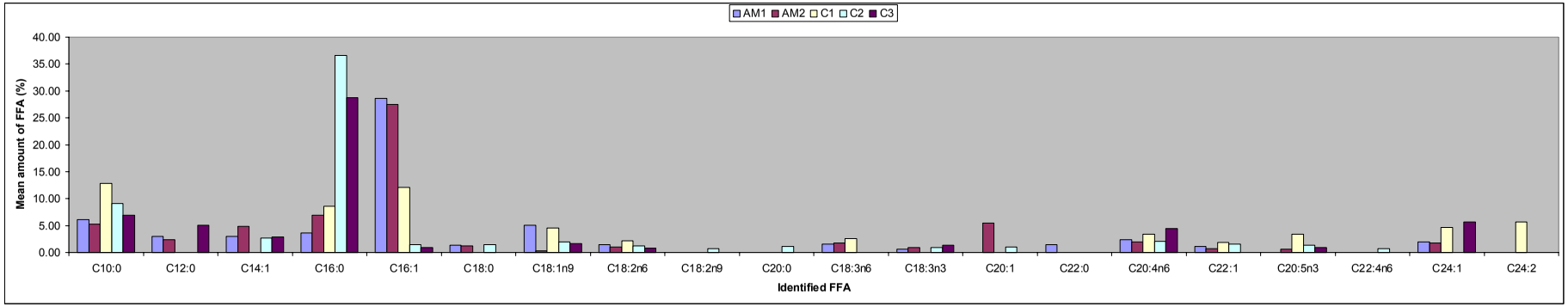
Table XIV – Free fatty acids (in µg/g muscle) in specific muscles

Muscles	C_1	C_2	C_3	Mean control horses	AM_1	AM_2	Mean AM affected horses	Ratio AM_1 vs controls	Ratio AM_2 vs controls	Ratio Mean AM vs controls
M. masseter			3 877.79	3 877.79		4 671.38	4 671.38		1.20	1.20
M. brachiocephalicus	1 454.31	179.70	1 616.59	1 083.53	2673.79	2 515.43	2 594.61	2.47	2.32	2.39
M. triceps brachii						2 367.70	2 367.70			
M. longissimus dorsi	146.85			146.85	848.75	2 472.86	1 660.81	5.78	16.84	11.31
M. quadriceps femoris	109.05	205.35		157.20	1 627.20	1 898.16	1 762.68	10.35	12.07	11.21
M. sacrocaudalis dorsalis			337.05	337.05						
M. semitendinosis	189.30	200.40	285.15	224.95		1 164.50	1 164.50		5.18	5.18
Mm. intercostales					2 470.50	4 552.62	3 511.56			
Interventricular septum					5 107.66		5 107.66			
Ventriclar free wall					5 877.24		5 877.24			

Legend: Grey = no corresponding samples between affected horses and controls; AM1 = case 1 affected with atypical myopathy ; AM2 = case 2 affected with atypical myopathy ; C1 = control horse 1; C2 = control horse 2; C3 = control horse 3

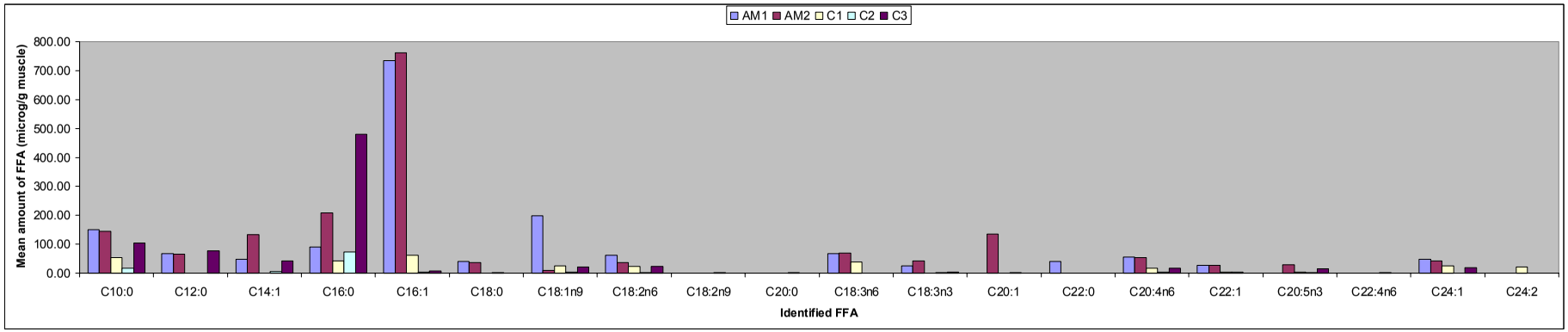
Figure 17 - Example of a chromatogram

Figure 18 - Mean of the identified FFA in % in individual horses



Legend : FFA = free fatty acids ; AM1 = case 1 affected with atypical myopathy ; AM2 = case 2 affected with atypical myopathy ; C1 = control horse 1; C2 = control horse 2; C3 = control horse 3.

Figure 19 - Mean of the identified FFA in µg/g muscle in individual horses



Legend : FFA = free fatty acids ; AM1 = case 1 affected with atypical myopathy ; AM2 = case 2 affected with atypical myopathy ; C1 = control horse 1; C2 = control horse 2; C3 = control horse 3.

Table XV – Identified free fatty acids (in µg/g muscle) in specific muscles

Identified FFA	Mean value for masseter in AM	Mean value for masseter in controls	Mean value for brachiocephalicus in AM	Mean value for brachiocephalicus in controls	Mean value for triceps in AM	Mean value for iliospinalis in AM	Mean value for iliospinalis in controls	Mean value for quadriceps in AM	Mean value for quadriceps in controls	Mean value for semitendinosus in AM	Mean value for semitendinosus in controls	Mean value for intercostales in AM	Mean value for in sacrocaud dorsalis in controls	Mean value for myocardium in AM
C10:0	184.58	263.95	116.08	94.82		107.15	16.29	97.12	21.65	56.86	18.22	255.48	23.12	224.61
C12:0	77.57	187.99	67.86	92.60		39.56		58.39		26.59	14.32	108.43	15.46	84.29
C14:1	112.85	116.49	70.29	19.20	353,85	41.43		42.03			9.18	98.17	5.77	
C16:0	366.04	454.49	169.55	169.61		182.84	8.31	52.28	47.03	42.56	37.57	242.69	29.23	115.33
C16:1	1435.70		764.80	72.00	222.03	456.45	6.06	469.89	8.41	434.00	9.21	1085.12		1355.04
C18:0	81.28		39.22	3.83		17.25		18.06		15.53	1.36	37.62		104.71
C18:1n9	20.27	29.07	75.14	37.05		13.78	6.06	126.07	4.79		6.12	39.91		575.01
C18:2n6	132.73	34.58	22.54	19.54		5.78		12.79		9.94	1.69	26.07		242.91
C18:2n9				1.78					1.48		0.82			
C20:0				2.11										
C18:3n6	196.27		17.22	37.78		2.02		17.70				43.42		205.18
C18:3n3	42.10		10.80	1.69		2.82					3.73			61.91
C20:1	22.80			1.92	246.31									
C22:0			33.87			7.84		14.37				75.82		70.90
C20:4n6	72.90	34.37	58.87	25.84		31.43	5.23	42.99	4.83	30.83	4.39	77.11	11.31	83.47
C22:1	30.99		21.12	2.55		13.02		19.62	3.62		3.48	49.08		46.07
C20:5n3	28.46			15.85			141.00		4.68		2.98			
C22:4n6									1.52					
C24:1	61.20	43.92	44.71	49.52		31.23	7.22	37.93	4.08	25.58	8.74		3.27	
C24:2				59.14			279.00		12.41		6.64			

Legend: AM: atypical myopathy affected horses; the highest amount of free fatty acid per muscle is coloured in yellow; red = AM; blue = control.

