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**Clinical and experimental investigation of the nervous
system of neonatal foals. Neuroendocrin transition from
foetal consciousness to perinatal life**

PhD Thesis

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List of Abbreviations

ACTH: Adrenocorticotrophic hormone

AM: Adrenomedullin

BBB: Blood brain barrier

CBC: Complete blood count

CSF: Cerebrospinal fluid

CNS: Central nervous system

CT: Computed tomography

CVID: Common variable immunodeficiency

DHEA: dehydroepiandrosterone

DHEAS: dehydroepiandrosterone-sulphate

FTPI: Failure of transfer of passive immunity

HIE: Hypoxic ischaemic encephalopathy

ICP: Intracranial pressure

HPA: hypothalamic-pituitary-adrenocortical

LC-MS: liquid chromatography mass spectrometry

MRI: Magnetic resonance imaging

NB: neurobehavioral

NMS: neonatal maladjustment syndrome

NSAID: Nonsteroidal anti-inflammatory drug

PCV: Packed cell volume

PCR: Polymerase chain reaction

PCV: Packed cell volume

RBC: Red blood cells

SIM: single ion monitoring

SRM: select reaction monitoring

TFC: turbulent flow chromatography

VMTH: Veterinary Medical Teaching Hospital

WBC: White blood cells

Summary

Unlike most mammalian neonates, foals reportedly exhibit unique behavioural characteristics in the immediate postnatal period. In addition, soon after birth, foals have to be capable to ambulate and escape from predators with an efficient manner, which requires a highly sophisticated and rapid maturation of the neuroendocrine and locomotor system. During this work, we have investigated the neurological, behavioural and endocrinologic changes of healthy and sick newborn foals. For the first time, we also documented the complete neurologic exam findings of the neonatal foal in Hungarian language.

We have described a previously incompletely explored phenomenon called the squeeze induced somnolence (SIS). While mimicking a manual restraint with a device, foals became recumbent with a relaxed, somnolent behaviour and electroencephalographic recordings revealed patterns consistent with slow wave sleep. Plasma adrenocorticotrophic hormone (ACTH), dehydroepiandrosterone sulphate, and androstenedione concentrations significantly increased during restraint, similarly to the foals' tolerance to noxious stimuli; however, the latter was independent of the concentration of circulating β -endorphin. We concluded that SIS by its immobilizing effect maybe an evolutionary strategy for the newborn to survive during parturition and prepares the neuroendocrin system for the postnatal life.

In a different study, we have retrospectively collected our clinical data from a 25-year period reviewing electronically almost 70.000 medical records of which 1.000 belonged to newborn foals. We were able to determine that infectious neurologic disease is very seldom in foals. In contrast to previous assumptions, we have found that septicemia of neonatal foals rarely results in meningitis. These findings guided us to non-infectious causes: the most common of which is neonatal maladjustment syndrome (NMS).

We were able to experimentally induce a temporary status compatible with NMS by the intravenous administration of allopregnanolone. We have found that infusion resulted in obtundation, lack of affinity for the mare and decreased response to external stimuli. We concluded that infusion of this steroid metabolite to a healthy neonatal foal resulted in neurobehavioural alterations compatible with those observed in foals with NMS. These findings suggested that increased progestagen concentrations may be responsible for some of the behavioural changes observed in foals with NMS.

We have further investigated the role of progestagens in foals with NMS. In a multi-centre clinical study, healthy foals showed a significant decrease in progestagen concentrations over the first 48 hours of life. Foals with NMS and sick, non-NMS foals had significantly increased progesterone, pregnenolone and androstenedione concentrations compared with healthy

foals. Progesterone and pregnenolone concentrations of sick, non-NMS foals decreased significantly over 48 h, whereas concentrations in NMS foals remained increased. We have concluded that pregnane concentrations of ill, neonatal foals remain increased following birth, reflecting a delayed, or interrupted, transition from intra- to extra-uterine life.

Lastly, we have investigated the role of adrenomedullin (AM) in healthy and critically ill foals, a substance associated with the hypophyseal-pituitary-adrenal axis. AM is a polypeptide with diverse biologic effects on the cardiovascular system that increases markedly in septic humans and laboratory animals. We have found that AM was not significantly different between septic and sick non-septic foals, but critically ill foals had significantly increased AM compared to healthy controls. In critically ill foals, AM was not predictive of survival but showed promise as a marker of health in neonatal foals.

In conclusion, our series of investigations have elucidated that newborn foals respond to restraint by a unique way manifesting in slow wave sleep and increase in pain tolerance; their neuroendocrin system is sensitive to non-infectious pathologic conditions likely caused by progestagens of adrenal origin; which are essential in the pathogenesis of NMS and alteration of behaviour. The pharmacological intervention into the steroid production is an intriguing future direction.

1. Introduction

Foals are particularly prone to perinatal disease and the neurologic abnormalities are often the first clinical signs one can appreciate. Many physiologic processes can deviate during delivery since the mare's placenta separates very early and the second stage of labour only lasts about 20-30 minutes (Knottenbelt 2003). While the cattle can deliver live calf after 2-4 hours spent in second stage of labour, a foal will likely be stillborn after 1 hour, and be already very compromised after 30 minutes (Madigan 1997). Additionally, since the mare's placenta prevents passage of immunoglobulins in utero, newborns are highly dependent on colostrum intake and timing of first suckle. Considering the fractious nature of foals, the rigid birth environment, the extreme caloric and fluid needs of newborn foals, it is not surprising that foal mortality has been always the highest among domesticated animals (Platt 1973, Cohen 1995). There are common, lay rules for assessment of normal behaviour and neurologic status of foals. The 1-2-3 rule is considered one of those: normal foal stands within an hour, suckles within 2 hours and in 3 hours the meconium is passed (Mayhew 1988). On the other hand, it is often difficult to separate abnormal behaviour from transient findings and from an overt reaction given to external stimulus especially that response to neurological testing of apparently healthy newborn foals differs significantly from the adults (Adams and Mayhew 1984). Among others, premature foals for example (foals born prior to 320 days of gestation) are often hyperreactive and hyperreflexive (Rossdale and Leadon 1979, Madigan 1997). Foals born after prolonged gestation (foals born after 360 days of gestation) are usually weaker, slower and may exhibit various neurological deficits including abnormal behaviour, aimless wondering, inadequate suckle, vocalization, orientation problems, head-tilt and nystagmus. There is another subset of term foals that appear normal in the first 24 hours then become flaccid and obtunded progressively (Bernard et al 1995, Rossdale 1972).

Pioneers in the field of equine neonatal neurology including Rossdale, Ousey, Silver, Palmer, Mayhew, Madigan and a few others have investigated and described the neurologic exam and special neurologic findings in foals (Jeffcott and Rossdale 1979, Adams and Mayhew 1984, Bernard et al. 1995, Palmer and Rossdale 1976). Thus, we now recognize that foals show stereotypic behaviour patterns that will disappear later in life. The most common stereotypic behaviour of foals is that they predictably collapse and become flaccid during a particular type of physical restraint (Mayhew 1988). This phenomenon previously referred to by authors as "flopping reaction" or "reflex relaxation" occurs in most newborn foals that are physically restrained (Jeffcott and Rossdale 1979). During restraint the foal may snap its teeth vigorously and urinate. These signs may represent normal submissive behaviour. Foals also respond to external stimulation with exaggerated movements and have a more angular head position and assume a base wide stance. In general, the foals' gait is dysmetric, while in lateral recumbency

they have increased extensor tone, hyperreflexive tendon reflexes, crossed extensor reflexes as well as recumbent extensor thrust reflexes in all four limbs (Adams and Mayhew 1984, Rosedale 1972).

The previous explains why normal and abnormal findings can be difficult to discern initially and that every foal, born from dystocia; or exhibit pre-, or dysmaturity, will have a high likelihood to show some form of neurologic deficit. Furthermore, most neurologically abnormal neonates will be categorized as “dummy”, hypoxic-ischaemic or maladjusted regardless of the findings and the inciting cause, which highlights that in reality how little is known about the pathologic and humoral processes of neurologic diseases in foals.

Objectives and Hypotheses

The objectives of our research included

- (1) to investigate the vital, humoral and electroencephalographic characteristics of the phenomenon occurring in foals during physical restraint;
- (2) to retrospectively investigate causes, clinical and clinicopathologic signs of meningitis in neonatal foals;
- (3) to evaluate the effects of intravenously administered allopregnanolone in a neonatal foal;
- (4) to evaluate serum steroid profile of healthy foals and foals with clinical disease especially those with NMS;
- (5) and to evaluate the plasma concentration of adrenomedullin in healthy and clinically ill foals.

Our first hypothesis was that, physical restraint of neonatal foals will result in vital, electroencephalographic, and humoral changes consistent with sleep and analgesia.

Our second hypothesis was that infectious neurologic disease, more specifically bacterial meningitis occurs in neonatal foals secondary to sepsis.

Our third hypothesis was that the infusion of allopregnanolone in a healthy neonatal foal would induce clinical signs compatible with NMS.

Our fourth hypothesis was that the serum steroid profile of foals with NMS is different than that of healthy control foals.

Our fifth hypothesis was that plasma adrenomedullin is increased in septic neonatal foals compared to sick non-septic and healthy control foals, and that it is predictive of survival.

2. Literature review

2.1. Physical restraint

Shortly after being born, healthy neonatal foals are active, agile and responsive to the environment, however they predictably collapse and become flaccid during a particular type of physical restraint (Adams and Mayhew 1984, Jeffcott and Rosedale 1979, Madigan 1997). This phenomenon, previously referred to by authors as “flopping reaction” or “reflex relaxation” occurs in most newborn foals that are physically restrained. During the procedure, the handler places one arm around the chest and the other one around the buttocks and compresses the foal’s body. The foal typically sinks to the ground and remains immobile during the restraint. Visual observations of this phenomenon provided the basis for speculations that this manoeuvre may be a form of induced narcolepsy/cataplexy or catatonia, which might persist throughout the neonatal period due to the delayed loss of intrauterine protective mechanisms (Adams and Mayhew 1984, Mayhew 1988). Similar, although less dramatic, behavioural responses have been described in a number of animal and human physical restraint studies; however, the exact pathways have not been extensively investigated to date. In rabbits, gentle but firm pinching of the skin with padded clips will lead initially to arousal, followed by decreased muscle tone, altered mentation, and the synchronization of EEG activity (Kumazawa 1963). In cats, rubbing and gentle pinching of a paw will decrease tonic activity in the dorsal column nuclei and somatosensory cortex (Melzack et al. 1969). In a primate experiment, baby monkeys introduced to fear stimuli preferred to cling and compress themselves against a soft cloth mother surrogate which provided contact comfort, and “deep touch” over a wire surrogate that provided milk (Harlow and Zimmermann 1959). Sheep become relaxed, while entering the “squeeze machine” to receive drugs during pharmacological studies (Grandin 1987). The reactions of cattle to being restrained by a strong pressure initially causes them to relax, but will lead to struggling and discomfort when they habituate (Grandin 1987). Infant swaddling, which has been a commonly applied procedure in several cultures from different eras, relies on the compression and restraint created by a blanket wrapped around the child’s body (Lipton et al. 1965). Temple Grandin, a renowned animal welfare scientist, constructed the “squeeze machine” for autistic individuals with hypersensitivity based on conventional cattle chutes (Grandin 1992). Physical restriction has been hypothesized to reduce the sympathetic stimulation to the ascending reticular activating system (ARAS) and decrease the level of arousal (Giacoman 1971). Animal restraint, in the form of physical restriction, may also cause the sympathetic and parasympathetic tone to become disproportionate, thus having a profound effect on consciousness and physical tone.

2.2. Septicaemia in foals

Neonatal septicaemia is considered as one of the most common causes of morbidity and mortality in equine neonates (Cohen 1995, Hoffmann et al. 1992, Corley et al. 2005, Hurcombe et al. 2008). The mortality rate ranges from 45 to 76% despite advancements in therapeutic and intensive care provision at referral institutions (Hurcombe et al. 2008, Stewart et al. 2002, Gayle et al. 1998). Definitive diagnosis of bacterial sepsis is obtained through microbial culture. Unfortunately, culture and antimicrobial susceptibility results are usually not available until more than 72 hours after submission and, consequently, empirical treatment is often instituted prior to diagnosis. Early antimicrobial treatment, although warranted in suspect cases, may impede the sensitivity of culture as a diagnostic test. In addition, false negative blood culture results may be due to low numbers of circulating bacteria or a relatively low volume of blood, and varying blood culture systems may yield inconsistent results (Lorenzo-Figueras et al 2006, Wilson and Madigan 1989). In one study, only 40% of gram negative infections were detected by antemortem blood culture relative to postmortem culture (Wilson and Madigan 1989). Given the relatively poor sensitivity of microbial culture for diagnosis of sepsis, a weighted scoring system is currently used as a diagnostic tool to identify sepsis; when tested prospectively, this sepsis score demonstrated a sensitivity of 93% and a specificity of 86% (Brewer and Koterba, 1988). However, another study reported a 67% sensitivity and a 76% specificity, suggesting that the scoring system's utility may vary based on the study population and geographic location (Corley and Furr, 2003). Furthermore, the sepsis score failed to predict bacteraemia in 48% of foals (Stewart et al. 2002). Finally, clinicopathologic findings have been associated with sepsis in foals, but lack of test specificity limits their usefulness in contributing to a diagnosis of sepsis (Corley et al. 2005, Hurcombe et al. 2008, Furr et al. 1997, Peek et al. 2006, Rohrbach et al. 2006). Early and accurate diagnosis of sepsis is pivotal to optimal care of critically ill patients, because delay in treatment is an independent risk factor for non-survival, with treatment costs higher than those of other critically ill foals (Gayle et al. 1998) In addition, accurate prognosis for survival is important given the rigors and expense of treatment. Thus, a simple, rapid and reliable test that provides an early diagnosis of sepsis and predicts survival has the potential to greatly reduce morbidity, mortality and medical cost in affected foals. Adrenomedullin (AM) is a 52-amino acid vasodilatory peptide that was first isolated from human pheochromocytoma patients (Kitamura 1993), and is synthesized by endothelial cells, vascular smooth muscle cells and the adrenal medulla (Kitamura et al. 1993, Hinson et al. 2000, Eto 2001). Adrenomedullin is a potent vasodilator, which along with its positive inotropic properties, helps to maintain perfusion to individual organs (Gibbons et al. 2007, Nishikimi et al. 2003). Adrenomedullin also has anti-inflammatory and bactericidal properties (Allaker et al. 1999, Isumi et al. 1999) Taken together, AM is beneficial in mitigating the systemic pro-inflammatory response that occurs in septic patients. Sepsis and septic shock leading to end-organ damage is associated with robust increases in plasma AM concentration (p[AM])

compared to other pathological conditions, both in experimental and clinical trials (Hirata et al. 1996, Nishio et al. 1997, Koo et al. 2001). Plasma [AM] has been shown to increase 50 fold in septic humans compared to healthy controls (Hirata et al. 1996, Nishio et al. 1997, Ueda et al. 1999).

2.3. Infectious and non-infectious neurologic diseases in foals

Neonatal foals may have infectious or non-infectious neurologic disease. Infectious neurologic disease in foals might be a consequence of septicaemia and hematogenous spread of a bacterial infection. A former study found that 8-10% of septicaemic foals develop bacterial meningitis (Platt 1973). Apart from this study, there is no literature specifically evaluating incidence of infectious neurologic disease, more specifically bacterial meningitis associated with neonatal septicaemia. Hematogenous spread could originate from colonization via the mucous membranes or from distant septic foci that reached access to the systemic circulation (Santschi and Foreman 1989, Morris et al 1987). Meningeal inflammation is exacerbated by bacterial cell wall components and characterized by production of prostaglandins, leukotrienes, cytokines, and attraction of leucocytes (Quagliarello and Scheld 1997, Webb and Muir 2000). Meningeal inflammation may lead to increased blood-brain barrier permeability, vasculitis, central nervous system (CNS) oedema and secondary inflammation of tissues adjacent to the meninges (Quagliarello and Scheld 1997, Webb and Muir 2000). Proliferating bacteria in the subarachnoid space may penetrate through the pores of the arachnoid villi, reaching the venous sinuses, entering the systemic circulation thus resulting in septicaemia (Quagliarello and Scheld 1992).

2.4. Neonatal maladjustment syndrome and neurosteroids

The most common non-infectious neurologic disease is neonatal maladjustment syndrome (NMS) that manifests within the first 72 h of life (Bernard et al. 1995). The proposed mechanisms include hypoxic and ischaemic events prior to, during and shortly after parturition (Palmer and Rossdale 1976). Affected foals exhibit neurological dysfunction such as seizures and altered states of consciousness, behaviour and response to stimuli (Bernard et al. 1995, Ringger et al. 2011). However, hypoxic and ischaemic injury is not always identified upon histopathological evaluation, and long-term neurological deficits have been reportedly rare. Foetal corticosteroids, through activation of the hypothalamo-pituitary-adrenocortical (HPA) axis, contribute to the maturation of many organs and regulate the transition between intra- and extrauterine life (Rossdale 2004). Rossdale et al. (1995) reported increased concentrations of progestagens in neonatal foals that rapidly decrease over the following 48 h

after birth (Houghton et al. 1991, Rossdale 2004). Foals with NMS have been reported to have persistently increased concentrations of plasma progestagens (Houghton et al. 1991, Rossdale et al. 1995, Rossdale 2004). Progestagens called neurosteroids can cross the blood–brain barrier and have neuromodulatory effects (Mellon and Griffin 2002, Naert et al. 2007). It is proposed that in a subset of foals the signs of NMS may not be the result of hypoxia, and that these neurosteroids may play a role in the aetiology and clinical manifestations of foals with NMS. Certain steroidal compounds, predominantly 5-alpha-reduced pregnanes, appear to have important neuromodulatory roles (Baulieu 1998, Mellon and Griffin 2002, Robel and Baulieu 1994). These steroids are synthesised de novo in glial cells from cholesterol or blood-borne steroid precursors (Robel and Baulieu 1994) and are potent allosteric modulators of the GABA-A receptor; low concentrations cause weak enhancement of GABA activity and high concentrations cause complete non-competitive inhibition (Baulieu 1998). Infusion of certain 5-alpha-reduced pregnanes into rats and mice (Naert et al. 2007, Zhu et al. 2001) leads to anaesthesia or marked behavioural effects suggesting that these pregnanes cross the blood–brain barrier and exert neuromodulatory effects.

3. Experiments

3.1. Experiment 1.

As published in American Journal of Veterinary Research 73. (2012) 1881–89:

” Evaluation of squeeze-induced somnolence in neonatal foals.”

Authors: Tóth B, Aleman M, Brosnan RJ, Dickinson PJ, Conley AJ, Stanley SD, Nógrádi N, Williams CD, Madigan JE.

The purpose of the study reported here was to test the hypothesis that application of a restraint device would result in behavioural, EEG, and humoral changes consistent with sleep and analgesia in neonatal foals.