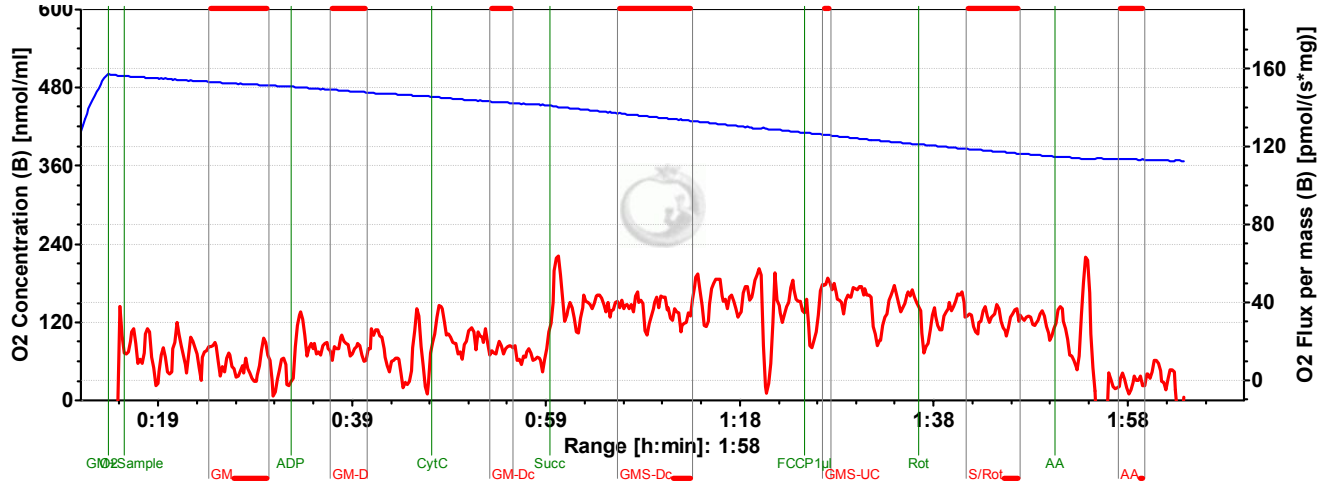
3. HIGH RESOLUTION RESPIROMETRY

From 0.6 to 4.1 mg wet weights of sample obtained by micro-biopsy were used per chamber of the oxygraph. The two muscle samples of the AM affected horse (AMa and AMb) showed a severely diminished mitochondrial respiration compared to those of the control horses. In all steps of the titration protocol, they showed a much lower O_2 flux. The most important increase in mitochondrial respiration in the AM affected horse was encountered when succinate was added as a substrate (respiratory stimulation by convergent electron flow from Complexes I+II following addition of Complex I+II substrates; Figures 20a,b and 21a,b, 22; Table XVI).

Little differences were noted between the two different samples of the affected horse (AMa and AMb). However, the respiratory capacity in the active coupled state (*i.e.* coupled OXPHOS capacity with Complex I substrates; see Table II) was slightly increased in AMb *vs*. AMa. Nevertheless, the oxygen flux remained largely below the minimum flux found in control horses following addition of Complex I substrates in presence of ADP (*i.e*. 7.9 *vs*. 23.7 $pmol \cdot s^{-1} \cdot mg^{-1}$). When the ETS was uncoupled from the OXPHOS, the respiration increased in AMa as in all control horses, showing a limiting factor by the OXPHOS. In contrast to AMa, AMb did not increase its respiration during uncoupling (Figure 22 and Table XVI).

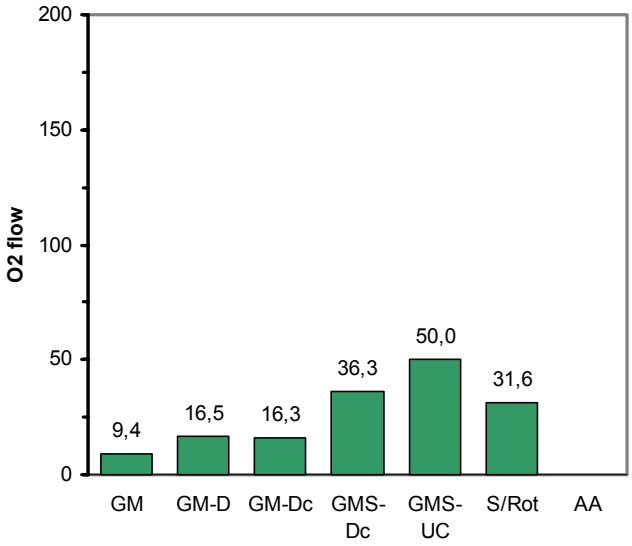
In the control horses and the AM affected horses, stimulation of respiration by adding ADP in the medium showed coupling (*i.e.* OXPHOS is functional), whereas the absence of the cytochrome *c* effect shows intactness of the outer membrane (Figure 22 and Table XVI).

Figure 20a - Respiration of permeabilized muscle fibers sampled from atypical myopathy affected horse since 3 days



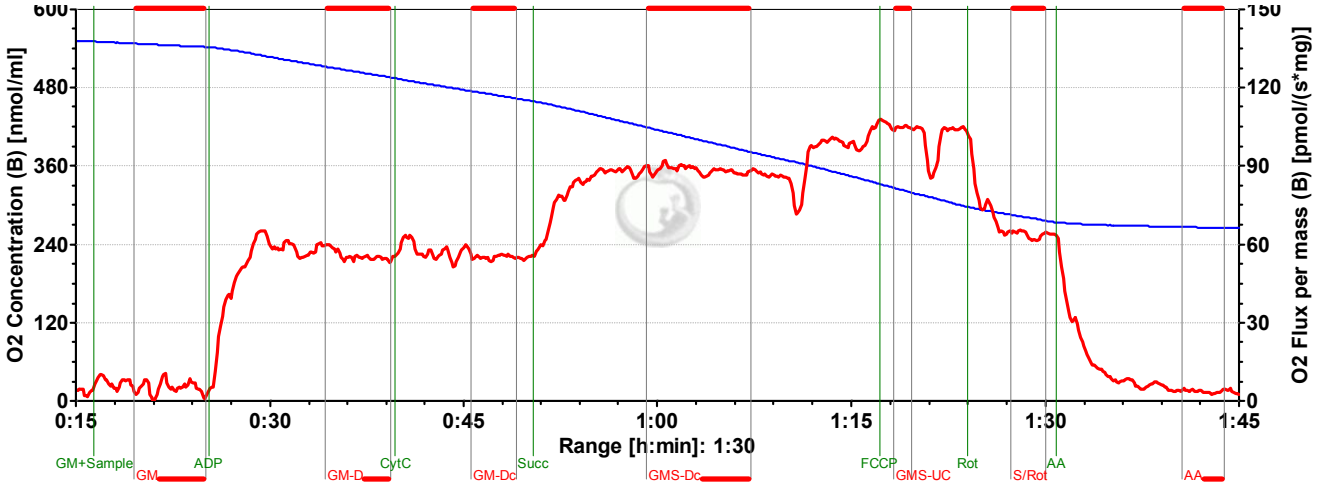
Legend: Oxygen concentration ([µM] blue line) and oxygen flux per mg wet weight of muscle ([pmol·s^{-1}·mg^{-1}] red line) in permeabilized fibers from atypical myopathy affected horse skeletal muscle with the standard titration protocol (see Table II). Events are labelled in green.

Figure 20b – Oxygen flux at steady state during the different steps of the respiration study performed with permeabilized fibers sampled from atypical myopathy affected horse since 3 days



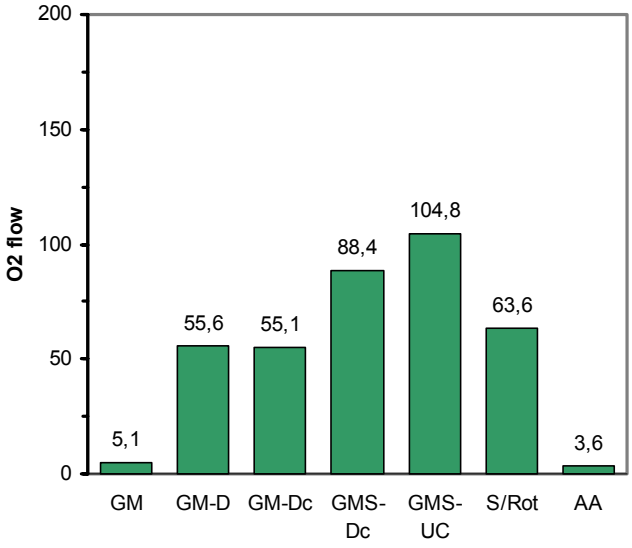
Legend: See Table II for explanation of the abbreviations.

Figure 21a - Respiration of permeabilized muscle fibers sampled from a control horse



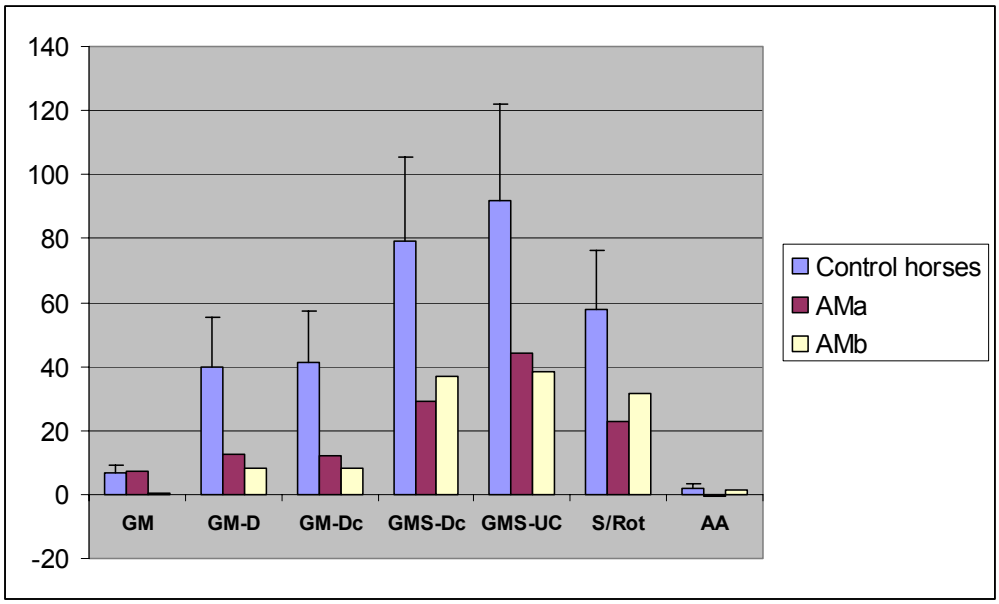
Legend: Oxygen concentration ([µM] blue line) and oxygen flux per mg wet weight of muscle ([pmol·s^{-1}·mg^{-1}] red line) in permeabilized fibers from control horse skeletal muscle with the standard titration protocol (see Table II). Events are labelled in green.

Figure 21b – Oxygen flux at steady state during the different steps of the respiration study performed with permeabilized fibers sampled from a control horse



Legend: See Table II for explanation of the abbreviations.

Figure 22 - Oxygen flux per mg wet weight of muscle (pmol·s^{-1}·mg^{-1})



Legend: AMa, AMb: atypical myopathy affected horse sampled at 3 and at 10 days, respectively, after the first clinical signs occurred; See Table II for explanation of the remaining abbreviations.

Table XVI - Comparison of the oxygen flux per mg wet weight of muscle (pmol·s^{-1}·mg^{-1}) between the horses

	GM	GM-D	GM-Dc	GMS-Dc	GMS-UC	S/Rot	AA
Control horses							
Mean	6.8	39.9	41.1	79.4	91.8	57.7	1.9
SD	2.6	15.3	16.2	26.1	30.1	18.6	1.5
Minimum	3	23.7	22.1	44.8	65.8	36.9	-0.2
Maximum	10.8	76.2	81.2	143.4	164.9	98.3	3.7
Atypical myopathy affected horse							
AMa	7.3	12.7	11.9	29.1	44.1	23	-0.7
AMb	0.6	7.9	8.3	37.1	38.3	31.5	1.6
Ratios versus controls							
AMa		32%	29%	37%	48%	40%	
AMb		20%	20%	47%	42%	55%	

Legend: AMa, AMb: atypical myopathy affected horse sampled at 3 and at 10 days, respectively, after the first clinical signs occurred; SD = standard deviation; See Table II for explanation of the remaining abbreviations.

DISCUSSION

Most results of this epidemiological study on European cases were in accordance with previous studies (Votion *et al.*, 2007b; Votion *et al.*, 2008) on confirmed Belgium cases. However, some important differences have been remarked. Firstly, the outbreaks during this study period were less devastating than previously reported outbreaks (Puyalto-Moussu *et al.*, 2004; Votion *et al.*, 2004) with a larger number of survivors (the overall survival rate during the studied period was 28% *vs* 15% (Puyalto-Moussu *et al.*, 2004; Votion *et al.*, 2004)). This study also describes fewer horses found dead before showing clinical signs (0% *vs* 5%), fewer recumbent horses (64% *vs* 96%) and a longer survival time (until 10 days *vs* 3-72 H) than in previous reports (Hosie *et al.*, 1986; Whitwell *et al.*, 1988; Brandt *et al.*, 1997; Votion *et al.*, 2007b). These differences might be explained by a less severe affection of horses during the study period compared to other years, and/or by geographic differences. Indeed, geographic differences concerning survival rate were revealed, demonstrated by the high survival rate in France during the autumn of 2006. They might be explained by different pasture characteristics and management between countries.

Secondly, also demographic differences have been remarked. In the past, mostly young horses were reported to be affected by AM (Hosie *et al.*, 1986; Whitwell *et al.*, 1988; Brandt *et al.*, 1997; Palencia and Rivero, 2007; Votion *et al.*, 2007b; Votion *et al.*, 2008), but the current study population consisted of as well young as old horses and no risk factor for young horses was demonstrated in this study. In a previous study (Votion *et al.*, 2008), age was presumed to be a confounding factor for the increased risk for stallions, but in the current study without the presence of an age predisposition the sex predisposition still remains visible; stallions were more and mares less at risk for the development of the condition. Poor or normal body condition could neither be confirmed as a risk factor for the development of AM as previously described (Votion *et al.*, 2007b; Votion *et al.*, 2008), however obesity was found to be a positive prognostic factor on the outcome. Some affected horses were extremely muscular. This parameter was not established on every case, but some horses seen at Liege University were in very good body condition and were extremely muscular considering their age. The present study described for the first time clinically affected donkeys and zebra's. To date, no proof has been provided for AM affecting other species than equidae although a deer was present in the LP group. Equidae are known to be more susceptible for certain toxins (*i.e.* mycotoxins and clostridial toxins) than other species (Green *et al.*, 1994; Osweiler, 2001; Renninger and Hooser, 2003).