Materials and Methods

Animals: Following approval by the University of California (UC) Davis Institutional Animal Care and Use Committee, neonatal foals born at the Center for Equine Health at UC Davis were evaluated for use in the study. The inclusion criteria were as follows: foals had to be term (>330 days gestation) with a normal, uncomplicated delivery and had to be healthy without apparent neurological deficits based on neurological and physical examination.

Restraint device and technique: A 20-foot-long, 0.5-inch diameter soft linen rope was used to construct the restraint device. A modified rope squeeze technique was applied, adopted from an earlier publication (Leahy and Barrow, 1953). The technique has been used in cattle and adult horses for restraint; however, to the best of the authors' knowledge, the use of this technique in foals had not been previously described. As the first step, a bowline knot was used to secure the rope around the neck to prevent tightening of that segment which could result in pressure on the trachea and/or the jugular veins (Figure 1). Two half-hitch knots were used to loop the rope around the thorax and abdomen 5-10 inches from each other perpendicular to the vertebral column. The half hitch knots were positioned directly on the dorsal thoracolumbar area. To propagate the phenomenon, a designated person stood behind the foal and pulled on the rope producing a generalized squeezing of the foal, while a second person was holding the foal and assisted with lying it down. Tension was maintained on the rope (by the person holding the rope) until the experiment was completed.

‘Vital parameters’ and mentation: ‘Vital parameters’ were recorded on the foals before and during restraint. Heart rate and respiration rate were determined following auscultation with a

stethoscope. Three measurements, taken at 5 minute intervals, were recorded both during the baseline and restraint periods. Means were calculated for each period. A single rectal temperature was measured with a digital thermometer prior to and at the end of the restraint period. Behaviour was recorded based on categorical visible changes in mentation and body position (bright and active or sleepy and recumbent).

Electroencephalography (EEG): EEG recordings were performed on a 32-channel telemetry unit (WEE-1000 AirEEG, Nihon-Kohden Corp, Tokyo, Japan) an acquisition system (EEG-9100 Neurofax, Nihon-Kohden Corp, Tokyo, Japan.) and a notebook computer (Dell Inspiron, Dell Corp, Round Rock, Texas, USA) with acquisition, review and file utility software installed (EEG-9200, Nihon-Kohden Corp, Tokyo, Japan). Stainless steel needle electrodes were placed subcutaneously using a modified (reduced number of electrodes) protocol used in foals at the Veterinary Medical Teaching Hospital of UC Davis (VMTH) as described in detail elsewhere (Aleman et al. 2006). Three central electrodes (C3, left; Cz, midline; and C4, right), 2 auricular electrodes (A1, left; A2, right), and a ground electrode (Z) were applied (Figure 2). Electrodes impedance was determined acceptable at $\leq 10 \text{ k}\Omega$. A transverse bipolar montage was used to review and analyse all EEGs. The settings for EEG recording included a sensitivity of $70 \mu\text{V}/\text{cm}$, time constant of 0.1, and a high frequency cut-off of 35 Hz. No sedatives were used in this study to avoid alterations of consciousness. Electrode placement was attempted with the foal standing and prior to the application of pressure with the restraint device. When this was not possible, due to lack of patient cooperation, the electrodes were placed immediately after the foals were in lateral recumbency with the restraint device maintained. In those foals, the EEG recording started within <20 seconds from the onset of restraint and recumbency. The EEG study was performed continuously for a minimum of 15 minutes. The recordings were visually examined to determine state of consciousness using previously described criteria in humans and horses (Williams et al. 2008, Mysinger et al. 1985).

Analgesia and pain threshold testing: Pain threshold and analgesic effects of the restraint device were tested with a square pulse stimulator (S48 square pulse stimulator, Grass Technologies, West Warwick, RI, USA). For stimulation, 27 gauge 5/8-inch needle electrodes (Safelead F-E3-48, Grass Technologies, West Warwick, RI, USA) were positioned with one 50 mm lateral to the base of the tail and the other 50 mm towards the ground over the semitendinosus muscle. The stimulation rate was 50 pulse per second (pps) with pulse duration of 10 msec each. Voltage range was 0.1 V - 20 V with a ramp rate of 0.2 V/s. The experimental session was started at a stimulus intensity of 0.1 mV, with the anodal electrode positioned distal to the cathodal electrode; if no response could be elicited, the

current was gradually increased in steps of 0.2 mV/s until a response was observed. Positive conscious pain responses as dichotomous variables (yes or no) were either defined as purposeful avoidance movements of tail, limbs, trunk, head and neck, attempts to kick or turning the head toward the site of electrical stimulation at the time the stimulation was applied (Natalini and Robinson 2000). Responses were evaluated by 3 independent observers who were blinded to the voltage being delivered. The threshold stimulation was repeated at least 3 times 2 minutes apart at each of three different assessment periods: before restraint, during restraint and after restraint. At least 5 minutes of "equilibration" in or out of the restraint device were required before pain threshold testing. Measurements taken for each assessment period were averaged.

Blood sample handling and analysis: Venipuncture was performed on the left or the right jugular vein before (following 15 minutes of acclimatization) and 15 minutes after the restraint device had been continuously applied. Venous blood was collected into a 3 mL previously heparinized (1000 U/ml) syringes, closed with a plastic stopper and placed on ice immediately. Venous blood gas analysis was performed within 2 hours of collection with a commercially available blood gas machine (ABL-705 blood gas analyzer, Radiometer, Copenhagen, Denmark) and values (pH, PvO₂, PvCO₂) were corrected to the animals' rectal temperature. Blood was collected with a 20 g 1-inch needle into a 25 mL plastic syringe, transferred to serum, glass and plastic EDTA, and heparin tubes and then placed on ice until the experiments were finished. Samples were centrifuged for 10 minutes at 3400 rpm and stored at -80 °C (Revco ultra-low-temperature freezer, Thermo Fisher Scientific Inc, Franklin, MA, USA) until processing.

Measurements for ACTH and cortisol were performed with a solid-phase, two-site sequential chemiluminescent immunometric assay (IMMULITE 2000, Siemens AG, München, Germany) as described by others (Perkins et al. 2002). The technique uses monoclonal and polyclonal antibodies for the capture and detection, respectively, of ACTH and cortisol.

Samples for other circulating steroids (nandrolone sulphate, boldenone sulphate, 17-beta estradiol sulphate, testosterone sulphate, 1,4-androstadien-3,17-one, testosterone glucuronide, 19-norandrostenedione, boldenone, androstenedione, nandrolone, oestrone, testosterone, epi-nandrolone, epi-testosterone, progesterone, 6-alpha-hydroxy androstenedione, nandrolone glucuronide, 17-beta estradiol, 17-alpha estradiol, 17-hydroxy progesterone, 19-norepiandrosterone, dehydroepiandrosterone, 17-hydroxy pregnenolone, 5-alpha dihydronandrolone, 5-alpha-estran-3beta,17alpha-diol, 5-alpha dihydrotestosterone, 19-nor-androsterone, 5-beta dihydrotestosterone, 5-alpha dihydroprogesterone, pregnenolone, allopregnanolone, pregnanediol, and oestrone sulphate) were analysed by liquid chromatography mass spectrometry (LC-MS) utilizing on-line sample extraction by turbulent flow chromatography (TFC) and detection by Single Reference Material (SRM) on

a triple quadrupole mass spectrometer (Fisher Scientific Inc, Franklin, MA, USA). This method has been described in detail elsewhere (Yu et al. 2008).

Samples for β -endorphin measurements were collected in heparinized tubes. Tubes also contained 2.5 UI/ml of Bacitracin (Sigma B-0125, 50,000 U/g, Sigma-Aldrich Corp, St. Louis, MO, USA) and 1000 IU/ml Trasylol in 0.1 M PBS (pH=7.0 with 0.01% Thimerosal, 10,000 kIU/ml). Measurements were performed with β -endorphin radioimmunoassay (RIA) kits (S2013, Bachem Inc, St Helens, Merseyside, UK) that have been previously validated for use in horses (Bossut et al. 1983).

Study design and statistical analysis: Animals served as their own controls in this prospective, interventional, pilot study. Following 15 minutes acclimatization time spent with the foal, pre-restraint blood samples and measurements were taken followed by application of the restraint device. While EEG was being performed, fifteen minutes into the restraint procedure blood samples were collected. Analgesia testing was performed after the EEG electrodes were removed. Paired comparisons were made between pre and post restraint values for each individual animal. Statistics were performed with a commercially available program (Minitab, version 15, Minitab Inc, State College, PA, USA).

Distribution of the data was tested with the Kolmogorov-Smirnov normality test. Wilcoxon signed ranked tests were used to compare values pre and post restraint. Fisher's exact test was used to test the difference for dichotomous categorical variables (gender, physiologic vs. supraphysiologic level of ACTH). Spearman rank correlation analysis was performed to reveal any association between plasma β -endorphin and pain threshold levels, plasma ACTH and androstenedione levels and plasma ACTH and DHEA-sulphate levels. Behaviour was not evaluated statistically. A *P*-value < 0.05 was considered to be significant in all tests.

Results

A total of 8 foals were used in the study. There were 4 males and 4 females. Foals had a median age of 2.5 days (range: 1-4 days). All 8 foals were Quarter Horses. Data are summarized in Tables 1 and 2. Variables were measured in at least 6 foals.

Mentation and arousal: All 8 foals were lively, active, and behaved normally prior to restraint. Somnolence became apparent in all foals within seconds after the restraint device was attached and pressure was applied (Figure 3). Three foals exhibited a few seconds of what appears to the authors as apparent dream like behaviour such as unconscious whinnying, rolling and kicking out with their limbs after 10-12 minutes into the restraint. After the restraint

device was removed all foals stood up immediately, stretched their limbs and approached their dams.

Vital parameters: Foals exhibited significantly lower heart rate, respiratory rate, and rectal temperature during the restraint ($P=0.014$ for each parameter) (Table 1).

Electroencephalography findings: EEG recordings were attempted in all 8 foals, however application of electrodes and recording was only possible in 4 foals due to lack of compliance. However, the EEG from one foal was excluded due to excessive movement artefacts. Statistical analysis was not performed on the data. One of the foals (foal 1) had 2 EEG studies (first EEG: using restraint device [rope], second EEG: [under manual restraint]). The rope was removed from the foal and allowed to recover for 4 minutes. A second EEG under manual restraint was not part of the study. However, it was performed in this foal because at the time the foal stood up after being released from the first study, the needle electrodes remained in place and EEG recording continued. The foal was manually restrained (no rope) and induced to recumbency. The EEG electrodes were maintained from the first study through the end of the second study. Foals 2 and 3 each had one recording with the restraint device. Recording of the EEG from the standing position to recumbency was possible in 2 foals (Figure 3A). All 3 foals displayed sleeping behaviour alternating with periods of wakefulness, which manifested on the EEG as sleep spindles, vertex sharp waves, slow waves, and K-complexes (consistent with slow wave sleep [SWS]), and high frequency waves (consistent with wakefulness) (Figure 3B-E). No rapid eye movement sleep (REM-sleep) was seen at the beginning of the recording with only 1 foal displaying observed REM-sleep (not shown due to movement artefacts during recording) several minutes into the recording as previously mentioned.

Analgesia and pain threshold testing: Foals exhibited a significantly increased pain threshold during restraint compared to unrestrained ($P=0.036$) (Table 1). There was no significant correlation between the percent of elevation in pain threshold levels and the percentile elevation in β -endorphin levels during the restraint ($P=0.34$).

Venous blood gas: Venous blood pH was not significantly different between restrained and unrestrained states ($P=0.83$). Venous partial pressures of carbon-dioxide ($PvCO_2$) and oxygen (PvO_2) were not significantly different during restrained and unrestrained states ($P=0.06$, $P=0.4$, respectively) (Table 1).

ACTH: ACTH levels were significantly elevated during the restraint compared to baseline levels ($P=0.014$). All 8 horses had physiologic levels of ACTH (<9 pmol/L) prior to restraint, while during restraint, 5 of 8 foals had supraphysiologic levels ($P=0.03$) (Table 1).

Cortisol: Serum levels increased during restraint in most foals compared to levels at rest; however, the difference was not significant ($P=0.08$) (Table 1).

Serum steroid metabolites: Thirty-four different steroid metabolites were measured, of which 27 did not have detectable serum levels either before or during restraint. Seven metabolites

listed below had measureable serum levels prior to and during the restraint period (Table 2). Steroid metabolites with 0 median values pre or post restraint were only tested statistically if either values were different from 0. The serum levels of DHEAS were significantly increased during restraint ($P=0.042$). There was no significant linear correlation between the percentile elevation in plasma ACTH and DHEAS levels during restraint ($P=0.56$). The serum levels of androstenedione were also significantly increased during restraint ($P=0.042$). There was a significant positive correlation between the percentile elevation in androstenedione and ACTH levels during the restraint ($P=0.04$). The serum levels of 17-OH pregnenolone, dehydroepiandrosterone (DHEA), epitestosterone, pregnanediol, and pregnenolone were not significantly altered during restraint (Table 2).

Discussion

We have shown that application of the described restraint device induces foals to lie down, and remain in lateral recumbency. Physical restraint appears to decrease the foals' voluntary motor activity; and also triggers somnolence as observed by the authors in all foals along with recorded periods of wakefulness/drowsiness, and late onset slow wave sleep (delta waves, K-complexes) in 3 foals. The latter findings are in agreement with the previous descriptions of SWS in humans, and horses (Berger et al. 1988, Williams et al. 2008, Mysinger et al. 1985). Human infants also exhibit slow wave activity on the electroencephalogram (Rosen and Satran 1965); decelerations in heart rate, and diminished brain metabolism (Mann et al. 1972) when increased pressures are applied by maternal pushing in the second stage labour (similarly to the restraint). However, the methods and design of our study preclude making further comparisons on these similarities.

The foals from this study did not exhibit cataplexy as proposed by Adams and Mayhew (Adams and Mayhew 1984). Cataplexy (loss of muscle tone often associated with a strong stimulus) is considered a pathologic finding in all species (Dauwilliers et al. 2007). Cataplexy is also accompanied by narcolepsy and manifests in acute onset of REM-sleep (rapid eye movements as well as a high frequency waves on EEG) (Zarcone 1988). The appearance of sleep spindles, K-complexes, vertex sharp waves and slow waves is not consistent with REM-sleep (Williams et al. 2008). Further, this phenomenon is a well-recognized event of young foals induced by restraint (squeeze induced somnolence [SIS]).

It must be noted that Mysinger et al. described low frequency waves and sleep spindles in awake neonatal foals (Mysinger et al. 1985). However, those foals were also under restraint at the time of recording. Thus, the impact of restraint on EEG characteristics remains unclear, since neither Mysinger nor our research group obtained sufficient EEG data without restraint

in neonatal foals. Unfortunately, the EEG data obtained in our study was limited by the low number of foals and lack of recording in 2 of 4 EEG studies while the foals were being restrained and recumbency induced. Further, to the best of our knowledge EEG of awake, unседated, unrestrained neonatal foals have not been described to date. Therefore, our findings are not sufficient to draw conclusions regarding the relationship between SIS and brain wave activity.

Although foals exhibited significant increase in pain thresholds during the restraint, there was unlikely sufficient analgesia for surgical procedures causing moderate or severe pain. A study measuring responses to noxious electrical stimuli in horses receiving epidural opioids showed threshold responses higher than 40 V (Natalini and Robinson 2000), in contrast to threshold values <13 V reported during this study. It is unknown whether the analgesia provided would be sufficient for minor procedures. Although circulating β -endorphin was also elevated during restraint, there was no significant positive correlation in the percentile increase of β -endorphin and pain threshold level during restraint, therefore the role of endorphin in this phenomenon remains undetermined and its contribution to pain attenuation is unknown. Although endogenous opioids are also potent psychotropes and anxiolytics (Akil et al. 1984), it is unlikely that the detected levels of β -endorphin alone could have caused this profound change in consciousness and physical tone based on reported normal values in adult horses (Pell and McGreevy, 1984). It is important to note that there have been no reference values for plasma β -endorphin established in neonatal foals.

Although activation of the HPA-axis and production of cortisol may only take minutes during stress in adult horses (James et al. 1984), significant elevation in cortisol levels were not found, which can be explained as a detectable increase in circulating cortisol levels in neonatal foals may require up to 30 minutes according to independent studies (Hart et al. 2009, Rosedale et al. 1982). Additionally, the small sample size may also preclude making further conclusions on this, however we found significant elevation in two steroid metabolites during restraint. One was DHEAS, a neurosteroid and an agonist of sigma-1 receptors, which exhibits potent neuromodulatory effects (Baulieu 1998). The potential role of DHEAS in the development of this phenomenon remains to be determined.

Limitations of this study include the lack of standardization regarding pressure exerted on the foals. It is possible that the different degree of compression contributed to the variability of the results. Grandin's hug machine delivers 30-80 psi pressure depending on the age of the subject; and nearly the whole lateral body surface is squeezed (Grandin 1992). Therefore, it is

possible that a more profound response could have been obtained with increasing the pressure or surface area.

EEG examinations were not performed in all animals due to lack of compliance; however, the recordings that were obtained were consistent among all 4 EEGs in 3 foals. It must be also noted that EEG evaluations were attempted prior to placement of the restraint device but were unsuccessful in most cases, since unrestrained and unsedated foals did not tolerate placement of the electrodes and movement artefact resulted in uninterpretable recordings. It should be mentioned that Mysinger et al. used disc electrodes and were able to obtain multiple tracings from foals, both in lateral recumbency and under standing restraint (Mysinger et al. 1985). The degree of restraint in the aforementioned study is not well described (Mysinger et al. 1985). Due to multiple difficulties to place and maintain surface electrodes (appropriate contact, sweat, thick dense hair, cooperation in unsedated foals), and expense; surface electrodes were not used in our study.

In conclusion, SIS may activate the HPA-axis, lower vital signs and cause dormancy. This may be a remnant manifestation of complex autonomic responses persisting throughout early perinatal period, where both the activation of HPA-axis and the quiescence could be essential to the transition from foetus to newborn (by preventing malpositioning during birth). It is possible that this technique could be used as a method of restraint for performing minor procedures in young foals. We did not detect adverse events to SIS restraint in this study; thus, use of the restraint device could be a safe alternative to mild sedation. Although the EEG data were not definitive in part due to the small number of foals, it did not support narcolepsy/cataplexy as the mechanism for the observed behavioural response and collapse of the foals upon restraint. Further studies with a larger group of foals are warranted to elucidate additional physiologic and receptor pathways that are involved in SIS.

Tables

Parameter	Sample size (n)	Pre median (range)	Restraint median (range)	P-value*
Heart rate (/min)	8	98 (92-112)	89.3 (69.3-94)	<i>0.014</i>
Respiratory rate (/min)	8	64 (45.3-84)	36.7 (33.3-45.3)	<i>0.014</i>
Rectal temperature (°C)	8	38.7 (38-39.2)	38.5 (37.8-39)	<i>0.014</i>

Venous blood pH	7	7.38 (7.34-7.42)	7.37 (7.33-7.41)	0.830
PvCO₂ (mmHg)	7	52.9 (51.1-53.6)	53.6 (52.1-58.1)	0.060
PvO₂ (mmHg)	7	37.9 (30.8-42.7)	39.9 (31.4-45.3)	0.400
ACTH (pmol/L)	8	5.08 (4.42-9.83)	11.35(4.97-45.1)	0.014
B-endorphin (pg/mL)	6	14.2 (2.5-46)	47.1 (9.8-76.2)	0.036

Table 1. Table shows the parameters evaluated during the experiment.

Medians and ranges are reported. *The Wilcoxon Signed Rank test. The difference was not significant between pre and post restraint values.

Steroid metabolite	Sample size (n)	Pre median (range)	Restraint median (range)	P value*
17-OH pregnenolone (ng/mL)	8	32 (5.5-92.7)	31.40 (5.95-94.8)	0.620
Androstenedione (pg/mL)	8	76.5 (0-598.2)	109.9 (37.1-823.1)	0.042
Cortisol (µg/dL)	8	2.2 (1.3-4.2)	2.9 (2.1-13.3)	0.080
DHEA (ng/mL)	8	9.09 (0.74-34.6)	12.35 (0.9-28.9)	0.270
DHEA-sulphate (pg/mL)	8	3224 (0-18750)	4889 (2270-27288)	0.042
Epitestosterone (pg/mL)	8	0 (0-128.9)	66.9 (0-148.8)	0.090
Pregnanediol (ng/mL)	8	0.32 (0-2.9)	0.175 (0-4.1)	0.590
Pregnenolone (ng/mL)	8	36.2 (14.6-60.5)	39.95 (16.1-75.8)	0.230

Table 2. Table shows circulating steroid levels during the experiment. A total of 34 different steroid metabolites were measured, of which 7 had measureable serum levels prior to and during the restraint period (Table 2). Steroid metabolites with 0 median values pre or post restraint were only tested statistically if either values were different from 0. Medians and ranges are reported. *The Wilcoxon Signed Rank test.

Figures



Figure1. Restraint device (rope). Note rope position in a standing foal prior to applying pressure.

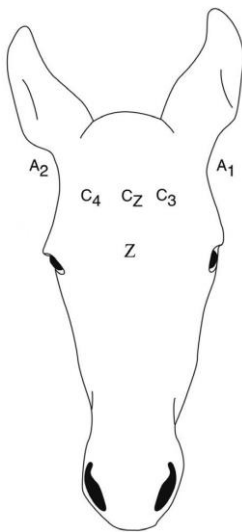
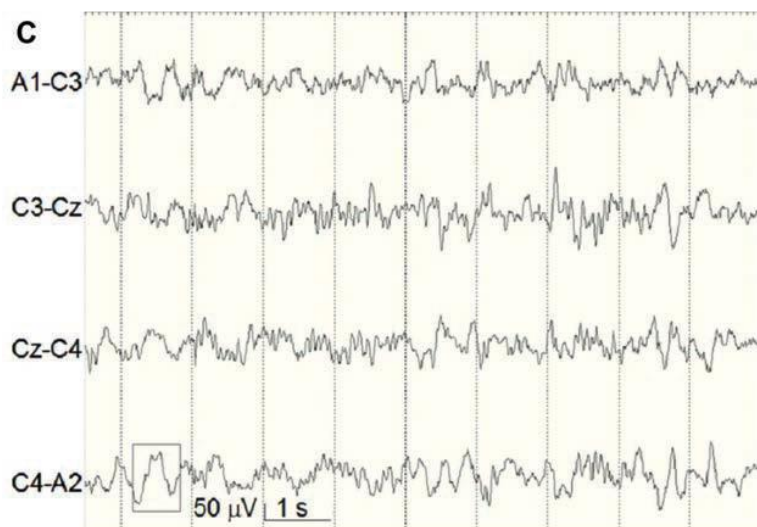
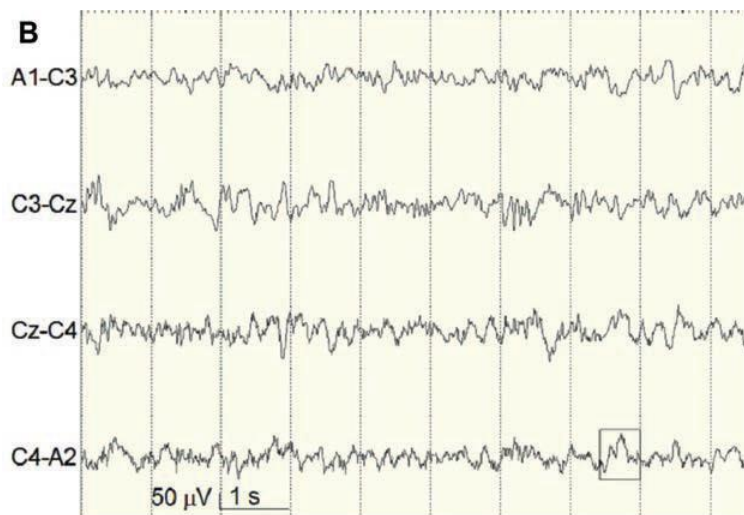
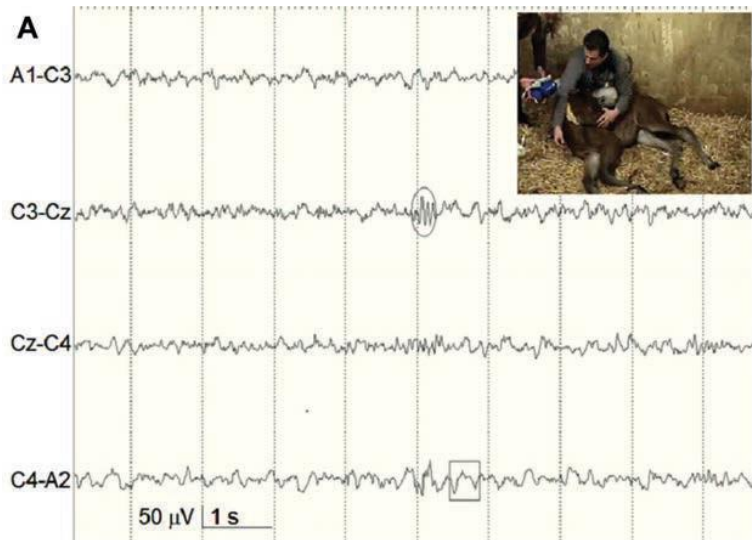


Figure 2. Schematic diagram of the EEG needle electrode placement. Three central electrodes (C3, left; Cz, midline; and C4, right), 2 auricular electrodes (A1, left; A2, right), and a ground electrode (Z) were applied.



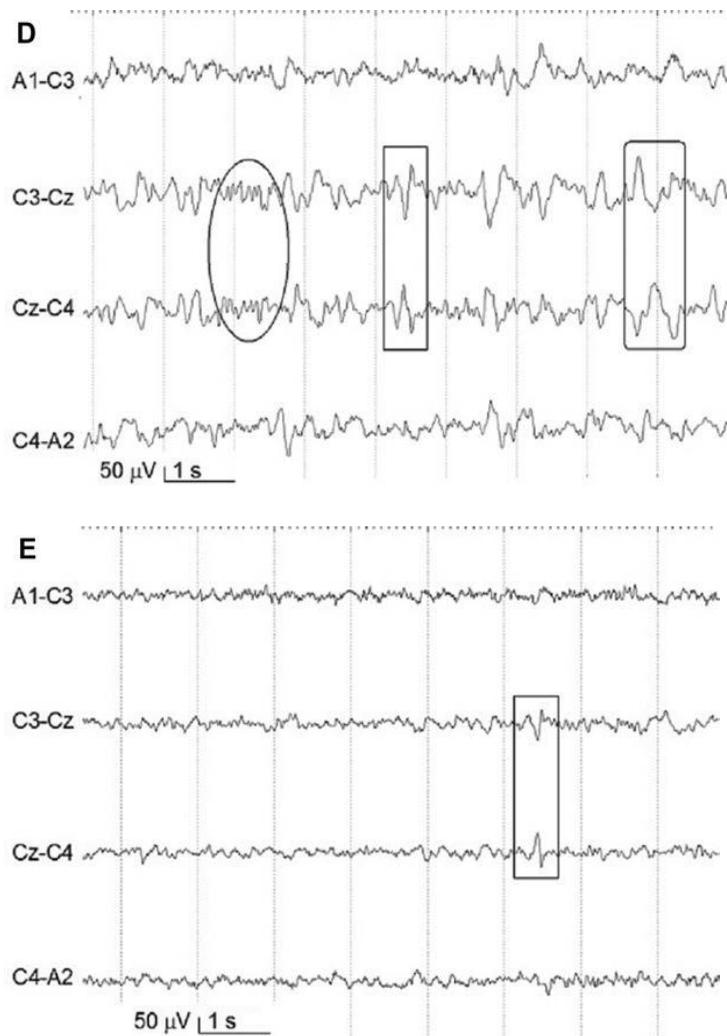


Figure 3. Representative EEG recordings obtained from 2 neonatal foals during manual restraint or restraint achieved by application of pressure via the rope restraint device. The EEG needle electrodes were placed as shown in Figure 2. A—Recording obtained from a foal (foal 1) during the period of transition from standing to recumbency induced via manual restraint. Notice the presence of sleep spindles (oval outline; 14.3 Hz, 71.8 μ V) at C3-Cz and a slow wave (square box; 64.8 μ V, 227 milliseconds) at C4-A2. The photograph at the top right corner of the image illustrates the point during restraint at which the tracings were obtained. B—Recording obtained from foal 1 during pressure application via the rope restraint device. Notice the slow wave (square box; 120 μ V, 358 milliseconds). C—Recording obtained from foal 1 during manual restraint. A slow wave (square box; 150.5 μ V, 481 milliseconds) and multiple sleep spindles (11 Hz) are present. D—Recording obtained from another foal (foal 2) during pressure application via the rope restraint device. Notice the presence of sleep spindles (oval outline; 11.5 Hz, 51.5 μ V), a vertex sharp wave (rectangular outline at centre; 160.6 μ V, 198 milliseconds), and K-complex (rectangular outline on right; 197.6 μ V, 522 milliseconds). E—Recording obtained from foal 2 in a period of observed drowsiness that occurred during pressure application via the rope restraint device. Notice the vertex sharp wave (rectangular

outline; 92.6 μ V, 174 milliseconds). Recorded findings for a third foal were similar to those for foal 2.

3.2. Experiment 2

As published in Journal of American Veterinary Medical Association. (2012) 580–587:
"Meningitis and meningoencephalomyelitis in horses: 28 cases (1985-2010)"

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The purpose of the study reported here was to describe the history, clinical signs, clinicopathologic and postmortem findings, causative agents, treatment, and outcome in a large population of horses with meningitis and meningoencephalomyelitis (infectious and non-infectious)

Materials and Methods

Horses with a definitive diagnosis of meningitis based on cytological analysis of CSF or postmortem macroscopic and histopathological examination were included in the study. The electronic medical database of the William R. Pritchard Veterinary Medical Teaching Hospital was searched from the years 1985 to 2010, using the words meningitis, meningoencephalitis, and meningoencephalomyelitis under the clinical, laboratory and pathological diagnosis fields. Medical records were reviewed and data extracted included signalment, presenting complaint, history, clinical signs, laboratory work, diagnostic procedures, outcome and histopathological examination. Descriptive statistical analysis was performed on the data using a commercially available statistical software. (Minitab 15, Minitab Inc, State College, PA, USA) Means, medians, ranges, minimums, maximums, standard deviations, and standard errors were calculated.

Results

Horses: Twenty-eight horses met the inclusion criteria. Twenty-two horses had confirmed infectious meningitis or meningoencephalomyelitis (19 bacterial, 15 through microbial isolation in CSF or tissue, 3 through the observation of bacteria in CSF, and 1 through a polymerase chain reaction in CSF; 1 fungal observed in CSF; and 2 parasitic upon histopathological evaluation). Four horses had suspected bacterial meningitis based on the presence of neutrophilic pleocytosis with degenerate neutrophils. Two horses had non-infectious meningitis; 1 associated to myelography, and 1 with chronic granulomatous meningitis of undetermined cause.

Signalment and history: Breed distribution consisted of 8 Thoroughbreds (32.4%), 7 Quarter Horses (25.9%), 6 Arabians (21.4%), 2 Paints (7.4%), 2 mixed breeds (7.4%), 1 Morgan (3.7%), and 1 American Miniature Pinto (3.7%). Twenty-two horses were older than 1 year of age (median 5 years, range 1 to 21 years), 4 foals were weanlings (4 to 7-months old), and 2 were neonatal foals (2 and 20 days old). Twelve horses (42.9%) were intact males, 10 (35.7%) were females, and 6 (21.4%) were geldings. Nine (32.1%) of the 28 horses had a previous history of head or cervical trauma (5 days to 2 weeks before presentation). One of the horses with head trauma had a draining non-healing wound in the ventral aspect of the mandible for 1.5 months. Eight (28.5%) of the 28 horses had longstanding (1 week to 2 months) undefined neurological problem. The remaining horses had acute colic episodes (n = 2, 7.1 %), chronic nasal discharge (n = 2, 7.1%; 2 months in 1 case, unknown length in the other), chronic weight loss for several weeks (n = 2, 7.1%), and 1 each horse (3.6%) had one of the following: 1 month history of *Streptococcus. equi subspecies equi* upper respiratory tract infection, *Corynebacterium pseudotuberculosis* infection in the nasal cavity and in the nasomaxillary sinuses, and pyogranulomatous cellulitis of the prepuce. One horse received an intramuscular injection in the neck, developed stiffness 4 to 5 days later and was presented 2 weeks following the initial signs. Both neonatal foals had septicemia, 1 was a premature foal with failure of passive transfer. The other foal was originally seen as a 2-day old for failure of passive transfer, discharged and represented for lethargy and neurological signs as a 20-day old foal. The foal developed septic thrombophlebitis at the previous catheter site. Both foals developed septic polyarthritis, thrombosis at multiple sites, and multiorgan failure.

Physical examination: The median rectal temperature in 28 horses was 37.9 °C (35.5-41.1°C; reference range: 37.5-38.2°C). Fever (temperature above 39 °C,) on presentation was observed in 4 (14.3%) of 28 cases. Median heart rate in 25 horses was 60 beats per minute (range: 32-96 bpm; reference range 28-40 bpm). Neonatal foals were excluded from this part of the descriptive statistics because of physiologically higher resting heart and respiratory rates. Nine (36%) of 25 horses exhibited tachycardia on presentation (>48 bpm in adults, >80 foals). Median respiratory rate in 25 horses (neonatal foals excluded) was 20 breaths per minute (range: 16-48 bpm; reference range 8-16 bpm). Seven of 25 horses (31.8%) had tachypnoea (>20/bpm) on presentation.

Neurological examination: Mentation was normal in nine of 28 (32.1%) cases. Obtundation and stupor were seen in 18 (64.3%) and 2 (7.1%) of the cases, respectively. Two (7.1%) horses

presented with seizures, and 2 apparently blind (running into objects and lack of a menace response). Nineteen (67.9%) of 28 horses were ambulatory and 9 (32.1%) were recumbent. Symmetrical ataxia and tetraparesis was seen in 16 (57.1%) of 28 cases, while 1 (3.6%) horse exhibited symmetrical ataxia of the pelvic limbs and paraparesis. Mean grade of ataxia was 3.05 on the thoracic limbs and 3.74 on the pelvic limbs according to a published grading scale (Lunn and Mayhew, 1989). Other gait abnormalities included hypermetria in 4 (14.3%) of the 28 cases; however it was not specified which limbs were affected. Extensor rigidity of the thoracic limbs was found in 1 (3.6%), and 1 horse was reluctant to move. Cranial nerve deficits were found in 20 (71.4%) of 28 cases. Head tilt, nystagmus and strabismus were observed in 13 (46.4 %), bilaterally absent menace response in 9 (32.1%), bilateral absence of pupillary light reflexes in 7 (25%), unilateral facial nerve paralysis in 3 (10.7%), and dysphagia in 1 (3.6%) of the cases. Segmental reflexes were difficult to evaluate and therefore not reported. Apparent cervical pain (horse appearing stiff, standing with neck extended, reluctance to flexion, and moving away from the examiner upon palpation) was noted in 9 (32.1%) of 28 cases. Hyperaesthesia (exaggerated response upon tactile stimuli) was apparent in 5 horses (Table 3).

Clinical pathology: The haematological abnormalities included haemoconcentration (n=4/25, 16%), anaemia (n=4/25, 16%); median PCV 35% (range: 26-59%; reference range: 30-46%); leucocytosis (n=12/26, 46.2%), or leukopenia (n=2/26, 7.7%); median white blood cell count 11,600/ μ L; range: 2,000-33,200/ μ L (reference range: 5,000-12,500/ μ L); neutrophilia (n=15/24, 62.5%), or neutropenia (n=3/24, 12.5%); median neutrophil count 8,160/ μ L; range: 900-25,900/ μ L (reference range: 2,600-8,600/ μ L); lymphopaenia (n=13/24, 54.2%); median lymphocyte count 1,500/ μ L; range: 171-5000/ μ L (reference range: 1,600-7,000/ μ L); and hyperfibrinogenaemia (n=12/24, 50%); median fibrinogen concentration 600 mg/dL; range: 200-1,100 mg/dL (reference range: 100-400 mg/dL). The serum biochemical abnormalities included hypoalbuminaemia (n=8/21, 38.1%); median albumin concentration: 2.95 g/dL; range: 2.1-4.1 g/dL (reference range: 2.7-4.1 g/dL); and hyperglobulinaemia (n=2/21, 9.5%); median globulin concentration: 3.8 g/dL; range: 2.4-6.2 g/dL (reference range: 1.6-5 g/dL). No other biochemical abnormalities were found. Total lymphocyte count including T and B populations, lymphocyte stimulation test, and immunoelectrophoresis for IgG, IgM, and IgA were comparable to a control horse, in one mare with *Halicephalobus gingivalis* meningoencephalomyelitis. However, these tests were performed 1 month earlier to the onset of neurological signs (Table 3).