New clinical aspects of AM have been demonstrated by studying the HP/CC group: hyperthermia was more often present than in previous studies (Votion *et al.*, 2007b), quite some cases showed a delayed transit (mucus covered faeces, colon impaction) and some males showed a penile prolapse. The presence of stiffness and high CK activities were important clinical parameters to differentiate between AM from conditions resembling AM. Since the first measured CK activities in AM affected horsed were not always

that much increased, it is recommended in case of low CK activity at the beginning of the clinical signs to repeat the analysis 24H later. In literature, one horse had been described to be stabled for a day when the first clinical signs of AM were noted (Votion *et al.*, 2007b). The present study showed two horses being stabled for 2 days before showing clinical signs. Thus it can be suggested that the causative agent needs several days to exert its action. Moreover, this study confirmed that horses need to be on the specific pasture for at least one week to be affected by AM (Votion *et al.*, 2007b).

Also some differences concerning management of horses and their pastures and pasture characteristics were revealed: the presence of trees was identified as a new risk factor, and several previously determined risk or protective factors were not confirmed by this study (no risk factor: the presence of a slope on pasture; no protective factor: regular vaccination and deworming). Unfortunately, for several other parameters the statistical analysis was impossible due to the poor amount of answers.

This is the first study describing survivors of AM. Unexpectedly, they recover uneventful and show surprisingly little muscle wastage regarding the severity of the myopathy. Knowing that AM affected horses have a chance to survive without apparent sequellae is of great importance for equine practitioners with respect to diagnosis, prognosis and treatment. Previously, normal respiration with normal PaO_2 levels had been identified as a possible positive prognostic factor, although on fatal cases (Votion *et al.*, 2007b). In most described cases of the current study PaO_2 levels were not measured and normal respiration could not be confirmed as positive prognostic factor due to a lack of answers. Nevertheless, normal mucosae, remaining standing for most of the time and obesity were identified as positive prognostic factors, whereas recumbency was a negative prognostic factor. Serum activities of CK had a tendency to be higher in survivors than in non-survivors, confirming previous observations that CK is not a liable prognostic indicator (Votion *et al.*, 2007b). Differences between survivors and non-survivors concerning pasture characteristics and management can be interpreted as risk or protective factors. Indeed, an interesting difference between pastures was marked: pastures of survivors contained more dead wood than those of non-survivors. Dead wood was described in other reports to be frequently present on pastures where AM cases declare (Votion *et al.*, 2007b; Votion *et al.*, 2008), suggesting that it is of direct or indirect importance for the causative agent. It might be possible that survivors had been more in contact with the causative agent due to the presence of dead wood and have developed certain immunity.

Previous studies on affected cases have suggested a dysfunction in mitochondrial lipid metabolism (Cassart *et al.*, 2007; Votion *et al.*, 2007b; Westermann *et al.*, 2007; Westermann *et al.*, 2008b). In human medicine, several inborn disorders in mitochondrial lipid metabolism are described (Nyhan, 2005). Clinically, they present with myopathies and cardiomyopathies, and sometimes liver involvement is noticed (Nyhan, 2005). They can originate from the different steps in the carnitine transfer,

or from fatty acid β oxidation. During the β oxidation two enzymes may be deficient: acyl-CoA dehydrogenases and HAD. Problems can also occur when the electrons produced during the β oxidation enter the ETS with ETF and ETF:QO or when the produced acetyl-CoA enters the Krebs cycle (Nyhan, 2005). In any condition in which FFA accumulate, esterifications with carnitine and accumulation of acyl-carnitines takes place (Nyhan, 2005). Inborn errors cause not necessarily clinical problems in newborns and may only lead to the development of clinical signs later on in life (Nyhan, 2005). Since AM occurs only in pasturing horses of all breeds (Votion *et al.*, 2004; Votion *et al.*, 2007b; Votion *et al.*, 2008) and seen its seasonal character (Hosie *et al.*, 1986; Whitwell *et al.*, 1988; Palencia and Rivero, 2007; Votion *et al.*, 2007b), the metabolic problem in AM would probably not concern an inborn error, but an acquired one.

Indeed, the present study demonstrated a severe problem in fatty acid metabolism. A smaller amount of triglycerides and a larger amount of monoglycerides were visualised at the TLC in affected muscles compared to control muscles. Triglycerides consist of FFA bound to glycerol and limited amounts are stored in muscle cells as energy source (Lehninger *et al.*, 1993d; MacLeay, 2004). Seen the important energy deficit in affected horses (as suggested by the accumulation of AMP metabolites in urine and muscle glycogen depletion (Westermann *et al.*, 2008a)), the stored triglycerides probably were degraded into FFA and glycerol with monoglycerides as intermediates. Also a severe increase in muscular FFA was found in the affected group. In normal muscle cells a small amount of FFA is stored (Nawrocki *et al.*, 1999). In AM affected horses, the important increase in sarcoplasmatic FFA probably is derived from degraded intracellular stored triglycerides, maybe even from catabolism of structural lipids (phospholipids of membranes), and most certainly from the FFA liberated in the bloodstream by adipocytes secondary to the energy demand. The latter corresponds to the frequently encountered hyperlipaemia (Delguste *et al.*, 2002; Votion *et al.*, 2004; Votion *et al.*, 2007b). Since the FFA cannot be metabolised further, they accumulate in the sarcoplasm, giving rise to dramatic amounts of lipid droplets visualised on histology by Oil red O staining (Cassart *et al.*, 2007). Strangely enough, no difference was present in the amount of total muscular lipids between the groups. The increase in FFA (in µg/gram muscle) was too small to make a difference on total lipids (in mg/gram muscle). Nevertheless, still important lipid accumulations are visible on histology in affected horses in contrast to normal horses. The affinity for neutral lipids (FFA and their derivatives) and the poor coloration of structural fats and fats of other classes of the Oil red O staining (Bayliss High and Lake, 1990) and a possible increase in FFA from catabolism of structural lipids might account for this phenomenon. It would be interesting to perform specific histological stainings for different lipid classes on future muscle samples of affected horses to confirm this hypothesis.

The present study shows no significant changes between *the FFA profiles* of the chromatograms of the different horses. This means that they have the same FFA in their muscles, however occurring in

different percentages and quantities. The minor differences in profiles between the horses can probably be explained by different feeding practices as the composition of accumulated lipids in skeletal muscle has been reported to be highly reflective of dietary fat (Andersson *et al.*, 2002; Perez-Palacios *et al.*, 2007). In human beings however, it is rare to find long chain FFA longer than 20 carbons (Kotani *et al.*, 2000). Their presence in all of the studied horses might be a species specific finding.