Cerebrospinal fluid analysis: CSF collection was performed in 23 (82.1%) out of the 28 horses. Sixteen (69.6%) of 23 samples were collected at the atlanto-occipital space (cisterna magna), 5 (21.7%) at the lumbosacral space, and 2 (8.8%) from both locations. Four (17.4%) of 23 cases had more than one CSF collection performed during the treatment course. The CSF appeared normal in 4 (17.4%) and abnormal in 19 (82.6%) horses upon macroscopic evaluation. The abnormalities included xanthochromia with or without cloudiness (n=14/19, 73.7%, Figure 2), serosanguinous (n=4/19, 18.2%), and fibrin clotted (n=1/19, 4.5%). The cytological evaluation of the CSF was abnormal in 20 cases, as follows. The total nucleated cell count had a median value of 1,224/ μ L (range 0-240,000/ μ L; reference range <5/ μ L). Percentage of neutrophils had a median of 87% (range 0-98%; reference range: 0%), large mononuclear cells had a median of 3% (range 0-24%; reference range: 10-30%), and small mononuclear cells had median of 7% (range 0-83%; reference range: \geq 70%). Eosinophils were not observed in any of the CSF samples. Red blood cells in the CSF had a median of 280/ μ L (range: 0-245,000/ μ L; reference range: 0/ μ L). Total protein concentration of the CSF had a median value of 282 mg/dL (range: 45-3,900 mg/dL; reference range: <70 mg/dL). The cytological interpretation of the CSF was moderate to marked suppurative inflammation as indicated by neutrophilic pleocytosis in 20 (87%) of 23 cases. Thirteen (65%) of the 20 horses had degenerate neutrophils on cytology. Two horses had normal CSF cytology. Intra- and extracellular bacteria were seen in 8 (34.8%) of the 23 CSF samples. Encapsulated yeasts compatible with *Cryptococcus neoformans* were observed in a CSF sample from 1 horse. Creatine kinase (CK) concentration in the CSF was measured in 9 horses and had a median value of 5 IU/mL (range: 0-102 IU/mL; reference range: <8 IU/mL). Glucose levels of CSF were measured in 6 cases with a median value of 30 mg/dL (range: 11-82 mg/dL; reference range: 30-70 mg/dL). Gases, electrolytes, and lactate were measured in CSF in 1 horse and showed lactic acidosis (pH 7.296, reference range 7.35-7.40; L-lactate: 13 mmol/L, reference range < 2 mmol/L), the rest of the parameters were within reference range. Glucose in this horse was 11 mg/dL and had extracellular cocci and coccobacilli on CSF. Culture of 2 consecutive CSF samples 2 days apart failed to isolate organisms in this horse. D-lactate was measured in a CSF sample collected prior to euthanasia and revealed 30 mmol/L, control horse 0 mmol/L). Antemortem microbial culture of CSF yielded organisms in 4 of 21 cases (19%). The organisms included *E. coli* (n=1/4 horses, sensitive to multiple antimicrobials), *Capnocytophaga canimorsus* (n=1/4, sensitivity report not available), *Corynebacterium pseudotuberculosis* (n=1/4, sensitive to multiple antimicrobials), *Fusobacterium sp.* and *Bacteroides sp.* (n=1/4, sensitivity report not available). No bacterial growth was reported in 17 (81%) of the 21 cases (Table 3).

Diagnostic imaging: Radiographic examination of the skull (n=14), cervical vertebral column (n =2) and thorax (n=1) were available. Skull radiographs showed abnormalities in 12 horses as follows. Fracture of the cranium was found in 7 cases; cribriform plate (n=3), basisphenoid bone (n=1), sphenopalatine bone (n=1), multiple fractures of the cranium (n=2), and tooth root abscess of second premolar and mandibular fistula with severe osteomyelitis (n=1). Pneumocephalus was evident in a horse with multiple fractures of the cranium. Radiographic alterations consistent with sinusitis in 4 cases (ventral and dorsal conchal, frontal, and maxillary sinuses), and blood within multiple sinuses in 1 case with multiple fractures were observed. Two of the horses with severe frontal sinusitis had pathological fractures of the cribriform plate. Two of the horses with maxillary sinusitis also had tooth abscesses. Cervical vertebral column radiographs showed atlantooccipital subluxation in 2 horses, and thoracic radiographs revealed pleuropneumonia in 1 case.

Computer tomography was available in 3 cases and revealed cerebellar coning at the foramen magnum (n=1), fracture of the cribriform plate and pneumocephalus (n=1) and right sided otitis media/interna with remodeling and suspected osteomyelitis of the temporal bone (n=1). Magnetic resonance imaging (MRI) was performed immediately after euthanasia in 1 horse. Sagittal and transverse T1, T2, PD, and FLAIR weighted images were obtained and revealed submeningeal hyperintensity in the cerebrum, brainstem and cerebellum on T2 and FLAIR sequences. On the sagittal images, there was coning of the caudal aspect of the cerebellum with protrusion of the cerebellum through the foramen magnum. These findings were compatible with cerebellar herniation, and severe submeningeal oedema compatible with meningitis.

Management and outcome: Treatment was initiated in 17 (60.7%) of 28 cases. Nine horses were hospitalized for more than 24 hours (median 6 days, range 3-15 days). The remaining horses were hospitalized less than 24 hours when either died or were euthanized due to severity of disease. Medical management consisted of intravenous polyionic fluids, broad spectrum antimicrobials, anti-inflammatory drugs (most horses received nonsteroidals, and 5 horses received a single dose of dexamethasone), sedatives, gastroprotectants, and nursing care. A variety of antimicrobials at high doses were used and included ampicillin, potassium penicillin, aminoglycosides, trimethoprim sulfamethoxazole, third and fourth generation cephalosporins, chloramphenicol, rifampin, and metronidazole. The mortality rate was 96.4% (n=27/28). Seven horses died and 20 were euthanized. The horse with cryptococcal meningitis was euthanized. A bone biopsy was performed in a mare with a draining tract in the mandible and revealed severe chronic granulomatous osteomyelitis with intralesional nematodes consistent with *Halicephalobus gingivalis*. The mare was treated with antimicrobials, NSAIDs and 1 dose of dexamethasone, ivermectin, fenbendazole, and bone debridement. This horse

was discharged after 1 month of hospitalization, came back for a recheck a month later and discharged. Two weeks after discharge, the horse developed neurological signs and was euthanized by the referring veterinarian.

Postmortem findings: Neuropathological evaluation was performed in 24 horses and revealed moderate to severe, diffuse, suppurative meningitis in 18 horses (75%), and moderate to severe, diffuse meningoencephalomyelitis in 6 horses (25%). Two of the horses with meningoencephalomyelitis had *Halicephalobus gingivalis* migration and leucoencephalomalacia, and one other had a displaced fracture at T6 with extradural compressive myelopathy and discospondylitis at the level of T6-T7. Osteomyelitis of fractured bones was observed in 3 horses. One of the horses diagnosed with meningitis also had pituitary abscessation. Two horses with normal antemortem CSF cytology and no bacterial growth had evidence of chronic multifocal inflammation of the meninges of undetermined cause (n=1), and diffuse subacute moderate meningitis that was suspected to be associated with a myelogram performed 2 days prior to euthanasia (n=1). Postmortem microbial culture was performed in 23 cases, of which 13 (56.5%) yielded bacterial growth. The following organisms were isolated on a bacterial culture in 13 horses: *Streptococcus equi subspecies zooepidemicus* (n=3, 13%), *Escherichia coli* (n=2, 8.3%), mixed growth (n=2, 8.3%, *Pasteurella caballi*, *E. coli*, *Peptostreptococcus*, *Fusobacterium*, *Actinomyces sp.*, *Fusobacterium sp.*, *Bacteroides sp.*), and in 1 horse each (4.2%) of the following; *Cryptococcus neoformans*, *S. equi ssp. equi*, *Actinobacillus sp.*, *Proteus sp.*, *Klebsiella sp.*, *C. pseudotuberculosis*, and anaerobes (*Bacteroides sp.* and *Fusobacterium sp.*). Two of these horses also had the same bacterial growth on an antemortem CSF sample. One horse with *C. pseudotuberculosis* antemortem culture was discharged. More than 1 organism was isolated in 3 horses. The horse with an antemortem isolation of *Capnocytophaga canimorsus* did not have a necropsy performed. Disseminated hematogenous infection was evident upon postmortem examination in 8 horses (5 adults, 1 weanling, and 2 neonatal foals). The adult horses had pleuropneumonia, interstitial pneumonia, pericardial effusion, osteomyelitis, disseminated intravascular coagulation, and tooth root abscess. The weanling had previous upper airway *S. equi ssp. equi* infection. The pathogen was identified in an antemortem CSF sample through polymerase chain reaction testing.

Discussion

Meningitis and meningoencephalomyelitis were diagnosed in 28 equine patients from our institution in a 25-year period. Meningitis is a rare neurological disorder in horses as evidenced by a few isolated case reports and a retrospective study of 450 horses with neurologic disease over a 12-year period in which 2 and 3 horses ($5/450 = 1\%$) were diagnosed with fungal and

bacterial meningitis, respectively (Rumbaugh 1977, Flaminio et al. 2009, Timoney et al. 1983, Stuart et al. 1973, Tyler 1993, Newton 1998, Finno et al. 2006, Raphel 1982). In the present study, both the patients' age and gender distribution were different from the hospital population during the same time period, largely due to the high number of young Thoroughbred colts in training or racing which shifted the median age and the gender distribution. The authors are not aware of any aetiological factors that could predispose young male horses to meningitis, although due to their temperament they may be more likely to sustain head trauma with disruption of the blood brain barrier (BBB). Concurrent infection in the head such as sinusitis, tooth root abscess, osteomyelitis, and otitis media/interna was seen in 6 horses. Similarly, ascending infectious processes adjacent to the CNS have been reported in adult horses prior to the development of meningitis (Smith et al. 2006). Other concurrent infections included interstitial pneumonia and pleuropneumonia. Disseminated involvement from haematogenous spread was found upon histopathological (involvement of other organs) and microbiological evaluation in 8 horses. Disseminated bacterial infection in adult horses has been reported in the literature (Rumbaugh 1977, Stuart et al. 1973, Tyler 1993). In neonatal foals, bacterial meningitis has been reported secondary to septicaemia in 8 to 10% of septic foals (Platt 1973). Other possible route of infection is through the vasculature of the pituitary gland because of the lack of a BBB (Reilly 1997). One of the horses from this study had a pituitary abscess. Helminthic meningitis caused by *H. gingivalis*, a free-living saprophyte nematode, enters via wounds in the skin and mucous membranes, spreads locally to adjacent tissues including bones and hematogenously to distant sites such as the kidneys and CNS in horses (Bryant 2006). Traumatic events with disruption of the BBB or meninges in the head or vertebral column were documented in 11 horses in this study. Human studies have shown that the incidence of posttraumatic meningitis after moderate to severe head injury is 1.4%; while after compounded skull fracture it could be as high as 2-11% (van de Beek et al. 2010).

Vital parameters at admission were within reference range in the majority of cases. However, many clinical records were not detailed enough to determine recent NSAID or analgesic administration that could have potentially altered these findings. A wide variety of signs have been described in horses with meningitis including lethargy, fever, weakness, cervical pain, and those associated with other concurrent diseases (Pellegrini-Masini et al. 2006). Similar to reports in other species with meningitis, abnormal mental status (71.4%) was a common neurological alteration in these horses (Quagliarello 1992). However, mentation may be normal early in the course of the disease. The majority of the horses exhibited moderate to severe ataxia, which could have been the result of trauma to the long tracts within the spinal cord, brainstem or involvement of the vestibular system. Cranial nerve deficits were a common finding in affected horses (71.4%), similar to a canine study where deficits were reported to be

present in 56-72% of cases (Radaelli and Platt 2002). Vestibular dysfunction (46.4%) was a common presentation, and could have resulted from head trauma or spread of infection like in the case of the horse with otitis media/interna. Other signs reported in horses with meningitis or meningoencephalomyelitis include disorientation, head pressing, aggressive behaviour, seizures, reluctance to move, and compulsive circling. People and dogs with meningitis exhibit severe neck pain which is considered one of the most characteristic and early signs of meningitis (Quagliarello 1992, Radaelli and Platt 2002). Affected individuals avoid voluntary movements such as neck flexion that would slow down CSF flow to the central canal of the spinal cord and consequently increase intracranial pressure (ICP) (Quagliarello 1992). Neck pain may be more difficult to assess in horses, especially in the recumbent animal. In this study, apparent cervical pain was noted in a third of the cases.

The most important haematological findings were leucocytosis, neutrophilia, lymphopaenia, and hypoalbuminemia. Neutrophilia and hyperfibrinogenaemia are the most commonly reported laboratory abnormalities in horses with infectious meningitis (Pellegrini-Masini et al. 2006). Combined variable immunodeficiency is an acquired disorder of adult horses manifested as recurrent fevers and bacterial infections (Flaminio et al. 2009, Pellegrini-Masini et al. 2006). In a study of 14 horses with common variable immunodeficiency (CVID), 6 horses had meningitis which in 1 case 3 episodes of infectious meningitis were documented (Flaminio et al. 2009). The disease is characterized by persistent severe B cell lymphopaenia or depletion as determined by B-cell markers CD19-like, CD21, and IgM; and hypo- or agammaglobulinaemia (IgG and IgM). In addition, serum IgA concentrations decline over time as shown by serial immunological testing (Flaminio et al. 2009). The horses from the current study had no history of recurrent fevers or infections. Further, none of the horses with lymphopaenia (lymphocytes 171-1,500/ μ L) had hypoglobulinaemia (globulins 2.4-5 g/dL). Immunological testing to investigate which population of lymphocytes was depleted in these horses was not performed. Hypoalbuminemia (2.1-4.1 g/dL) was not severe and there were no clinical manifestations such as oedema. Globulins within reference range (usually high end of reference range) to slightly increased values could have accounted for hypoalbuminaemia.

Both in human and veterinary medicine CSF analysis is considered to be the antemortem gold standard for the diagnosis of meningitis (Radaelli and Platt 2002, Pellegrini-Masini et al. 2009) The majority of the cases had an abnormal CSF upon macroscopic evaluation. Xanthochromia was the most common macroscopic abnormality that reflected blood-brain barrier leakage or damage (Quagliarello 1992, Webb and Muir 2000). Bloody discolouration can be due to blood contamination, intrathecal haemorrhage, haemorrhagic infarct or diapedesis also consistent with disruption of the blood-brain barrier (Quagliarello 1992, Webb and Muir 2000) The elevated protein in the CSF in these horses could be attributed to protein leakage from the

disruption of the BBB and local production as the result of inflammation. Neutrophilic pleocytosis and the high percentage of degenerate neutrophils in the samples supported an infectious process. Furthermore, one third of the CSF samples contained intra- or extracellular bacteria. However, neutrophilic pleocytosis in the absence of degeneration and bacteria does not rule out bacterial meningitis; as shown in 2 horses on which CSF analysis at different times did not reveal neutrophilic degeneration in the first CSF but showed an increased neutrophil count with degeneration (2/2) and organisms in the second CSF (1/2). Furthermore, a study reported 1 horse with CVID and presumed bacterial meningitis that had an initial lymphocytic pleocytosis that became neutrophilic in a subsequent CSF (Pellegrini-Masini 2006). Infectious organisms were observed in almost half of the CSF samples, bacterial in 8 and fungal in 1 horse. Similar to other reports in horses, marked elevation in CSF protein and profound neutrophilic pleocytosis was seen in the horse with cryptococcal organisms (Seckel et al. 1982, Hart et al. 2008). One horse with *H. gingivalis* and bacterial meningoencephalomyelitis had elevated protein and severe neutrophilic pleocytosis. A second horse with *H. gingivalis* had normal CSF cytology on a first visit when no neurological signs were present. A CSF sample was not available when the horse developed neurological signs. Creatine kinase in CSF was elevated in half of the horses on which CK was measured and could suggest CNS trauma. Glucose in CSF lower than 50% of serum glucose concentration is a common finding in people with bacterial meningitis (Sigurdardottir et al. 1997). Glucose in CSF was low in 4 of 6 horses, and in 3 horses was lower than 50% of serum glucose. Increased lactate in CSF could be an indication of increased anaerobic metabolism (L-lactate) or presence of fermenting bacteria (D-lactate) such as *Klebsiella*, *E. coli*, *Lactobacillus*, and *Bacteroides* among others (Smith et al 1986, Drury et al. 1965). Mammals do not produce D-lactate and lack the enzymatic system for its metabolism (Smith et al. 1986). In one horse with bacterial meningitis, CSF L-lactate was increased and D-lactate was detected at high levels, parameters that support sepsis.

Despite limitations to identify skull pathology with radiography, this modality was helpful to recognize fracture of cranial bones, pneumocephalus, alterations supportive of osteomyelitis, and sinusitis. Advanced imaging such as CT and MRI have increased diagnostic capabilities in equine medicine (Spoormakers et al. 2003, Cornellise et al. 2001). Computed tomography with three-dimensional reconstruction provides detailed imaging of bone structures; however intracranial lesions may be missed (Lacombe et al. 2010). In one horse, the tract of penetrating trauma to the frontal sinus and cribriform plate was clearly defined with CT. Other example includes the horse with otitis media/interna which skull radiographs did not reveal abnormalities but upon CT evaluation, soft tissue thickening and fluid within the tympanic bulla, remodeling with suspected osteomyelitis of the temporal bone involving the acoustic meatus were evident.

Magnetic resonance imaging is considered an excellent diagnostic modality for meningitis in humans and small animals, as the disease causes characteristic imaging changes. The cost and set up of available MRI machines for equine patients frequently limit its use in equine neurology (Radaelli and Platt 2002, Spoormakers et al. 2003). Furthermore, the requirement of general anaesthesia in the moderate to severely neurologically compromised horse increases the risk of injury during the anaesthetic recovery.

Medical management was limited in this study due to the apparent advanced stage and severity of disease, safety concerns, and cost that resulted in death or euthanasia in less than 24 hours from admission in most cases. Drug penetration to the central nervous system could be challenging due to the unique BBB characteristics including in-situ transport mechanisms that may remove drugs from the CNS (Quagliarello 1997, Webb and Muir 2000, van de Beek et al. 2010). Drugs with high lipid solubility, small molecular weight and low protein binding are more likely to penetrate the BBB (Mitchell et al. 2007). Rapid bactericidal killing in CSF requires drug concentrations that exceed the minimum bactericidal concentration (not minimum inhibitory concentration) by 10-20 fold (Scheid, 1987). Bactericidal drugs are preferred; however, based on the successful outcome in few mature horses treated with chloramphenicol suggests that bacteriostatic drugs may also be effective (Mitchell et al. 2006). Antimicrobials that readily penetrate the BBB include imipenem, trimethoprim/sulphonamide, fluorinated quinolones, metronidazole, chloramphenicol, rifampin, and macrolides (Pellegrini-Masini 2006, Mitchell et al. 2007). States of inflammation may facilitate drug penetration as in the case of penicillins, selected cephalosporins (cefotaxime, ceftriaxone, ceftazidime), and vancomycin. Favourable and unfavourable outcomes have been reported in horses with various antimicrobials (Pellegrini-Masini et al. 2006). Environmental factors of infected meninges such as pH and inflammatory proteins may alter penetration, availability, and activity of antimicrobials (Quagliarello et al. 1997). Specific dosing regimens and duration are unknown in horses with bacterial and fungal meningitis but maximal doses if tolerated (adverse effects, toxicity) for long-term may be indicated. Duration of treatment in horses with successful outcome has ranged from 3 weeks to a few months (Pellegrini-Masini et al. 2006). The sole survivor in this study was mildly obtunded, tetraparetic but ambulatory and had moderate cranial nerve deficits. The horse was referred as soon as the neurological deficits became apparent. Early recognition and referral may have played an important role in the favourable outcome in this horse. Successful treatment with long-term (197 days) fluconazole for cryptococcal meningitis has been reported (Hart et al 2008). However, because fluconazole is fungistatic, a short course of fungicidal treatment prior to fluconazole may be recommended. There have been reports of horses appearing neurologically worse after the initiation of therapy (Hart et al. 2008, van de Beek et al. 2010). This was also observed in a few horses from this

report. This may be due to the killing of organisms and local production and release of inflammatory cytokines. Therefore, the use of NSAIDs is indicated prior and during the early stages of antimicrobial treatment. It has been proposed that early treatment with dexamethasone in the acute stages of bacterial meningitis in adult people improves outcome (De Gans and van de Beek 2002). However, recent studies have demonstrated that dexamethasone as an adjunctive therapy does not reduce neurological disabilities or death (van de Beek et al. 2010). A few horses from this study were administered dexamethasone prior to referral or during the early antimicrobial treatment. Due to the retrospective nature of this study and high fatality rate, recommendations on the use of steroids are not possible.