However, important differences have been noted in *quantities of FFA*, where AM affected muscles contained more FFA than control muscles. In controls, palmitic acid (C16:0) was almost always the most abundant FFA. Palmitic acid is the precursor of other long-chain FFA (Lehninger *et al.*, 1993c) and has been reported to be a major FFA in human plasma (Mehta *et al.*, 1998) and in skeletal muscle tissue of rats (Gorski *et al.*, 1998). An explosive increase in palmitoleic acid (C16:1) was demonstrated in affected muscles. Palmitoleic acid is also a major FFA in human plasma (Mehta *et al.*, 1998) and is the desaturated version of palmitic acid, containing a double bond between C9 and C10. Palmitoleic acid has been reported to increase in human plasma with dyslipaemia (Zak *et al.*, 2000) and in plasma, liver and muscle tissue of obese rats (Fukuchi *et al.*, 2004). Besides these relations with pathologies of lipid metabolism, changes in muscular FFA content and composition of blood FFA correlate with insulin resistance and impaired glucose disposal in skeletal muscle. However, contradictory results with respect to the effects of palmitoleic acid on insulin action have been reported; it may promote insulin resistance (Fukuchi *et al.*, 2004), or enhance insulin-stimulated glucose transport (Dimopoulos *et al.*, 2006). Interestingly, impaired insulin sensitivity influenced FFA desaturase activity (Vessby *et al.*, 2002). In AM, pathological metabolic situations like hyperlipaemia, hyperglycaemia and a suspicion of insulin resistance are present (Delguste *et al.*, 2002; Votion *et al.*, 2004; Votion *et al.*, 2007b). Thus the increase of palmitoleic acid is in accordance with observations in human beings and rats, although they reflect more chronic processes. Nevertheless, a significant increase of FFA has been already observed after 7 hours fasting, showing the possibility of fast occurring changes in plasmatic FFA (Kotani *et al.*, 2000).

The muscles with the highest amount of FFA, as well in affected as control horses, are all muscles known to have a high amount of fiber type I (*M. masseter*, *Mm. intercostales* and myocardium). Interestingly, the *M. masseter* showed the lowest increase in muscular FFA when comparing control horses to affected horses. In rats, the same phenomenon has been observed secondary to a pathological increase in plasma glucose: *more important amounts* of FFA in muscles with a high proportion of type I fibers, but a *less important increase* of FFA by these muscles (Nawrocki *et al.*, 1999). These type I fibers depend largely on aerobic metabolism of glucose and fatty acids for their energy (MacLeay, 2004). As mentioned in the introduction, AM affects mostly type I fibers, being respiratory and postural muscles (Brandt *et al.*, 1997; Cassart *et al.*, 2007; Palencia and Rivero, 2007).

Recently, the metabolic defect occurring in AM was defined as MADD based on characteristic profiles of organic acids and acyl-carnitines in urine, plasma and muscle tissue, and the finding of deficiencies of short and medium chain acyl-CoA dehydrogenases and isovaleryl dehydrogenase (Westermann *et al.*, 2007; Westermann *et al.*, 2008b). The acyl-carnitines measured in these studies are acyl-fatty acid chains bound to carnitine that already partially have been cut in smaller chains in the mitochondria. In a normal functioning β oxidation the chains are cut in portions of 2 carbons. Due to the deficiency they are cut in larger pieces, resulting in an increased amount of short and medium chain acyl-carnitines. The deficient cutting explains the presence of uneven acyl-carnitine chains, usually not present in mammals, and the binding of carnitine with short chain fatty acids (FFA smaller than 12 carbons can enter the mitochondria freely (Lehninger *et al.*, 1993e)). The latter studies looked at fatty acid metabolism by a very different approach than the present study, where all muscular FFA have been measured. The chloroform, used to dissolve the lipids in the present study, breaks connections between lipids and proteins, thus including the acyl-fatty acids chains, which are now deconnected from carnitine, in the measured muscular FFA. Due to the different approach between these studies, not the same lipid classes were studied and thus not the same lengths of fatty acid chains were increased.

Additional metabolic deficiencies described about AM are: increased uric acid excretion in urine due to degradation of AMP (demonstrating ATP depletion), glycogen depletion, increase of glycolytic enzymes except a very low activity of the enzyme phosphoglycerate mutase (PGAM) in one horse (Westermann *et al.*, 2008a). The amount of mitochondria was not changed (concluded by a normal amount of citrate synthase) (Westermann *et al.*, 2008a), although the overall oxidative potential of myofibers was weaker as demonstrated by NADH tetrazolium reductase (NADH-TR) (Cassart *et al.*, 2007) and the histochemical assay for succinate dehydrogenase (SDH) (Palencia and Rivero, 2007). Moreover, with electronic microscopy also ultrastructural changes of the mitochondria have been visualised (Cassart *et al.*, 2007). Results of the histochemical staining with NADH-TR are related to the mitochondrial respiratory Complex I and/or mitochondrial alterations, whereas weak staining with SDH suggests biochemical defects associated with the mitochondrial respiratory Complex II and/or mitochondrial alterations.

With HRR, a severely diminished activity of the mitochondrial respiration of the affected horse was found in every titration step. This means that Complex I and Complex II showed lowered activity, although Complex I seemed more severely affected than Complex II. On the contrary, in a previous report, the activity of Complex II was reported to be the most decreased (59% of control horses) compared to the activities of the other complexes (75% of control horses) (Westermann *et al.*, 2008a). However, the spectophotometric methods used to measure the activity of the complexes assess activity of individual complexes rather than in an integrated manner like HRR does.

Westermann and collaborators (2007, 2008b) hypothesised a FAD-dependent dysfunction seen the decreased activity of several dehydrogenases, which all utilized FAD as cofactor (including acyl-CoA dehydrogenases and isovaleryl dehydrogenase). In this hypothesis, the diminished activity of Complex II would result from a dysfunction of its FAD prosthetic group (electrons move from succinate to FAD, then to coenzyme Q which passes electrons to Complex III). Such FAD-dependent dysfunction might result from a riboflavin deficiency. Riboflavin, also known as vitamin B_2, is a precursor in the synthesis of FAD and flavin mononucleotide (FMN), another flavin nucleotide, both required as coenzymes by flavoproteins. No riboflavin deficiency was present in AM affected horses, but the possibility of a competition between riboflavin and a toxin can not be ruled out (Westermann *et al.*, 2008b). However, not all FAD-dependant enzymes are deficient in AM; the activities of ETF and the ETF:QO were measured to be normal (Westermann *et al.*, 2007). Moreover, the switch of palmitic acid to its desaturated form palmitoleic acid demonstrated by the present study suggests that FFA desaturases, which are also FAD dependant (Lehninger *et al.*, 1993c), function correctly. It is worth noting that Complex I itself is a flavoprotein, but which uses FMN rather than FAD as cofactor. Several flavoproteins are even known to impair electron transfer in the ETS, some of them demonstrating a stronger inhibition activity for Complex I than Complex II (Hodnick *et al*., 1994). Further researches should explore the presence of these compounds in samples collected on AM cases and/or in their environment.

In time, small alterations were visible in the affected horse. AMb showed an increase in O_2 flux by Complex II, whilst no change was found in Complex I compared to AMa. This means that at 10 days after the first signs of AM were noticed, the horse started to regain some, although minor, activity of Complex II, while at a clinical point of view he had severely deteriorated. In the future, HRR can potentially be used as a clinical guide for decision making in AM, since ameliorations in mitochondrial respiration measured by HRR will sooner be visible than clinical improvement. The time between muscular sampling and obtaining results is reasonable for clinical decision making. Despite, it cannot be excluded that even when the mitochondrial respiration ameliorates, negative secondary effects continue to cause damage possibly leading to the death of the horse. Further research with a follow up in time of several cases affected with AM should test this hypothesis. Survivors of AM have been described in the epidemiological part of the study and recovery was noted after 2 to 30 days (mean 11.1 ± 6.9 days) since the onset of clinical signs. It can be postulated that survival occurs when the action of the etiological cause of AM has elapsed, is destroyed or inactivated, or when its pathological effects has been bypassed by the body. This acute, severe myopathy resembles certain clostridial toxin and/or mycotoxin induced diseases, where also a certain time span is needed to overcome the effects of the toxin and where recovery can be uneventful as well (Osweiler, 2001; Renninger and Hooser, 2003; Van Galen *et al.*, 2008a).