Nineteen horses were confirmed to have bacterial meningitis/meningoencephalomyelitis with 4 additional suspects based on neutrophilic pleocytosis with degenerate neutrophils. Antemortem bacterial isolation has been documented to be of low yield in various species including horses (Pellegrini-Masini et al. 2006, Radaelli and Platt 2002, Pellegrini-Masini et al. 2005, Finno et al. 2006). From this study, antemortem microbial culture yielded bacterial organisms in 4 of 19 confirmed cases suggesting a low sensitivity. Polymerase chain reaction proved to be useful for the diagnosis of streptococcal meningoencephalomyelitis in 1 colt on which bacterial culture revealed no growth (Finno et al. 2006). Cryptococcal organisms are usually visualized upon cytological evaluation of CSF samples as it was the case in 1 horse from this study and others documented in the literature (Hart et al. 2008, Seckel et al. 1982). Postmortem culture of CNS tissue had a higher sensitivity for the isolation of organisms than the antemortem CSF culture. The following organisms have been reported to cause suppurative meningitis, meningoencephalitis/myelitis, or intracranial abscessation in adult horses. Bacterial organisms include *S. equi ssp. equi*, *S. equi ssp. zooepidemicus*, *S. suis*, *Actinomyces sp.*, *Corynebacterium pseudotuberculosis*, *E. coli*, *L. monocytogenes*, *Sphingobacterium multivorum*, *Staphylococcus aureus*, coagulase negative *Staphylococcus sp.*, *Actinobacillus equuli*, *Pasteurella caballi*, and *Klebsiella pneumoniae* (Rumbaugh 1977, Smith et al. 2002, Pellegrini-Masini et al. 2005, Allen et al. 1987, Mitchell et al. 2007, Timoney et al. 1987, Pusterla et al. 2007, Ford and Lokai 1980, Devriese et al. 1990, Emerson et al. 1968, Aleman et al. 1998). In foals, *Listeria monocytogenes*, *Salmonella agona*, *S. equi ssp. equi*, *S. equi ssp. zooepidemicus*, *Staphylococcus aureus*, *E. coli*, and *Klebsiella pneumoniae* have been reported to cause suppurative meningitis (Pellegrini-Masini et al. 2006, Morris et al. 1987, Finno et al. 2006, Stuart et al. 1973). In this study, 5 bacterial species not previously reported were identified and included *Proteus sp.*, *Peptostreptococcus sp.*, *Capnocytophaga canimorsus*, *Fusobacterium sp.*, and *Bacteroides sp.* Pathogens causing respiratory disease such as *Streptococcus equi* subspecies *equi* and *zooepidemicus* have been the most commonly reported organisms causing bacterial meningitis in non-neonatal foals and adult

horses. Four of our horses were infected with streptococcal species. Cases of bacterial meningitis due to *S. equi ssp. zooepidemicus* and *S. equi ssp. equi* in adults and children that have had close contact with horses have been rarely reported (Downar et al. 2001, Elsayed et al 2003). Pathogens causing bacterial meningitis in neonatal foals are usually those causing bacteraemia/septicaemia as in the case of 1 neonatal foal (*E. coli*) from this study (Platt et al. 1973). The value of blood culture in adult horses with bacterial meningitis has not been investigated but it may be warranted as haematogenous spread is one of the presumed routes of infection into the CNS. Parasitic meningoencephalitis in horses has been documented to be caused by *Halicephalobus gingivalis* and *Trypanosoma evansi* (Bryant et al 2006, Seiler et al. 1981). Cytological evaluation of CSF has been reported to reveal *T. evansi* in horses (Seiler et al. 1981). In this study, 2 horses were infested with *H. gingivalis* and identified upon histopathological evaluation.

Major limitations to this study include the retrospective nature of the report. Additional information would have been useful such as complete biochemical analysis of CSF (electrolytes, glucose, D- and L-lactate, gases, pH), immunological testing (subpopulations of lymphocytes, immunoglobulin profiles), and advanced diagnostic imaging (CT and MRI). Second, clinicians may have a different approach and interpretation of neurological signs, using different diagnostic and treatment modalities, estimating prognosis, and recommending euthanasia.

Important points from this study include the following. Meningitis is confirmed to be a rare disorder in horses, which is most commonly associated with infection that can expand from local or haematogenous routes. Penetrating trauma with disruption of the BBB or meninges is also a common way for entry of organisms. Bacterial, fungal and parasitic meningitis or meningoencephalomyelitis can occur in horses. Non-infectious meningitis appeared to be less common; however transient meningitis such as secondary to a myelogram is possible but seldom recognized or documented. Common neurological abnormalities include alterations in mental status, cranial nerve deficits, ataxia and vestibular dysfunction. Leucocytosis, neutrophilia, lymphopaenia, and hyperfibrinogenaemia are the most common haematological abnormalities in the case of infectious meningitis, mainly bacterial. Transient immunodeficiency and CVID must be considered in horses with no known history of trauma or ascending/haematogenous infection. History of recurrent infections and marked lymphopaenia with hypo- or agammaglobulinaemia should prompt the clinician to investigate for CVID in adult horses, not evaluated in this study. Neutrophilic pleocytosis is characteristic of meningitis. Bacterial meningitis cannot be ruled out in the absence of degenerate neutrophils or organisms in a CSF sample. Polymerase chain reaction for common bacterial organisms and the use of lactate (L- and D-lactate) measurement should be considered as a diagnostic aid in CSF

samples when bacterial meningitis is suspected and no bacterial growth is observed. Despite a low yield of bacterial isolation from CSF samples, CSF microbial culture should not be discouraged. In this study, 6 pathogens not previously reported were identified, 3 of which were isolated from antemortem CSF samples. Infectious meningitis or meningoencephalomyelitis is a potentially fatal disease. However, treatment should not be discouraged as cases with successful outcome have been documented. Early recognition and aggressive treatment may improve survival in affected horses.

Variable	No. of horses	Mean \pm SD	Median (range)	Reference range
Physical				
Heart rate (beats/min)*	26	56.9 \pm 17	60 (32–96)	28–40
Respiratory rate (breaths/min)*	26	24.8 \pm 10.8	20 (16–48)	8–16
Rectal temperature ($^{\circ}$ C)*	26	38.2 \pm 0.8	37.9 (33.3–41.1)	37.5–38.2
Hematologic				
PCV (%)	25	37.1 \pm 9.14	35 (25.9–59)	30–46
WBC count (cells/ μ L)	25	14,118 \pm 7,642	11,600 (2,000–33,200)	5,000–11,600
Neutrophil count (cells/ μ L)	24	11,014 \pm 7,328	8,170 (900–25,900)	2,600–6,800
Lymphocyte count (cells/ μ L)	24	1,705 \pm 1,205	1,500 (171–5,000)	1,600–5,800
Fibrinogen (mg/dL)	24	541.7 \pm 244.8	600 (200–1,100)	100–400
Serum biochemical				
Albumin (g/dL)	20	2.9 \pm 0.6	2.9 (2.1–4.1)	2.7–4.2
Globulin (g/dL)	20	3.9 \pm 1.1	3.8 (2.4–6.2)	1.6–5.0
CSF cytologic				
Total nucleated cell count (cells/ μ L)	23	20,991 \pm 1,224	1,224 (0–240,000)	<5
RBC count (cells/ μ L)	23	27,959 \pm 65,380	280 (0–245,000)	0
Total protein (mg/dL)	23	697 \pm 1,177	282 (45–3,900)	<70
Neutrophils (%)	23	74.1 \pm 30.2	87 (0–98)	0
Small mononuclear cells (%)	23	14.8 \pm 22.5	7 (0–83)	50–70
Large mononuclear cells (%)	23	5.9 \pm 6.9	3 (0–24)	10–30
Eosinophils (%)	23	0	0	0
Organisms [†]	23	NA	NA	NA
⁺CSF biochemical				
Creatine kinase (U/L)	9	26.2 \pm 38.4	5 (0–102)	0–8
Glucose (mg/dL)	6	41.1 \pm 14.6	30 (11–82)	40–70
pH	1	7.296	NA	7.35–7.40
l-lactate (mmol/L)	1	13	NA	<2
d-lactate (\square mol/L)	1	300	NA	0
CSF bacterial culture				
Before death [‡]	23	NA	NA	NA
After death [‡]	23	NA	NA	NA

*Data from 2 neonatal foals were excluded from these values because of physiologically higher resting heart rate, respiratory rate, and rectal temperature, compared with older horses. [†]On cytologic examination of CSF from 23 horses, bacteria were observed in CSF of 8 horses and fungi were observed in CSF of 1 horse. [‡]Microbial culture of CSF from 23 horses yielded bacterial growth for 4 horses before death and for 13 horses after death.
NA = Not applicable.

Table 3. Physical and clinicopathologic findings in 28 horses with meningitis and meningoencephalomyelitis.

3.3. Experiment 3.

As published in Equine Veterinary Journal 44, Suppl. 41 (2012) 109–112:” Allopregnanolone infusion induced neurobehavioural alterations in a neonatal foal: Is this a clue to the pathogenesis of neonatal maladjustment syndrome?”

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The aim of this study was to evaluate if the infusion of allopregnanolone in a healthy neonatal foal would induce clinical signs compatible with NMS.

Materials and methods

A healthy neonatal 50 kg Quarter Horse colt from the research herd at the School of Veterinary Medicine, University of California, Davis was selected for the infusion. The foal was born from a healthy Quarter Horse mare with a normal gestational length without complications, and normal observed parturition except that the placental passage was prolonged. Although retention of the placenta for 7 h post birth is not considered normal by most standards, the placenta was evaluated and determined to be complete and normal. Further, the mare did not display any signs or complications associated with placental retention. The foal was deemed healthy based upon complete physical and neurological examinations immediately post birth. The foal exhibited normal adaptive behaviour with righting reflex, and time to stand and suckle within normal limits. Repeat physical and neurological examinations at age 6 h, immediately prior to infusion, were also normal.

Intravenous catheters were placed aseptically in the right jugular vein for sample collection and in the left jugular vein for infusion of allopregnanolone. Allopregnanolone (5 alpha-pregnan-3 alpha-ol-20-one) was dissolved in an ethanol-based solution to a total concentration of 9 mg/ml. Infused dose and concentration of allopregnanolone in this foal were determined based on concentrations reported in *in vivo* studies in the modulation of the HPA axis in male rats (Naert *et al.* 2007). An initial bolus of 0.05 mg/kg bwt i.v. of allopregnanolone was given followed by a constant rate infusion (CRI) of 0.02 mg/kg bwt/min using an infusion pump. Based on clinical effects of the initial dosage, a second bolus of 0.1 mg/kg bwt i.v. was given after 5 min and followed by a CRI of 0.04 mg/kg bwt/min for 5 min. The infusion was discontinued for 30 min to allow observation of any neurobehavioural (NB) alterations, and then a final bolus of 0.2 mg/kg bwt i.v. was given.

Neurobehavioural alterations were recorded and graded through a NB scoring system developed by the authors for the assessment of foals with NMS (Table 4. From preliminary work, foals with NMS had scores >8 from a range of 0 (normal foal) to 20 (comatose with paroxysmal activity) (JE Madigan, unpublished data). Mentation was defined as: normal if the foal was alert and responsive; quiet to obtunded if the foal was apparently lethargic but responsive to external stimuli (e.g. touch, sound); stuporous if level of consciousness was decreased but responsive to painful stimuli (e.g. pinching skin with haemostats); and comatose if the foal lost consciousness and was unresponsive to any stimuli. Paroxysmal activity was defined as abnormal events such as seizures or seizure-like activity, rhythmic limb movements, tremors or paddling. The NB scores were calculated at 5 min intervals throughout the infusion period. Following the infusion, the foal was observed hourly for the first 6 h and then at 12 h intervals for 2 days. Heparinised blood samples were collected at birth, age 6 h and at 15 min intervals during the infusion. Blood was immediately centrifuged following collection and plasma stored at -80°C until analysed by liquid chromatography mass spectrometry (LC-MS) utilising on-line sample extraction by turbulent flow chromatography (TFC) and detection by select reaction monitoring (SRM) on a triple quadrupole mass spectrometer. Samples were diluted 2:1 with water fortified with 4 internal standards: D3-testosterone, D3-boldenone, D7-androstenedione and D3-testosterone sulphate. Analytes were separated by liquid chromatography using a Thermo TLX-2 TFC system with a Thermo Cyclone P extraction column and an ACE C18 analytical column. Analytes were introduced by electrospray ionisation to a Thermo TSQ Vantage triple-quadrupole mass spectrometer operating in both negative and positive modes. Free concentrations of 34 steroids were monitored in one analytical method over a 24-min run time. Detection and quantitation was accomplished using 3 or more SRM transitions per compound for all compounds other than 17-hydroxy pregnenolone where single ion monitoring (SIM) was utilised. This method was validated and the following assessed for each analyte: linearity, limit of detection, limit of quantitation, accuracy, precision, matrix effects, extraction recovery and potential endogenous interferences. The following steroids were analysed: allopregnanolone, dehydroepiandrosterone (DHEA), 5- α dihydroprogesterone, 17-hydroxy pregnenolone, pregnenolone, pregnanediol and progesterone. A diagram of the relation of these neurosteroids is shown in Figure 4 For NB comparison, a second age-matched clinically healthy neonatal Quarter Horse colt was infused with 99.9% ethanol diluted with 0.9% saline to a final concentration of 5% ethanol without allopregnanolone. Infusion of this solution followed the same protocol (dosage [based on 5% ethanol] and rate) of administration as for the first foal. The study was approved by an Animal Care and Use protocol from University of California, Davis.

Results

Prior to the allopregnanolone infusion, the colt was bright, alert and responsive (NB score of 0). Infusion of 0.05 mg/kg bwt allopregnanolone followed by a CRI at 0.02 mg/kg bwt/min resulted in signs of sedation and decreased responsiveness to the environment (NB score of 14). Infusion of higher concentrations of allopregnanolone (0.1 and 0.2 mg/kg bwt) resulted in dramatic NB effects with the foal becoming recumbent, stuporous, unresponsive to the mare, environment, sound and tactile stimulation (NB score of 16). Clinical signs persisted during the constant rate infusion. Within 8 min of cessation of the infusion the foal began to show signs of increased responsiveness. By 15 min after cessation of the infusion or the final bolus the foal was standing but continued to show clinical signs of mild obtundation, reduced coordination and poor udder seeking ability (NB score of 9). The foal appeared normal by 30 min after infusion of the neurosteroid (NB score of 0). No long-term NB effects were observed following the infusion. The control foal's NB scores were unchanged throughout the infusion. Steroid concentrations from *Foal 1* are detailed in Table 5 these were not measured in the control foal due to lack of NB alterations, cost and probability of undetectable concentrations of neurosteroids based on preliminary work (JE Madigan, unpublished data). Due to an increase in dehydroepiandrosterone (DHEA) concentrations between birth and age 6 h, luteinising hormone (LH) concentrations at various time points were measured to investigate if this rise was due to production by the testis. However, there was no change in LH concentration.

Discussion

Infusion of allopregnanolone to a healthy foal in this study produced marked NB effects. This is consistent with the clinical use of certain steroidal drugs, such as alphaxalone, as anaesthetic agents in male rats (Naert *et al.* 2007). Allopregnanolone in other species has been shown to cross the blood–brain barrier and is thought to mediate its effects in the central nervous system (CNS) via the GABAA receptor (Zhu *et al.* 2001). Infusion of allopregnanolone in this healthy foal provided evidence that 5-alpha reduced pregnanes can cross the blood–brain barrier and have effects in the CNS. Allopregnanolone concentrations peaked in conjunction with maximum NB effects following infusion. The rapid recovery from NB alterations with no apparent residual deficits once the infusion was discontinued, suggested that allopregnanolone was quickly metabolised in this healthy foal. Similar rapid dampening effects in the CNS and recovery were observed with the use of the neurosteroid anaesthetic alphaxalone in ponies undergoing castration (Leece *et al.* 2009). As allopregnanolone is apparently metabolised rapidly, the clinical signs associated with NMS in foals would be expected to dissipate rapidly. However, clinical manifestations of NMS can last several days, suggesting ongoing persistent production and release of allopregnanolone or other

neurosteroids responsible for such observations. It is also unclear what triggers and stops these events in affected foals. Progesterone levels in this healthy foal decreased with age and are in agreement with the results of previous work (Holtan *et al.* 1991). The rise in DHEA between birth and age 6 h in this foal appeared to be neither testicular nor adrenal in origin as determined by constant levels of luteinising hormone and pregnanes, respectively, and was therefore deemed unlikely to be of biological relevance. Recently, higher plasma concentrations of progesterone, epitestosterone and androstenedione were found in NMS foals compared with foals with other disorders (J.E. Madigan and B. Tóth unpublished data). Findings from that work, along with the NB alterations induced by the infusion of allopregnanolone support our proposed hypothesis that NMS is in part a manifestation of persistent foetal HPA status mediated and sustained by elevated concentrations of progestagens as occurs naturally in the foetus (Warnes *et al.* 2004). The foetus must rapidly change from the quiet suppressed state *in utero* to one of arousal, and attempts to rise shortly after birth. A failure of the transition from the foetal HPA status to immediately post birth signals to engage the newborn into normal post foaling neurobehaviour may be the cause or involved in part in the pathogenesis of NMS. Further, the measured neurosteroids and altered neurological status in this study suggest that neurosteroids readily cross the blood–brain barrier and exert altering CNS effects compatible with NMS in affected foals. Certainly some foals suffer severe birth hypoxia and recover, and have been included in the broad description of NMS. However, the recovery from severe birth hypoxia would be expected to be slow and likely to have residual neurological deficits as documented in all other mammalian species suffering severe birth hypoxia (McAuliffe *et al.* 2006). We propose that ongoing production of pregnanes by the foal's brain and adrenal glands causes the clinical signs observed in foals with NMS and that rapid recovery of signs with no apparent residual deficits would be compatible with the decline of pregnane-mediated sedative type effects (Zhu *et al.* 2001). It is unclear how foals that are normal at birth develop NMS within the first 48 h of life. However, we speculate that a similar mechanism reported in neonatal sheep may occur whereby neonatal stress can increase allopregnanolone production by the brain and release of deoxycorticosterone from the adrenal glands, which the brain metabolises into 5 α -tetrahydrodeoxycorticosterone (TH-DOC), another CNS depressant (Hirst *et al.* 2008). Obtundation, seizures and hyperaesthesia are common signs of NMS. Whilst the infused neuroactive steroid allopregnanolone has a dampening effect in the CNS, others within the large spectrum of neurosteroids, including metabolites of allopregnanolone, have excitatory effects that may be associated with seizures and hyperaesthesia (Rogawski and Reddy 2004). Neurosteroid concentrations in clinical NMS are likely to be a far more complex condition than that represented by infusion of one compound. Further work is indicated to evaluate the role of pregnanes in foals with NMS.

Parameter	0	1	2	3
Mentation and reaction to stimuli	Normal, bright, alert, responsive	Mildly obtunded, slightly decreased or increased reactivity to stimuli	Moderately obtunded, moderately decreased or increased reactivity to stimuli	Severely obtunded with hyper-reactivity to stimuli, to comatose
Ability to stand	Stands unassisted	Stands with minimal assistance	Stands with marked support	Unable to stand
Bonding to mare	Actively bonds with and follows mare	Slightly reduced interaction with mare	Aimless wandering or periods of reduced responsiveness to mare	Unaware of mare
Ability to nurse	Latches on and nurses effectively	Searches out teat or bottle but does not nurse vigorously	Weak, ineffective suckling reflex	No suckling reflex
Seizures	None	None	None, limb stretching, paddling	Seizures
Ear position	Erect	Erect	Ears partially erect	Floppy, no tone

Table 4 Neurobehavioral scoring system. The scores for each parameter evaluated are added for a total score (minimum 0, maximum 18). For each parameter, a score of 0 = normal, 1 = mild, 2 = moderate, 3 = severely affected.

Age (hrs)	Time point	Neuro behavior score	DHEA	17-OH pregnenolone	Progesterone	Pregnenolone	5- α Dihydroprogesterone	Allopregnanolone	Pregnane diol
0	10 minutes after birth	0	37.940	1.295.070	3.126	3.074.455	ND	ND	110.674
6	Pre-infusion	0	86.93	91.877	880	1.279.409	ND	ND	67.134
6 $\frac{1}{4}$	Bolus 0.05 mg/kg IV, CRI 0.02 mg/kg/min	14	74.475	73.982	776	1.252.565	18.065	478.909	63.241
6 $\frac{1}{2}$	15 minutes post initial infusion	3	1.232.216	95.275	1.189	1.206.684	18.842	126.566	73.497
6 $\frac{3}{4}$	Bolus 0.2 mg/kg IV	15	56.910	57.581	646	1.144.544	28.406	466.208	79.574

Table 5. Steroid concentrations in a healthy foal infused with allopregnanolone. All concentrations are in pg/mL, neurobehavior score (0 = normal, minimum abnormal = 1, maximum abnormal = 18), DHEA = dehydroepiandrosterone, ND = not detected.

3.4. Experiment 4.

As published in Equine Veterinary Journal 45. (2013) 661–665:” Abnormal plasma neuroactive progestagen derivatives in ill, neonatal foals presented to the neonatal intensive care unit”

Authors: J. E. Madigan, E. F. Haggett, K. J. Pickles, A. Conley, S. Stanley, B. Moeller, B. Toth and M. Aleman

We propose that NMS may comprise of more than one phenotype: foals with hypoxia and ischaemia and foals with persistence of foetal hypothalamic-pituitary-adrenocortical (HPA) axis and increased pregnanes (pregnenolone, progesterone and metabolites) that recover rapidly with no apparent residual neurological deficits. The aim of this study was to determine the steroid profile of foals with NMS and compare it with that of foals with other neonatal diseases and healthy control foals.

Materials and methods

Foals

The NMS foal group (n = 32; 15 colts and 17 fillies) and the other neonatal disease foal group (n = 12; 4 colts and 8 fillies) comprised foals admitted to the University of California, Davis Veterinary Medical Teaching Hospital in 2008 and Rossdale and Partners, Newmarket, UK in 2010 and 2011. To be included as a foal with NMS other disorders with a similar clinical presentation, such as prematurity and sepsis, were ruled out based on a minimum database (published sepsis score, complete blood count, chemistry panel, blood gases, indirect blood pressure, central venous pressure, blood culture, urinalysis, abdominal ultrasound and carpi, tarsi, thoracic and abdominal radiography) (Brewer and Koterba 1988). Foals with a sepsis score of 11 or greater were additionally classed as septic (Brewer and Koterba 1988). Historical knowledge of pre-, intra- or postnatal hypoxia was recorded. Clinical signs of NMS included altered mentation (obtunded, stuporous, comatose), decreased bonding to the mare, vocalisation, aimless wandering, hyper- or lack of reactivity to stimuli, seizures and abnormal ear position. Foals were subjectively scored by the attending clinician as mild-moderate if able to nurse and ambulate with help or severe if recumbent and unable to nurse, even with help. Foals in the other neonatal disease group (sick, non-NMS controls) were randomly selected based on client consent and availability of the authors for sample collection.

A third group of healthy control neonatal foals (n = 10; 4 colts and 6 fillies) was recruited from the 2009 and 2010 foal crops at the Center for Equine Health, University of California, Davis. Inclusion criteria for control foals included a term birth (>320 days gestation) with normal, uncomplicated delivery and physical examination. All foals were less than 48 h of age at

enrolment into the study. No attempts were made to standardise treatments given to the foals during hospitalisation. Outcome was recorded as survival to discharge. The study was approved by the University of California Institutional Animal Care and Use Committee and client consent obtained prior to enrolment in the study.

Sample collection and analysis

Heparinised blood was collected from healthy control foals at 0, 24 and 48 h following birth. Samples were collected from NMS foals and other neonatal disease foals after initial stabilisation and thereafter at the designated 24 and 48 h time points as appropriate. For foals presenting at birth, samples were collected within 2 h of parturition. Whole blood was immediately centrifuged after collection and plasma stored at -80°C until analysed. Plasma was analysed by liquid chromatography mass spectrometry (LC-MS) utilising online sample extraction by turbulent flow chromatography (TFC) and detection by select reaction monitoring (SRM) on a triple quadrupole mass spectrometer. Samples were diluted 2:1 with water fortified with 4 internal standards: D3- Testosterone, D3-Boldenone, D7-Androstenedione and D3-Testosterone Sulphate. Analytes were separated by liquid chromatography using a Thermo TLX-2 TFC system with a Thermo Cyclone P extraction column and an ACE C18 analytical column. Analytes were introduced by electrospray ionisation to a Thermo TSQ Vantage triple-quadrupole mass spectrometer operating in both negative and positive modes. Free steroid concentrations of 34 steroids were monitored in one analytical method over a 24 min run time. Detection and quantitation was accomplished using 3 or more SRM transitions per compound for all compounds other than 17-hydroxy pregnenolone where single ion monitoring (SIM) was utilised. This method was validated and the following assessed for each analyte: linearity, limit of detection, limit of quantitation, accuracy, precision, matrix effects, extraction recovery and potential endogenous interferences. The following steroids were evaluated: pregnanes including progesterone, 17-hydroxy-progesterone, 5 α dihydroprogesterone, pregnenolone, allopregnanolone and pregnanediol; androgens and oestrogens including nandrolone sulphate, boldenone sulphate, 17- β oestradiol sulphate, testosterone sulphate, 1,4-androstadiene-3,17-one, testosterone glucuronide, 19-norandrostenedione, boldenone, androstenedione, nandrolone, oestrone, testosterone, epinandrolone, epitestosterone, 6- α -hydroxyandrostenedione, nandrolone glucuronide, 17- β oestradiol, 17- α oestradiol, 19-norepiandrosterone, dehydroepiandrosterone (DHEA), DHEA-sulphate, 17-hydroxypregnenolone, 5- α dihydronandrolone, 5- α -estran-3- β -17- α diol, 5- α dihydrotestosterone, 19-nor-androsterone, 5- β dihydrotestosterone, oestrone sulphate. These steroids were chosen due to convenience of a pre-existing, extensive steroid panel.

Data analysis

Descriptive data are reported as median and ranges. Friedman tests were used for repeated measures analysis of steroid concentrations of healthy foals. Kruskal–Wallis tests were used for multiple group comparisons. Following a significant Kruskal–Wallis test, Mann–Whitney tests were used for non-paired 2 group comparisons with Bonferroni–Holm correction. Nonparametric tests were chosen based on the failure of the data to conform to normal distributions using a Kolmogorov and Smirnov test and inability to transform the data using conventional methods. Level of significance was set at $P < 0.05$.

Results

On presentation, 19 NMS foals were graded as mild-moderate and 13 as severe. Altered states of consciousness of foals with NMS ranged from mildly obtunded to stuporous to comatose. Several progestagens were detected in extremely low concentrations (data not shown). Five steroids (both pregnanes and androgens) were consistently identified among foal samples: progesterone, pregnenolone, androstenedione, DHEA and epitestosterone. Healthy foals showed progressive, significant decreases in these steroids over the first 48 h of life (progesterone $P < 0.0001$; pregnenolone $P < 0.0001$; androstenedione $P = 0.009$; DHEA $P = 0.006$; epitestosterone $P = 0.004$) (Fig 5). There was no significant difference in healthy foal pregnane or androgen profiles between genders. Compared with healthy foals, NMS foals showed increased concentrations of androstenedione ($P = 0.02$) and progesterone ($P = 0.04$) at 0 h (within 2 h of birth), androstenedione ($P = 0.0002$), DHEA ($P = 0.001$), epitestosterone ($P = 0.0004$), progesterone ($P = 0.0001$) and pregnenolone ($P = 0.0007$) at 24 h of age and androstenedione ($P = 0.0008$), DHEA ($P = 0.007$), progesterone ($P = 0.0001$) and pregnenolone ($P = 0.003$) at 48 h of age (Fig 5, Table 6). Sick control foals also had significantly increased concentrations of progesterone ($P = 0.001$) at 0 h, androstenedione ($P = 0.005$), DHEA ($P = 0.003$), progesterone ($P = 0.01$) and pregnenolone ($P = 0.0009$) at 24 h and androstenedione ($P = 0.004$), progesterone ($P = 0.0004$) and pregnenolone ($P = 0.0006$) at 48 h compared with healthy foals (Fig 1, Table 1). Compared with sick control foals, NMS foals had significantly higher concentrations of epitestosterone at 0 and 24 h of age ($P = 0.02$, $P = 0.002$, respectively). In contrast, sick control foals had significantly higher progesterone concentrations than NMS foals at 0 h ($P = 0.01$). While pregnane concentrations of sick control foals remained increased above those of healthy foals, their progesterone and pregnenolone concentrations decreased significantly ($P = 0.02$, $P = 0.04$, respectively) over 48 h. In contrast, steroid concentrations of NMS foals remained increased and showed a trend of increasing concentration over time (Fig 5). When considering the NMS foal population, foals with NMS alone had significantly higher DHEA concentrations than foals with NMS and another disease ($P = 0.02$). There was no significant difference in pregnane concentrations between mild-moderate and severely affected NMS foals (data not shown). When considering all ill foals

admitted to the neonatal intensive care unit (NICU) (i.e. collating NMS and sick control foal data), there was no significant difference in pregnane concentrations between survivors and nonsurvivors, septic and nonseptic individuals and foals with and without a known history of hypoxia (data not shown).

Discussion

The results of this study confirm that there are differences in the pregnane profiles of neonatal healthy foals, foals with NMS and foals with other clinical diagnoses. Pregnane concentrations of healthy neonatal foals declined rapidly, to essentially zero, within 48 h of birth in agreement with the study by Houghton et al. (Houghton et al. 1991). The foetal foal is subjected to high levels of progesterone and other progestagens *in utero* (Holtan et al. 1991), deemed important in providing tonic inhibition of foetal central nervous system (CNS) activity and damping movement to prevent maternal damage (Mellor et al. 2005). Injections of progesterone or its metabolites into the ovine foetal circulation in late gestation reduce foetal electroencephalograph, electrocorticograph and electrooculograph activity, breathing movements and behavioural arousal, while inhibition of placental progesterone enhance these parameters (Crenshaw et al. 1966, Crowley et al. 1997, Nicol et al. 1997, Nicol et al. 2001). The loss of placentally-derived precursors at birth and the switch to adrenal or other derived precursors causes this dramatic decline in pregnane concentrations shortly after birth in healthy neonates (Hirst et al. 2006). Apart from epitestosterone concentrations of sick control foals, foals presenting ill to the NICU (i.e. NMS and sick control foals) had higher concentrations of all measurable pregnanes than healthy controls within 2 h of birth. Pregnane concentrations of NMS foals remained increased over the 48 h time period in contrast to those of sick control foals that had significantly lower progesterone and pregnenolone concentrations at 48 h compared with birth. Serial blood sampling with continued elevation or increasing pregnane concentrations over 48 h of age may therefore prove useful in aiding diagnosis and possibly prognosis of NMS; however, further work is required to validate this possibility. These observations support the hypothesis of a delayed, or interrupted conversion from intra- to extra-uterine life in ill, neonatal foals, particularly those with NMS. This mechanism may be similar to that reported in foals of mares treated with the progestagen altrenogest, which have a slower adaptation to the extra-uterine environment (Neuhauser et al. 2007). These steroids are suspected to be of adrenal origin based on extensive studies of neonatal lamb neurosteroid production (Mellor et al. 2005). Neonatal foals in this study and that of Rossdale (Rossdale et al. 1997) showed endogenous rises in neurosteroid concentrations thus eliminating placental origin. The higher DHEA concentrations in NMS foals compared with foals diagnosed with NMS and another disease suggests different adrenocortical responses in these foal subsets. Pregnane profiles did not appear to differ between mild-moderate and severely affected foals

although it is likely that a larger population needs to be sampled to detect such differences. Furthermore, the categorisation used may have been inappropriate for finding such differences. In previous studies, pregnane concentrations decreased in foals with NMS as they displayed clinical improvement (Rossdale et al. 1995). This observation could not be validated by the current study as concentrations were only measured over the first 48 h of life. Pregnane concentrations were not significantly different between survivors and nonsurvivors in this study and, again, the short sampling period is likely to have precluded the ability to detect this finding. The effect of a known hypoxic episode on plasma pregnane profiles was examined due to the suggested aetiological role of hypoxia in NMS. Hypoxia did not appear to have an effect on pregnane concentrations; however, it is impossible to accurately evaluate this criterion, particularly with regard to intra-uterine hypoxia. Differences in concentrations of pregnenolone and pregnanediol between sick and healthy foals have previously been described (Rossdale et al. 1995). Pregnanediol was not consistently measurable in the current foal population whereas the androgens, androstenedione and epitestosterone have not previously been identified in foals with NMS (Rossdale 2004). It is likely that these analytes were simply not investigated in the analytical method originally developed (Houghton et al. 1991). The use of LC-MS allows better differentiation of the individual steroids than can be achieved by radioimmunoassay (Madigan et al. 2012). The cause of the increased plasma pregnane concentrations detected in ill neonatal foals cannot be elucidated from this study; however, the authors propose that these concentrations occur as a result of persistence of foetal signals for the *in utero* state of being quiet and non-ambulatory. Certain pregnanes, such as progesterone and their metabolites have neuromodulatory, anaesthetic and anxiolytic properties important for tonic inhibition of foetal CNS activity and damping foetal movement to prevent maternal damage (Mellor et al. 2005). The receptors in the foetal brain are more sensitive to these pregnanes, compared with the receptors in the adult brain (Crossley et al. 2000). Infusion of the neurosteroid pregnane allopregnanolone to a healthy neonatal foal induced obtundation, lack of affinity for the mare and decreased response to external stimuli (Madigan et al. 2012). These effects were short-lasting and associated with measurable concentrations of pregnanes (Madigan et al. 2012). These suggest that these steroids can cross the blood–brain barrier and exert neuromodulatory effects, which at high concentrations may have a dampening effect in the CNS with resulting alterations in states of consciousness, altered behaviour and responsiveness to stimuli, such as observed in NMS cases. Specific enzymes may be inhibited in these foals and the roles of 5 α -reductase, 3 β -hydroxysteroid dehydrogenase and 3 α -hydroxysteroid dehydrogenase need to be further evaluated. It has been suggested that the 5 α -reduction step may be critical in determining the quantity of 5 α -reduced pregnane metabolites either produced from progesterone within the foetal brain or derived from precursors entering the brain from the blood (Nguyen et al. 2003). The underlying cause of

any possible abnormal adrenal function is also not known; it may reflect a state of dysmaturity in which the foal fails to transition to extra-uterine life or may reflect hypoxic injury to the HPA axis (Rossdale et al. 1997). Another potential reason for persistence of foetal hormones is a failure of normal events of parturition that are essential for the transition from the *in utero* foetal cortical status to extra-uterine behavioural status. Regulation of the neuroactive steroid content in the foetal ovine brain is independent of adrenal steroidogenesis and hypothalamic–pituitary factors (Nguyen et al. 2004); however, in the neonate, concentrations of some neurosteroids and their precursors in the peripheral circulation dramatically affect concentrations in the brain (Nguyen et al. 2003). Lastly, another possible mechanism would be the reversion to foetal cortical status when adverse post birth circumstances occur. The syndrome of reversion to foetal circulation is a well known and accepted consequence of adverse birth and post birth events, which is seen in both maladjusted foals and those with other neonatal diseases and causes the neonate to revert to mechanisms that regulated the cardiovascular system *in utero*. It is also possible that the increased pregnanes are acting in a neuroprotective role as has been reported in other species. Stress (hypoxia, endotoxin) in the neonatal period increases neurosteroid concentrations in the brain of newborn lambs (Hirst et al. 2006, Nguyen et al. 2003), suggested to represent an endogenous protective mechanism. Similarly, acute, but not chronic, hypoxic stress during pregnancy increases foetal neurosteroid concentrations (Hirst et al. 2006). Indeed, inhibition of neurosteroid synthesis increases asphyxia-induced brain injury in late gestation foetal sheep (Yawno et al. 2007). Phenotypical characteristics of ‘maladjusted foals’ may have more than one aetiology (hypoxic/ischaemic vs. nonhypoxic/ischaemic). We speculate that the nonhypoxic foal is the one that lacks the normal transition from synthesis to inhibition of specific neurosteroids for readiness for birth (from foetal to neonatal neurosteroid profile). This may explain why some affected foals have a relatively fast recovery with no remaining long-term neurological deficits and no apparent or known hypoxic events prior, during or shortly after birth. Plasma concentrations of progestagens were measured in this study but ideally concentrations in brain tissue, known to be much higher than those in the peripheral circulation, should have been measured. Neurosteroids and their precursors are known to cross the blood–brain barrier (Wang et al. 1997) and are extremely potent such that small concentrations can have large local effects in neuronal tissue. Further, many steroids are metabolised to other compounds prior to exerting their effects. In conclusion, specific alterations in pregnane profiles were detected between healthy control foals and ill, neonatal foals presenting to NICU. The anaesthetic and sedative properties of these pregnanes may account for the behavioural alterations seen in maladjusted and ill foals. These differences may reflect a delayed or interrupted transition from foetal to neonatal HPA status. Repeated measurements of these pregnanes over time may be useful for distinguishing between foals with NMS and other neonatal disorders. Increased pregnane

concentrations may cross the blood–brain barrier and be responsible for some of the behavioural and neurological alterations observed in foals with NMS.