With the present epidemiological and pathophysiological study, some new aspects concerning treatment and prevention can be proposed. Treatment has already been set out in the introduction and only new aspects will be discussed. Seen the severe defects in mitochondrial lipid metabolism occurring in AM affected horses, but not in carbohydrate metabolism, it might be beneficial to enhance carbohydrate metabolism by supplementing carbohydrates and stimulating insulin sensitivity. Carbohydrate supplementation can be performed by adapting nutrition or by glucose administration. Most horses keep a good appetite; some of them are even craving for food (Votion *et al.*, 2007b). In those cases, a reduced quantity of fat and a high quantity of carbohydrates (for example concentrates with molasses, grains, corn, fruits, honey, sugar water, sugar blocks, fresh grass) might be beneficial. Small amounts of food frequently over the day should prevent peaks in hyper- and/or hypoglycaemia and fasting should be avoided at all times. Exaggeration, however, should not be encouraged since carbohydrate overload can cause deleterious effects (endotoxaemia, gas distension, diarrhoea, laminitis). Especially horses not used to digest large amounts of carbohydrates are at risk for these complications (Jones, 2004). Increasing carbohydrate content is contradictory to nutritional advice for several other equine myopathies, proposing a decrease of carbohydrates and an increase of lipid content (MacLeay, 2004). If horses are not eating well, or when hyperlipaemia and hyperglycaemia maintain, glucose can be administrated orally or intravenously (Barton, 2004). At the same time, glucose also supports the affected hepatic metabolism (Hosie *et al.*, 1986; Whitwell *et al.*, 1988; Brandt *et al.*, 1997; Votion *et al.*, 2004) by decreasing the need for hepatic gluconeogenesis (Barton, 2004). Seen the hyperglycaemia (Hosie *et al.*, 1986; Whitwell *et al.*, 1988; Brandt *et al.*, 1997; Votion *et al.*, 2007b), the diminished or depleted intrasarcoplasmatic glycogen stores (Westermann *et al.*, 2008a), the increased activity of carbohydrate metabolism (Westermann *et al.*, 2008a) and the energetic dysbalance, a certain degree of insulin resistance is suspected to occur in AM-affected horses. To stimulate insulin sensitivity and thus the glucose uptake in skeletal muscle, insulin can be administrated under regular control of the serum glucose levels (Bertone and Horspool, 2004). Several human products, as chromium (III) or metformin, have been described to enhance insulin sensitivity, but conflicting results of efficacy are reported in horses (Firshman and Valberg, 2007; Anderson, 2008). On the contrary, corticosteroids and alpha2-agonists induce insulin resistance (Firshman and Valberg, 2007) and thus should be avoided. Heparin can be administrated to further control the hyperlipaemia by potentiating lipoprotein lipase activity (Barton, 2004). This might increase triglyceride removal from the blood back into adipose tissue, but probably lipoprotein lipase is already at maximum rate, as is the case in hyperlipaemic poneys, and additional benefit of heparin can be questioned (Barton, 2004).

Following the hypothesis of MADD due to a riboflavin blockage, a supplementation of affected horses with this vitamin B2 (via B-complex vitamin supplementation) might be usefull (Westermann *et al.*, 2007; Westermann *et al.*, 2008b). The daily requirements of a healthy mature horse for riboflavin are

12 mg, and good-quality hay, alfalfa or pasture normally contain largely enough (Rooney, 2004). Dependant on the severity of AM, the requirements of riboflavin in affected horses probably exceed those for healthy horses.

Special attention should be given to the digestive system as several horses with colic signs and diminished transit were reported in this study and recently by Sherlock and Mair (2008) (Sherlock and Mair, 2008). Passage of electrolytes and paraffin per nasogastric tube and offering good quality of fibers might avoid further problems.

Some preventive methods have been highlighted by this study. Seen that horses can be affected by the condition until two days being stabled, one can estimate horses being save from the condition once they have been stabled for at least 3 days. When not enough boxes are available to stable all horses during an outbreak, it is advised to give priority to not working horses, young and older horses and to stallions. Complementation of horses with food during the risk periods of AM decreases the risk at AM.

This study has several limitations. Since also non-confirmed or non-infirmed cases were included in the epidemiological study and since the files of the studied cases were not always complete, categorizing the horses was sometimes difficult. Probably, some horses were AM affected, but did not end up in the CC or HP category due to lacking information. The reverse is also possible: cases not being AM affected, but nevertheless entering the HP group because of the presence of a myopathy induced by another cause. A second limitation is the underestimation of the real number of European cases. One explication for this underestimation is the rapid death of AM cases (Hosie *et al.*, 1986; Whitwell *et al.*, 1988; Brandt *et al.*, 1997; Votion *et al.*, 2007b), which complicates establishing a clinical diagnosis. Another important reason for the underestimation of the disease is that not every AM affected case has been communicated. Co-grazing horses can be subclinically affected and are per definition grazing on the same premises as affected horses, which makes them less ideal objects for a control group regarding pasture characteristics and management, but more ideal concerning demographic data. To demonstrate other differences between groups and with more certainty, a larger study population is required. Especially for the HRR other cases should be used to confirm the findings of this study.

In conclusion, the epidemiological part of this study demonstrates a less devastating outbreak than previously reported, together with some new clinical presentations of the condition. The pathophysiological part confirmed the severe mitochondrial dysfunctions in the β oxidation of the FFA and in Complex I and II of the ETS. Further research is necessary to find the cause of AM and to better understand its pathophysiology in order to establish an effective treatment and prevention.

REFERENCES

ANDERSON R.A. Chromium and polyphenols from cinnamon improve insulin sensitivity. *Proc. Nutr. Soc.*, 2008, 67, 48-53.

ANDERSSON A., NALSEN C., TENGBLAD S., VESSBY B. Fatty acid composition of skeletal muscle reflects dietary fat composition in humans. *Am. J. Clin. Nutr.*, 2002, 76, 1222-1229.

ANONYMOUS Atypical myoglobinuria: a new disease in horses? *Vet. Rec.*, 1985, 116, 86-87.

BARTON M.B. Disorders of the liver. In: 2nd edn., Sellon, D.C., Reed, S.M., Bayly, W.M. (Eds.), Equine internal medicine, Saunders: Philadelphia, 2004, 951-994.

BAYLISS HIGH O.B., LAKE B.D. Lipids. In: 3rd edn., Stevens, A., Bancroft, J.D.(Eds.), Theory and practice of histological techniques, Churchill Livingstone : New York, Edinburgh, 1990, 213-242.

BEDRY R., BAUDRIMONT I., DEFFIEUX G., CREPPY E.E., POMIES J.P., RAGNAUD J.M., DUPON M., NEAU D., GABINSKI C., DE WITTE S., CHAPALAIN J.C., GODEAU P. Wild-mushroom intoxication as a cause of rhabdomyolysis. *N. Engl. J. Med.*, 2001, 345, 798-802.

BERTONE J., HORSPOOL L.J.I. Equine clinical pharmacology. Saunders: Edinburgh ; Toronto, 2004, 397.

BRANDT K., HINRICHS U., GLITZ F., LANDES E., SCHULZE C., DEEGEN E., POHLENZ J., COENEN M. Atypical myoglobinuria in grazing horses. *Pferdeheilkunde*, 1997, 13, 27-34.

CASSART D., BAISE E., CHEREL Y., DELGUSTE C., ANTOINE N., VOTION D., AMORY H., ROLLIN F., LINDEN A., COIGNOUL F., DESMECHT D. Morphological alterations in oxidative muscles and mitochondrial structure associated with equine atypical myopathy. *Equine Vet. J.*, 2007, 39, 26-32.

DAGNELIE P. Statistique théorique et appliquée. In : 2nd edn., Larcier, D.B. (Eds), Inférence statistique à une et à deux dimensions , 1998, 1-659.

DELGUSTE C., CASSART D., BAISE E., LINDEN A., SCHWARZWALD C., FEIGE K., SANDERSEN C., ROLLIN F., AMORY H. Atypical myoglobinuria in grazing horses: an outbreak in Belgium. *Annales De Medecine Veterinaire*, 2002, 146, 235-247.

DIMOPOULOS N., WATSON M., SAKAMOTO K., HUNDAL H.S. Differential effects of palmitate and palmitoleate on insulin action and glucose utilization in rat L6 skeletal muscle cells. *Biochem. J.*, 2006, 399, 473-481.

FINNO C.J., VALBERG S.J., WUNSCHMANN A., MURPHY M.J. Seasonal pasture myopathy in horses in the midwestern United States: 14 cases (1998-2005). *J. Am. Vet. Med. Assoc.*, 2006, 229, 1134-1141.

FIRSHMAN A.M., VALBERG S.J. Factors affecting clinical assessment of insulin sensitivity in horses. *Equine Vet. J.*, 2007, 39, 567-575.

FUKUCHI S., HAMAGUCHI K., SEIKE M., HIMENO K., SAKATA T., YOSHIMATSU H. Role of fatty acid composition in the development of metabolic disorders in sucrose-induced obese rats. *Exp. Biol. Med.*, 2004, 229, 486-493.

GERBER V., STRAUB R., FREY J. Equine botulism and acute pasture myodystrophy: new soil-borne emerging diseases in Switzerland? *Schweiz. Arch. Tierheilkd.*, 2006, 148, 553-559.

GNAIGER E., KEIZNETSOV A.V., SCHNEEBERGER S., SEILER R., BRANDACHER G., STREURER W., MARGREITER R. Mitochondria in the cold. In: Heldmaier, G., Klingenspor, M. (Eds.), Life in the cold: Springer, Heiderlberg, Berlin, New York, 2000, 431-442.

GORSKI J., NAWROCKI A., MURTHY M. Characterization of free and glyceride-esterified long chain fatty acids in different skeletal muscle types of the rat. *Mol. Cell. Biochem.*, 1998, 178, 113-118.

GREEN S.L., LITTLE C.B., BAIRD J.D., TREMBLAY R.R.M., SMITHMAXIE L.L. Tetanus in the horse - a review of 20 cases (1970 to 1990). *J. Vet. Intern. Med.*, 1994, 8, 128-132.

GRENIER B. Utilisation des ODDS dans les tests associés ou séquentiels. In : Masson (Eds.), Décision médicale: analyse et stratégie de la décision dans la pratique médicale: Paris, 1990, 102-106.

GUYTON A.C., HALL J.E. Lipid metabolism. In: 11th edn., Guyton A.C., Hall J.E (Eds.), Textbook of medical physiology, Elsevier Saunders: Philadelphia, 2006a, 840-851.

GUYTON A.C., HALL J.E. Metabolism of carbohydrates, and formation of Adenosine Triphosphate. In: 11th edn., Guyton A.C., Hall J.E (Eds.), Textbook of medical physiology, Elsevier Saunders: Philadelphia, 2006b, 829-839.

GUYTON A.C., HALL J.E. Sports physiology In: 11th edn., Guyton A.C., Hall J.E (Eds.), Textbook of medical physiology, Elsevier Saunders: Philadelphia, 2006c, 1055-1066.

HOSIE B.D., GOULD P.W., HUNTER A.R., LOW J.C., MUNRO R., WILSON H.C. Acute myopathy in horses at grass in East and South East Scotland. *Vet. Rec.*, 1986, 119, 444-449.

JONES S.L. Inflammatory diseases of the gastrointestinal tract causing diarrhoea. In: 2nd edn., Sellon, D.C., Reed, S.M., Bayly, W.M. (Eds.), Equine internal medicine, Saunders: Philadelphia, 2004, pp 884-913.

KOTANI A., FUSE T., KUSU F. Determination of plasma free fatty acids by high-performance liquid chromatography with electrochemical detection. *Anal. Biochem.*, 2000, 284, 65-69.

LEHNINGER A.L., NELSON D.L., COX M.M. The Citric Acid Cycle. In: 2nd edn., Lehninger, A.L., Nelson, D.L., Cox, M.M. (Eds.), Principles of biochemistry, Worth Publishers: New York, 1993a, 447-478.

LEHNINGER A.L., NELSON D.L., COX M.M. Glycolysis and the catabolism of hexoses. In: 2nd edn., Lehninger, A.L., Nelson, D.L., Cox, M.M. (Eds.), Principles of biochemistry, Worth Publishers: New York, 1993b, 400-445.

LEHNINGER A.L., NELSON D.L., COX M.M. Lipid biosynthesis. In: 2nd edn., Lehninger, A.L., Nelson, D.L., Cox, M.M. (Eds.), Principles of biochemistry, Worth Publishers: New York, 1993c, 642-687.

LEHNINGER A.L., NELSON D.L., COX M.M. Lipids. In: 2nd edn., Lehninger, A.L., Nelson, D.L., Cox, M.M. (Eds.), Principles of biochemistry, Worth Publishers: New York, 1993d, 240-267.

LEHNINGER A.L., NELSON D.L., COX M.M. Oxidation of fatty acids. In: 2nd edn., Ed: Lehninger, A.L., Nelson, D.L., Cox, M.M., Principles of biochemistry, Worth Publishers: New York, 1993e, 479-505.

LEHNINGER A.L., NELSON D.L., COX M.M. Oxidative phosphorylation and photophosphorylation. In: 2nd edn., Lehninger, A.L., Nelson, D.L., Cox, M.M. (Eds.), Principles of biochemistry, Worth Publishers: New York, 1993f, 542-597.

LEHNINGER A.L., NELSON D.L., COX M.M. Principles of bioenergetics. In: 2nd edn., Lehninger, A.L., Nelson, D.L., Cox, M.M. (Eds.), Principles of biochemistry, Worth Publishers: New York, 1993g, 359-399.

LEMIEUX H., VOTION D.M., GNAIGNER E. Mitochondrial respiration in permeabilized muscle fibers: needle biopsy from horse skeletal muscle. *Mitochondrial Physiology Network 12*, 2007, 23, 1-4.

LETELLIER T., MALGAT M., COQUET M., MORETTO B., PARROTROULAUD F., MAZAT J.P. Mitochondrial myopathy studies on permeabilized muscle-fibers. *Pediatr. Res.*, 1992, 32, 17-22.

MACLEAY J.M. Diseases of the muskuloskeletal system. In: 2nd edn., Sellon, D.C., Reed, S.M., Bayly, W.M. (Eds.), Equine internal medicine, Saunders: Philadelphia, 2004, 461-522.

MEHTA A., OESER A.M., CARLSON M.G. Rapid quantitation of free fatty acids in human plasma by high-performance liquid chromatography. *J. Chromatogr. B. Biomed. Appl.*, 1998, 719, 9-23.

NAWROCKI A., GORSKA M., ZENDZIAN-PIOTROWSKA M., GORSKI J. Effect of acute streptozotocin diabetes on fatty acid content and composition in different lipid fractions of rat skeletal muscle. *Horm. Metab. Res.*, 1999, 31, 252-256.