Steroid (ng/ml)	Healthy controls (n = 10)	Sick controls (n = 12)	NMS (n = 32)	P value (Kruskal–Wallis)
Androstenedione	nd ^a (nd–0.51)	1.15 ^b (nd–7.65)	6.56 ^b (nd–57.34)	<0.0001
DHEA	7.68 ^a (nd–117.90)	101.14 ^{a,b} (nd–1412.60)	92.98 ^b (nd–511.06)	0.028
Epitestosterone	nd (nd–0.15)	nd (nd–13.20)	0.53 (nd–11.53)	0.1
Progesterone	nd ^a (nd–0.09)	6.09 ^b (1.97–14.28)	14.22 ^b (0.75–73.61)	<0.0001
Pregnenolone	103.76 ^a (5.6–313.20)	1119.40 ^b (248.26–3416.08)	1922.08 ^b (nd–15,917.33)	0.001

DHEA = dehydroepiandrosterone; nd = not detectable. Groups with differing superscripts are significantly different (Mann–Whitney).

Tabel 6. Median (range) serum steroid concentrations (ng/ml) in healthy, sick control and neonatal maladjustment syndrome (NMS) foals at 48 h of age

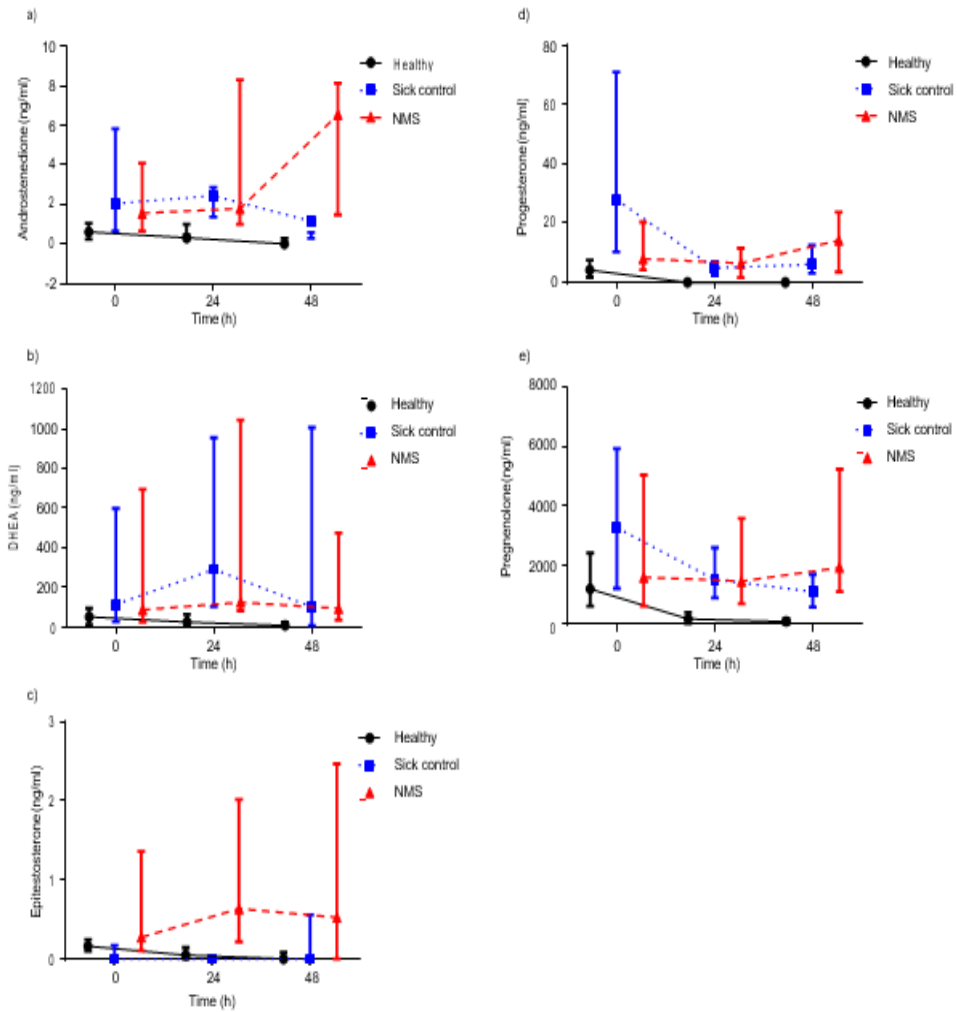


Fig 5. Median and interquartile range plasma steroid concentrations (ng/ml) of a) androstenedione, b) dehydroepiandrosterone (DHEA), c) epitestosterone, d) progesterone and e) pregnenolone for healthy, sick control and neonatal maladjustment syndrome (NMS) foals during the first 48 h of life.

3.5. Experiment 5

As published in Journal of Veterinary Internal Medicine 28 (2014) 1294–1300:

” Plasma adrenomedullin concentration in critically ill neonatal foals”

Authors: B. Tóth, N.M. Slovis, P.D. Constable, and S.D. Taylor

We hypothesized that p[AM] is increased in septic neonatal foals compared to sick nonseptic and healthy control foals, and that p[AM] is predictive of survival in septic neonatal foals. Based on these hypotheses, the objectives of the study were to compare p[AM] in septic, sick nonseptic, and healthy neonatal foals, and determine the prognostic value of p[AM] for survival in septic neonatal foals.

Materials and Methods

Data collection

This study was approved by the Purdue University Institutional Animal Care Committee. Critically ill neonatal foals < 1 week of age were recruited from Purdue University’s Veterinary Teaching Hospital and from Hagyard Equine Medical Institute during the 2011 and 2012 foaling seasons. Jugular venous blood was collected at admission for measurement of p[AM]. Critically ill foals were categorized as septic (sepsis score of > 11 and/or positive blood culture) or sick non-septic (sepsis score ≤ 11 and negative blood culture results when available). Foals euthanized due to financial constraints were excluded from the study.

Healthy control foals were recruited from the equine teaching herd at Washington State University’s College of Veterinary Medicine and from Gumz Farms, a privately-owned farm in Kentucky. To be included in the control group, foals had to have an uncomplicated birth following normal length of pregnancy (> 330 days), a normal physical examination (PE), blood IgG > 800 mg/dl at 24 hours of age.

Information obtained from critically ill foals included signalment (year of admission, age of foal at admission [hours], breed and gender), history of dystocia or caesarean section, evidence of prematurity / dysmaturity, PE findings at presentation including behaviour, mentation, rectal temperature, heart rate, respiratory rate, capillary refill time (seconds), and fecal consistency. Admission mean arterial pressure and blood lactate concentrations were also recorded when they were available.

Information obtained from healthy foals included history, signalment, PE findings, and blood IgG concentration at 24 hours of age.

Venous and arterial blood gas analysis (pH, partial pressure of carbon dioxide [PCO₂], partial pressure of oxygen [PO₂], and base excess) and haematologic data (PCV, MCV, MCHC, haemoglobin concentration, total white blood cell count, segmented neutrophil, band neutrophil count, presence of toxic neutrophils, lymphocyte, and platelet count) and plasma fibrinogen, were obtained. Biochemical findings (total CO₂, total calcium, phosphorus, sodium, chloride, potassium, magnesium, anion gap, glucose, creatinine, serum urea nitrogen, total protein, bilirubin, globulin and albumin concentrations and activity of alkaline phosphatase [ALP], creatine kinase [CK], aspartate aminotransferase [AST], gammaglutamyl transferase [GGT] activity) were also collected. Blood culture results (positive or negative), type of organism (Gram positive, negative, anaerobic or fungal) and species were also recorded when available. Treatments, complications during hospitalization, length of hospitalization, outcome, and hospital bill were also obtained. Additional diagnostic modalities performed in some foals included ultrasonography, radiography, rectal digital palpation, jaundiced foal agglutination testing, fecal bacterial culture, synovial fluid analysis, and cerebrospinal fluid analysis.

Laboratory and Analytical Methods

Blood samples for culture were obtained after aseptic preparation of the skin and removal of 20 – 30 mL of blood into a sterile syringe from a jugular vein or following sterile placement of an intravenous catheter. The sampling needle was discarded and a separate needle was used to transfer 3 - 7 mL of blood into a commercially available blood culture bottle (BBL SEPTI-CHEK TSB (Tryptic Soy Broth), Becton Dickinson Microbiology Systems, Beckon Dickinson and Company, Cockeysville, MA, USA).

For determination of p[AM], 7 - 10 ml blood was transferred into a plastic tube containing ethylenediaminetetraacetic acid (EDTA). Following centrifugation at 3200 rpm for 15 min at 4 °C, plasma was transferred into plastic tubes and placed into a - 20 °C or - 80 °C freezer. Samples in the -20 °C freezer were relocated to a -80 °C freezer within an acceptable time frame based on plasma AM stability (Nishio et al. 1997). Plasma AM concentration was determined using a previously validated, commercially available ELISA assay developed for equine AM (USCN Life Technologies, Wuhan, China). The company's reported validation data are the following: intra-assay CV < 10%; inter-assay CV < 12%; recovery 85-100%; and linearity 83-105%. (performed on serum, heparin and EDTA plasma) A standard curve was created for each ELISA plate during this study. Sample concentration was determined by plotting optical density to the standard curve. The intra-assay CV was 9.8% and the pooled inter-assay CV was 9.7% based on 5 plates and assay detection range was 12 – 1,000 pg/ml, with an assay sensitivity of 6.5 pg/ml. Values < 6.5 pg/mL were reported as 6.5 pg/mL.

For routine haematologic and biochemical analyses, blood was either collected from a jugular vein or from a jugular catheter upon admission. In some instances, blood was also collected into heparinized 3-mL plastic syringes for immediate blood gas analysis. Venous blood samples were collected as described above, and arterial samples were collected from either the dorsal metatarsal or the transverse cubital artery. Collected blood was placed into glass tubes containing EDTA for haematology, and sodium citrate for fibrinogen concentration and heparin for serum biochemistry. Haemograms were performed with a commercial automated multichannel blood cell-counting system (Cell-Dyn 3500R, Abbott Diagnostics, Abbott Park, IL, USA) with differential counts performed by manual cytologic examination of a blood smear. Serum biochemistry was performed with commercial automated analyzers (Vitros 5,1 FS Chemistry System, Ortho-Clinical Diagnostics, Inc, Rochester, NY, Olympus AU400 Beckman Coulter, Inc, Brea, CA, USA). Blood IgG concentration was determined semi-quantitatively with a commercially available concentration immunoassay technology test (SNAP Foal IgG Test, Idexx Laboratories, Inc, Westbrook, ME, USA). Plasma fibrinogen concentration was measured with a commercial automated analyzer (Stago STA Compact, Diamond Diagnostics, Holliston, MA, USA) or with heat precipitation tests. Arterial and venous blood pH, PCO₂, and PO₂ were measured within 15 minutes of blood collection with an automated blood gas analyzer (Olympus AU400 Beckman Coulter, Inc, Brea, CA) Blood L-lactate was measured with a commercially available lactate analyzer (Accutrend, Roche Diagnostics, Raritan, NJ).

Statistical Analysis

Statistics were performed with commercially available software (SAS 9.3, SAS Institute Inc, Cary, NC, US). A Distribution of data was tested with the Shapiro-Wilk normality test. Continuous data were expressed as median and range. Mann-Whitney U test was used to test the differences between critically ill and healthy foals, septic and sick non-septic foals, and survivors versus non-survivors. The best cut-offs for p[AM] identified in a multivariate logistic regression model to be associated with health status was performed by use of receiver operating characteristic (ROC) curve analysis. It was considered as non-informative (area under the curve [AUC], 0.50), less accurate (AUC, 0.50–0.70), moderately accurate (AUC, 0.71 – 0.90), or perfect (AUC, 1.00). For all analyses described above, a P-value < 0.05 was considered significant.

Results

Study population

A total of 151 neonatal foals (90 sick, 61 healthy) < 1 week of age were included. Forty-six percent of hospitalized foals (42/90) were classified as septic and 54% (48/90) as sick non-septic. The median age of septic and sick non-septic foals was 10 and 8 hours, respectively, with the median age of all critically ill foals at admission of 10 hours (0-168 hours). For healthy control foals, the median age was 24 hours. The breeds represented by hospitalized foals included Thoroughbred (75/90), Quarter Horse (8/90), Standardbred (3/90), Warmblood (1/90), Clydesdale (2/90) and Arabian (1/90). Healthy foals consisted of Arabians (30/61) and Quarter Horses (31/61). Of the hospitalized foals, 54% (49/90) were colts and 46% (41/90) fillies. In the healthy foal group, 54% (33/61) were colts and 46% (28/61) fillies. The overall survival rate in the septic and sick non-septic groups were 30/42 (71.4%) and 38/48 (79.2%), respectively with an overall survival rate of 75.5% (68/90).

Comparison of p[AM] in different groups

There was no significant difference in p[AM] either in septic versus sick, non-septic foals ($P=0.051$) or in survived versus non-survived foals ($P=0.71$). There was a highly significant difference between critically ill foals and healthy controls ($P<0.0001$) (Table 7 and 8; Figure 6). Plasma [AM] was compared within subgroups of critically ill foals based on historical findings or final diagnoses. No particular diagnosis was associated with a significantly different p[AM] (Table 9).

Association of clinical and clinicopathologic findings with outcome

Survivors had higher rectal temperatures ($P=0.048$). From the clinicopathologic variables survivors exhibited lower anion gap ($P=0.003$), total calcium ($P=0.023$), blood L-lactate ($P=0.025$) and MCV ($P=0.005$); and higher tCO_2 ($P=0.004$). Surviving foals had longer duration of hospitalization ($P<0.0001$) and higher hospital bill ($P<0.025$). (Table 10).

Clinical and clinicopathologic variables in septic and sick non-septic foals

Significant differences were detected in the septic versus sick non-septic groups. Septic foals had higher plasma L-lactate concentration ($P=0.004$) total serum bilirubin concentration ($P=0.01$), and higher incidence of failure of transfer of passive immunity (FTPI) ($P=0.02$). Septic foals also had a higher incidence of toxic changes ($P=0.03$) and band neutrophilia ($P=0.002$) (Table 11). There was no significant correlation between sepsis score and p[AM] ($\rho = 0.058$).

Receiver operating characteristic curve

A p[AM] cut-off value of 0.041 ng/mL provided an AUC of 0.75 with a sensitivity of 91.1% and a specificity of 54% to predict critical illness (Figure 7).

Discussion

We have found that p[AM] is not associated with sepsis or survival. There is a 6-fold increase in the median p[AM] in critically ill foals compared to healthy controls. Considering that the group of critically ill foals entailed approximately 20 different clinical diagnoses, we conclude that p[AM] is a marker of health rather than an indicator of a specific clinical entity in neonatal foals. There was also no significant correlation between sepsis score and p[AM], which may indicate a lack of true relationship between severity of sepsis and p[AM] in neonatal foals assuming that the conventional categorization using a weighted sepsis score and / or positive blood culture for sepsis in this study population was accurate.

Human studies revealed different degree of changes in p[AM] with various clinical diseases (Gibbons 2007), with up to 50-fold increase in septic patients compared to healthy individuals (Ueda et al. 1999). LPS and inflammatory cytokines during sepsis and systemic inflammation induce AM gene expression in various tissues; and during septic shock p[AM] culminates more than in any other pathological condition in human patients (Hirata 1996). Contrary to these facts, there was no significant difference in p[AM] between septic foals and non-septic foals, which may be due to the inaccuracy of the current sepsis status categorization. It is also possible that the aetiopathogenesis and disease course of equine neonatal sepsis is different compared to sepsis in humans. Experimental studies on laboratory animals show that there is a progressive and significant increase in p[AM] starting around 2 hours following a septic insult (Koo et al. 2001). Nearly half of the septic foals were less than 2 hours of age, which may have resulted in lower p[AM] values at the time of collection. This could explain why increased concentrations were not detected in the septic foal group. The increase in p[AM] is progressive and reaches plateau approximately 20-30 hours after the onset of septic insult (Koo et al,

2001), therefore it is possible that trends during disease course are more representative of diagnosis and prognosis than a single time point sampling.

There were no significant differences in p[AM] values in survivor versus non-survivor foals. This may be due to the lack of direct or indirect effect of p[AM] in survival. However, in human clinical sepsis studies, p[AM] appears to be not only a marker for evaluating disease severity, but also an early predictor correlating with subsequent organ dysfunction and outcome (Ueda et al. 1999). Since foals subjected to euthanasia due to financial reasons were excluded from the study, it is less likely that euthanasia biased the statistical results. Despite that decisions for euthanasia were based on highly trained and experienced clinicians' discretion, it is still possible that with continuous intensive care a fraction of the euthanized foals would have recovered and survived to hospital discharge.

Plasma [AM] was evaluated within the following subgroups: FTPI, dystocia, caesarean-section, prematurity and NMS). It is noteworthy that none of these entities were associated with a significantly different p[AM]. In human clinical trials, pregnancy complications (preeclampsia, preterm delivery, low birth weight) are associated with significant changes in p[AM] (Lenhart and Caron 2012), while in infants with birth hypoxia, p[AM] is increased (Di Iorio et al. 2004). Plasma [AM] has been linked to numerous other pathologic conditions including cardiovascular, renal and lung diseases (Nishikimi et al. 2003); however, these were not evaluated in this study due to statistically inadequate numbers.

Independent of p[AM], we found that survivors had longer hospitalization time and higher hospital bills. Of the clinical and clinicopathologic variables survivors exhibited lower anion gap, total calcium, plasma L-lactate and MCV; and higher tCO₂. These findings are in agreement with previous prospective and retrospective studies (Hoffman et al. 1992, Corley et al. 2005, Hurcombe et al. 2009, Dembek et al., 2013). The relationship between these variables and p[AM] was not further evaluated in this study.