NAWROCKI A., GORSKI J. Effect of plasma free fatty acid concentration on the content and composition of the free fatty acid fraction in rat skeletal muscles. *Horm. Metab. Res.*, 2004, 36, 601-606.

NOUT Y.S., REED S.M. Management and treatment of the recumbent horse. *Equine Vet. Ed.*, 2005, 17, 324-336.

NYHAN W.L. Disorders of fatty acid oxidation. In: 2nd edn., Nyhan, W.L., Barshop, B.A., Ozand, P.T. (Eds.), Atlas of metabolic diseases, Hodder Arnold, 2005, 241-299.

OSWEILER G.D. Mycotoxins. *Vet. Clin. North Am. Equine Pract.*, 2001, 17, 547.

PALENCIA P., RIVERO J.L.L. Atypical myopathy in two grazing horses in northern Spain. *Vet. Rec.*, 2007, 161, 346-348.

PEREZ-PALACIOS T., ANTEQUERA T., MURIEL E., MARTIN D., RUIZ J. Stereospecific analysis of phospholipid classes in skeletal muscle from rats fed different fat sources. *J. Agric. Food Chem.*, 2007, 55, 6191-6197.

PUYALTO-MOUSSU C., SAISON A., LECONTE D. Myoglobinurie atypique: épidémiologie de cas Français de myopathie aigue. *Pratique Veterinaire Equine*, 2004, 36, 29-35.

RENNINGER M.L., HOOSER S.B. Botulism. Robinson, N.E. (Eds.), Current therapy in equine medicine 5, Saunders: Philadelphia, 2003, 799-801.

ROONEY D.K. Applied nutrition. In: 2nd edn., Sellon, D.C., Reed, S.M., Bayly, W.M. (Eds.), Equine internal medicine, Saunders: Philadelphia, 2004, 235-272.

SHERLOCK C.E., MAIR T.S. Concurrent atypical myoglobinuria and colon displacement in a filly. *Equine Vet. Ed.*, 2008, 20, 228-233.

VAN DER KOLK H. Equine atypical myopathy. *Tijdschr. Diergeneeskd.*, 2006, 131, 934-934.

VAN GALEN G., DELGUSTE C., SANDERSEN C., VERWILGHEN D., GRULKE S., AMORY H. Tetanus in the equine species: a retrospective study of 31 cases. *Tijdschr. Diergeneeskd.*, 2008a, in press.

VAN GALEN G., SERTEYN D., AMORY H., VOTION D.M. Approche diagnostique des myopathies chez le cheval. *Le nouveau praticien equine*, 2008b, 15, 25-31.

VAN GALEN G., SERTEYN D., AMORY H., VOTION D.M. Atypical myopathy: New insights into the pathophysiology, prevention and management of the condition. *Equine Vet.Ed.*, 2008c, 20, 234-238.

VESSBY B., GUSTAFSSON I.B., TENGBLAD S., BOBERG M., ANDERSSON A. Desaturation and elongation of fatty acids and insulin action. *Lipids and Insulin Resistance: the Role of Fatty Acid Metabolism and Fuel Partitioning*, 2002, 967, 183-195.

VOTION D.M., AMORY H., DEMOULIN V., DESMECHT D., ROLLIN F., THIRY E., BAISE E., CASSART D., DELGUSTE C., PIAT E., SANDERSEN C., LINDEN A. Atypical Myopathy (*Atypical Myoglobinuria*). 2004 webpage: www.ivis.org/reviews/rev/votion/chapter.asp?LA=1 Accessed on 06/02/2008

VOTION D.M., DELGUSTE C., BAISE E., CASSART D., DESMECHT D., LINDEN A., ROLLIN F., SANDERSEN C., AMORY H. Presumption of atypical myopathy: Identification of critical clues for diagnosis from clinical cases referred to the Veterinary Faculty of the University of Liege during spring 2003. *Annales De Medecine Veterinaire*, 2003, 147, 183-193.

VOTION D.M., LEMIEUX H., MOUITHYS-MICKALAD A., SERTEYN D., GNAIGER E. 2007a Study of the mitochondrial respiration in the equine muscle with high-resolution respirometry: feasibility, preliminary results and potential applications. In: *First MiPsummer School on Mitochondrial Respiratory Physiology*, MiP Society, Schröcken, Austria. p 2.

VOTION D.M., LINDEN A., DELGUSTE C., AMORY H., THIRY E., ENGELS P., VAN GALEN G., NAVET R., SLUSE F., SERTEYN D., SAEGERMAN C. Atypical myopathy in grazing horses: A first exploratory data analysis. *Vet. J.*, 2008.

VOTION D.M., LINDEN A., SAEGERMAN C., ENGELS P., ERPICUM M., THIRY E., DELGUSTE C., ROUXHET S., DEMOULIN V., NAVET R., SLUSE F., SERTEYN D., VAN GALEN G., AMORY H. History and clinical features of atypical myopathy in horses in Belgium (2000-2005). *J. Vet. Intern. Med.*, 2007b, 21, 1380-1391.

VOTION D.M., NAVET R., LACOMBE V.A., SLUSE F., ESSEN-GUSTAVSSON B., HINCHCLIFF K.W., RIVERO J.L.L., SERTEYN D., VALBERG S. Muscle energetics in exercising horses. *Equine and Comparative Exercise Physiology*, 2007c, 4, 105-118.

WENCHICH L., DRAHOTA Z., HONZIK T., HANSIKOVA H., TESAROVA M., ZEMAN J., HOUSTEK J. Polarographic evaluation of mitochondrial enzymes activity in isolated mitochondria and in permeabilized human muscle cells with inherited mitochondrial defects. *Physiol. Res.*, 2003, 52, 781-788.

WESTERMANN C.M., DE SAIN-VAN DER VELDEN M.G.M., VAN DER KOLK J.H., BERGER R., WIJNBERG I.D., KOEMAN J.P., WANDERS R.J.A., LENSTRA J.A., TESTERINK N., VAANDRAGER A.B., VIANEY-SABAN C., ACQUAVIVA-BOURDAIN C., DORLAND L. Equine biochemical multiple acyl-CoA dehydrogenase deficiency (MADD) as a cause of rhabdomyolysis. *Mol. Genet. Metab.*, 2007, 91, 362-369.

WESTERMANN C.M., DORLAND L., VAN DIGGELEN O.P., SCHOONDERWOERD K., BIERAU J., VAN DER KOLK J.H. Creatine metabolism, carbohydrate metabolism, purine metabolism and oxidative phosphorylation in healthy horses and horses suffering from atypical myopathy, FVM, University of Utrecht, Utrecht, 2008a.

WESTERMANN C.M., DORLAND L., VOTION D.M., DE SAIN-VAN DER VELDEN M.G.M., WIJNBERG I.D., WANDERS R.J.A., SPLIET W.G.M., TESTERINK N., BERGER R., RUITER J.P.N., VAN DER KOLK H.J. Acquired multiple Acyl-CoA dehydrogenase deficiency in 10 horse with atypical myopathy. *Neuromuscul. Disord.*, 2008b, 355-364.

WHITWELL K.E., HARRIS P., FARRINGTON P.G. Atypical myoglobinuria - an acute myopathy in grazing horses. *Equine Vet. J.*, 1988, 20, 357-363.

ZAK A., ZEMAN M., TVRZICKA E., STIPEK S., BUCHTIKOVA M., POUSEK L., NOVAKOVA E. [Fatty acid composition and parameters of VLDL and LDL in persons with dyslipidemia]. *Cas. Lek. Cesk.*, 2000, 139, 18-23.