Further, we also found that septic foals had higher plasma L-lactate, total bilirubin and higher incidence of FTPI than sick non-septic foals. Septic foals also had a higher incidence of toxic changes and band neutrophils. Some of these findings are mutually inclusive due to the use of sepsis score for categorization of neonates. These findings also correspond to previously published data (Hoffman et al. 1992, Corley et al. 2005, Pusterla et al. 2006; Hurcombe et al. 2009, Dembek et al. 2013). Given that p[AM] was markedly different in critical illness, construction of a ROC curve using the most sensitive and specific cut-off concentration was aimed for prediction of critical illness in foals. This is an important approach as prediction of illness has not been previously attempted in equine neonatal medicine. The sepsis score developed by Brewer and Koterba was reported to have a 93% sensitivity and 86% specificity

to identify septic neonates at a particular study population in South-Eastern United States. The latter was reevaluated by Corley and Furr and resulted in conflicting data providing lower specificity, sensitivity and lower negative predictive value (Corley and Furr, 2003). Currently serum IgG is considered as one of the more useful and practical method of health evaluation in newborn foals (Metzger et al. 2006). In our study 35% of foals diagnosed with critical illness had normal IgG levels, which questions the sensitivity of serum IgG in early detection of perinatal diseases. Our ROC curve with the most sensitive and specific cut-off concentration enables to categorize 75% of foals as healthy or ill, thus based on our study, we propose that AM maybe a better indicator of health than serum IgG. The sensitivity and specificity of serum IgG compared to p[AM] in detecting perinatal illnesses would require further investigation.

One major limitation of the study includes lack of age-matched controls. It is likely that p[AM] undergoes dynamic changes during the early neonatal period, therefore sequential measurements and strict age matched controls may have provided accurate and more generalizable data for diagnosis and prognosis. Additionally, p[AM] may still be increased physiologically in the first few hours of age due to foaling stress similar to cortisol, however the latter returns to normal by 24 hours of age (Silver et al 1990). Also, another limitation is that transportation to hospital may have contributed to stress-induced AM release, while controls were not transported. It must be noted though that the time between leaving a farm and blood collection was usually less than 2 hours in the majority of cases due to the close proximity of breeding operations, which is shorter than the minimum amount of time needed to have significant increase in p[AM] compared to baseline according to experimental studies (Koo et al. 2001).

In summary, p[AM] appears to be a promising marker of perinatal health in newborn foals; however, it is not associated with sepsis, specific clinical entities, or survival based on this study population. Further studies are warranted to evaluate the prognostic value of serial p[AM] measurements in critically ill foals.

Selected group	Plasma adrenomedullin (ng/ml) median (range)
Healthy (n=61)	0.03 (0.01-0.54)
Sick (n=90)	0.17 (0.01-0.73)

Sick survived (n=68)	0.15 (0.01-0.73)
Sick died (n=22)	0.24 (0.02-0.72)
Sick non-septic (n=48)	0.16 (0.01-0.73)
Sick septic (n=42)	0.18 (0.01-0.73)
Blood culture negative (n=49)	0.17 (0.02-0.73)

Table 7. Descriptive statistical data of p[AM] in selected groups. Medians and ranges are presented in ng/ml.

Group 1. median (range) ng/mL	Group 2. median (range) ng/mL	P value
Healthy 0.03 (0.01-0.54)	Sick 0.17 (0.01-0.73)	<0.0001
Survival 0.15 (0.01-0.73)	Non-survival 0.24 (0.02-0.72)	0.051
Sick, non-septic 0.16 (0.01-0.73)	Septic 0.18 (0.01-0.73)	0.70

Table 8. Statistical comparison of p[AM] in selected groups using Mann-Whitney U test.

Groups based on historical finding or diagnosis	Remaining critically ill foals (90-n)	P value
FTPI (n=58)	32	0.38
Dystocia (n=41)	49	0.34
C-section (n=11)	79	0.57
Placentitis (n=26)	64	0.63
HIE (n=36)	54	0.74

Premature (n=19)	71	0.77
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Table 9. Comparison of p[AM] based on history or clinical diagnosis in the affected and unaffected foals

Variable of interest	Survived (n=68) median (range)	Not survived (n=22) median (range)	P value
Hospitalization (days)	5.5 (2-58)	2.5 (1-8)	<0.0001
Rectal temperature (°F)	38 (34-40)	37 (32-38.5)	0.025
Hospital bill (USD)	3500 (800-35000)	2550 (880-11000)	0.048
Admission sepsis score	10 (0-21)	12 (4-24)	0.09
CRT (sec)	2 (1-4)	2 (1-5)	0.13
Age (hrs)	10 (1-168)	4 (1-120)	0.22
Respiratory rate (breaths/min)	36 (16-140)	34 (12-68)	0.40
Heart rate (beats/min)	106 (64-180)	104 (72-160)	0.76
Anion gap (mmol/L)	12.0 (4.0-40.0)	17.5 (9.0-40.0)	0.003
Total CO₂ (mmol/L)	28.0 (10.0-35.0)	23.0 (5.0-32.0)	0.004
Calcium (mg/dL)	11.2 (8.4-17.9)	12.35 (10.1-19.9)	0.023
L-lactate (mmol/L)	5.0 (1.7-19.9)	8.4 (3.5-20.8)	0.025
Glucose (mg/dL)	118 (6-240)	73 (14-265)	0.06
Potassium (mmol/L)	3.9 (2.3-5.6)	4.1 (2.8-5.6)	0.08
Creatinine (mg/dL)	2.65 (0.8-29.7)	3.8 (1-33.3)	0.11
BUN (mg/dL)	20 (5-100)	21 (15-116)	0.17
Phosphorus (mg/dL)	5.2 (3.3-13.2)	6 (3.6-14.9)	0.20
Albumin (g/dL)	2.9 (1.9-3.8)	3.0 (2-3.8)	0.20

AST (U/L)	129 (39-2482)	111 (49-669)	0.46
Sodium (mmol/L)	137 (123-152)	137 (129-151)	0.62
Globulin (g/dL)	1.6 (0.7-3.9)	1.5 (0.8-4.3)	0.63
Total protein (g/dL)	4.4 (3.3-7.1)	4.5 (3.4-7.1)	0.75
ALP (U/L)	1957 (366-4362)	2160 (420-4200)	0.75
CK (U/L)	430 (49-4751)	411 (70-2364)	0.78
Bilirubin (mg/dL)	3.2 (0.9-7.7)	3.4 (0.8-34.0)	0.86
GGT (U/L)	23.5 (7-82)	20.0 (9-107)	0.89
Chloride (mmol/L)	98 (80-112)	98 (75-122)	0.89
MCV (fL)	40.0 (33.0-47.0)	42.4 (34.0-55.8)	0.005
RBC (M/ μ L)	9.7 (5.6-14.0)	8.9 (3.8-13.1)	0.13
Neutrophils (K/ μ L)	5.5 (0.2-22.5)	4 (0.1-26.5)	0.26
Haemoglobin (g/dl)	13.4 (7.9-42.3)	12.85 (4.5-16.2)	0.34
MCHC (g/dL)	34.8 (30.1-43.2)	34.3 (29.7-37.2)	0.38
WBC (K/ μ L)	6.65 (1.1-26.5)	5.7 (0.7-30.1)	0.44
PCV (L/L)	39.0 (14.9-50.8)	37.5 (15.0-49.8)	0.52
Toxic changes present	29	8	0.62
Fibrinogen (mg/dL)	300 (100-600)	269 (72-600)	0.66
Lymphocytes(K/ μ L)	1.3 (0.2-3.4)	1.2 (0.1-4.6)	0.77
Bands (K/ μ L)	0 (0-2.9)	0 (0-2.0)	0.84
Platelets (K/ μ L)	302 (44-506)	325 (76-625)	0.98

Table 10. Statistical comparison of clinical and clinicopathologic data in the survived vs. not-survived groups using Mann-Whitney U test.

Variable of interest	Sick, non-septic (n=48) median (range)	Sick septic (n=42) median (range)	P value
Admission sepsis score	9 (0-11)	14 (4-24)	<.0001
Hospital bill (USD)	2700 (800-35000)	3800 (1000-9700)	0.07
Hospitalization (days)	4 (1-13)	6 (0.2-58)	0.19
CRT (sec)	2 (1-3)	2 (1-5)	0.27
Rectal temp (F)	37.8 (34.3-40)	37.8 (32-39)	0.31
Resp. rate (breaths/min)	33 (20-120)	36 (12-140)	0.42
Age (hrs)	8 (1-72)	10 (1-192)	0.46
Heart rate (beats/min)	104 (72-180)	104 (64-180)	0.89
L-lactate (mmol/L)	5.0 (1.7-10.7)	6.6 (2.9-20.8)	0.004
FTPI present	25	33	0.01
Bilirubin (mg/dL)	2.6 (0.9-5.8)	3.5 (0.8-34.0)	0.02
Total CO ₂ (mmol/L)	27 (12-35)	25 (5-35)	0.083
Anion gap (mmol/L)	13.0 (4.0-36.0)	14.0 (6.0-40.0)	0.12
Potassium (mmol/L)	3.8 (2.3-5.6)	4.1 (2.9-5.6)	0.17
Glucose (mg/dL)	119 (6-226)	92.5 (12-265)	0.24
Creatinine (mg/dL)	2.9 (1-33.3)	3.7 (0.8-29.7)	0.39
GGT (U/L)	18 (9-82)	28 (7-107)	0.40
Albumin (g/dL)	2.9 (1.9-3.8)	2.9 (2.0-3.8)	0.48
Calcium (mg/dL)	11.7 (8.4-17.2)	11.7 (9.4-19.9)	0.49
Globulin (g/dL)	1.4 (0.8-3.9)	1.7 (0.7-4.3)	0.63
Total protein (g/dL)	4.5 (3.4-6.6)	4.4 (3.3-7.1)	0.65
Chloride (mmol/L)	98 (75-112)	98 (80-122)	0.70

ALP (U/L)	1770 (95-4200)	2197 (420-4362)	0.71
BUN (mg/dL)	20 (9-100)	21 (5-116)	0.76
AST (U/L)	120 (39-2482)	120 (45-1825)	0.78
CK (U/L)	474 (10-2707)	324 (68-4751)	0.95
Sodium (mmol/L)	137 (123-152)	137 (126-151)	0.97
Phosphorus (mg/dL)	5.5 (3.5-10.8)	5.3 (3.3-14.9)	0.98
Bands (K/μL)	0 (0-2.6)	0.02 (0-2.9)	0.002
Toxic changes present	14	23	0.03
PCV (L/L)	40.2 (22.8-50.8)	38.0 (14.9-49.8)	0.069
Haemoglobin (g/dL)	13.6 (8.4-16.2)	12.9 (4.5-15.9)	0.12
MCHC (g/dL)	34.9 (31.3-43.2)	34.4 (29.7-37.2)	0.21
Fibrinogen (mg/dL)	300 (100-500)	300 (72-600)	0.27
Neutrophils (K/ μ L)	5.5 (0.69-22.5)	3.8 (0.1-26.5)	0.45
WBC (K/ μ L)	6.7 (1.06-5)	5.8 (0.7-30.1)	0.46
RBC (M/ μ L)	9.6 (6.8-13.1)	9.2 (3.8-14.0)	0.50
Platelets (K/ μ L)	325 (128-625)	300 (44-582)	0.53
Lymphocytes(K/ μ L)	1.4 (0.1-3.4)	1.1 (0.2-4.6)	0.58
MCV (fL)	41.0 (33.0-46.0)	40.0 (34.0-55.8)	0.83

Table 11. Statistical comparison of clinical and clinicopathologic data in the sick, non-septic vs. septic groups using Mann-Whitney U test.

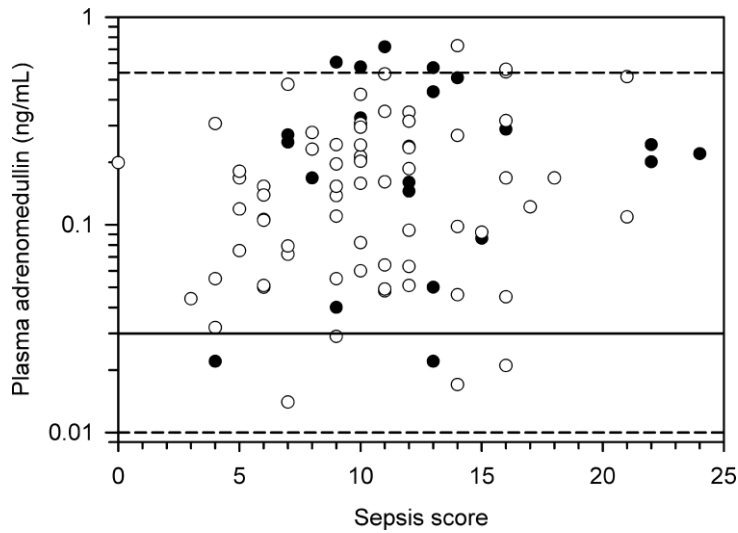


Figure 6. Scatterplot of relationship between plasma adrenomedullin concentration in critically ill neonatal foals ($n = 90$) and sepsis score on admission. The thick horizontal line is the median plasma adrenomedullin concentration in healthy neonatal foals, and the dashed horizontal lines show the 95% confidence interval for the plasma adrenomedullin concentration in healthy neonatal foals. Filled circles represent foals that did not survive and open circles represent foals that survived to discharge from the hospital.

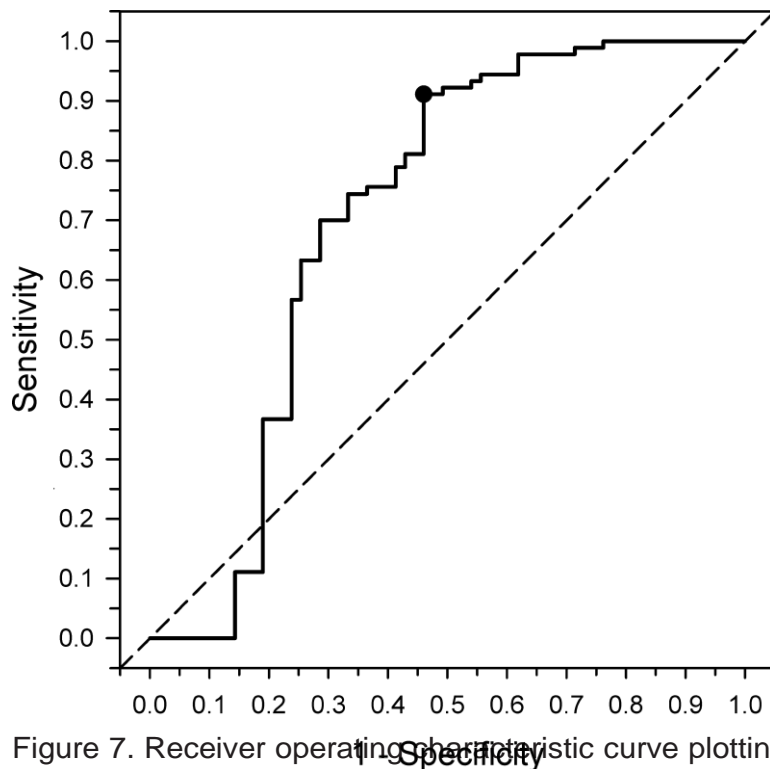


Figure 7. Receiver operating characteristic curve plotting test sensitivity against (1 – test specificity) at the optimal cutoff value for plasma adrenomedullin concentration (0.041 ng/mL) in differentiating healthy from critically ill neonatal foals. The dashed diagonal line

is the chance line (sensitivity [Se] = specificity [Sp] = 0.50; equivalent to tossing a coin, area under the curve = 0.50). The filled circle is the test Se (0.91) and Sp (0.54) at the optimal cutoff value for plasma adrenomedullin concentration. The area under the curve is 0.75.

4. Overview of new scientific results

1. In our 1st experiment, we have shown that application of the described restraint device induces foals to lie down, and remain in lateral recumbency. Physical restraint appeared to decrease the foals' voluntary motor activity; and also triggered somnolence as observed by the authors in all foals along with recorded periods of wakefulness/drowsiness, and late onset slow wave sleep (delta waves, K-complexes). The latter findings are in agreement with the previous descriptions of SWS in humans, and horses. We named this phenomenon based on its phenotypic characteristics: squeeze induced somnolence (SIS).
2. In our 2nd experiment, diagnoses of meningitis and meningoencephalomyelitis were made for 28 equine patients from University of California Davis Veterinary Medical Teaching Hospital during a 25-year period. The overall prevalence of disease for the study period at this hospital was 0.04% (28/70,000) and 0.2% (28/14,000) for horses with neurologic disease. The overall prevalence of disease in neonatal foals was 0.2% (2/1,000) and 0.5% (2/400) in foals with septicaemia. These data also show that foals with septicaemia have a higher likelihood to have meningitis than even adult horses with neurologic signs. In this retrospective study altered mental status, cranial nerve deficits, and gait abnormalities were identified as the most common neurologic signs. Additionally, 5 pathogens not previously described were isolated from a few horses of the present study, 3 of which were isolated from CSF samples obtained before death.
3. In our 3rd experiment, infusion of allopregnanolone to a healthy foal produced marked NB effects. Infusion of allopregnanolone provided evidence that 5-alpha reduced pregnanes can cross the blood-brain barrier and have effects in the CNS. Allopregnanolone concentrations peaked in conjunction with maximum NB effects following infusion. The rapid recovery from NB alterations with no apparent residual deficits once the infusion was discontinued suggested that allopregnanolone was quickly metabolised in this healthy foal. However, clinical manifestations of NMS can last several days, suggesting ongoing persistent production and release of allopregnanolone or other neurosteroids responsible for such observations. It remains unclear what triggers and stops these events in affected foals.

4. Our 4th study revealed that there are differences in the pregnane profiles of neonatal healthy foals, foals with NMS and foals with other clinical diagnoses. Pregnane concentrations of healthy neonatal foals declined rapidly, to essentially zero, within 48 h of birth. Apart from epitestosterone concentrations of sick control foals, foals presenting ill to the NICU (i.e. NMS and sick control foals) had higher concentrations of all measurable pregnanes than healthy controls within 2 h of birth. Pregnane concentrations of NMS foals remained increased over the 48 h time period in contrast to those of sick control foals that had significantly lower progesterone and pregnenolone concentrations at 48 h compared with birth. These observations support the hypothesis of a delayed, or interrupted conversion from intra- to extra-uterine life in ill, neonatal foals, particularly those with NMS.

5. In our 5th experiment, in which we investigated the role of a polypeptide of mainly adrenal origin, we have found that plasma adrenomedullin (pAM) was not associated with sepsis or survival. There was a 6-fold increase in the median p[AM] in critically ill foals compared to healthy controls. Considering that the group of critically ill foals entailed approximately 20 different clinical diagnoses, we concluded that p[AM] is a marker of health rather than an indicator of a specific clinical entity in neonatal foals. There was also no significant correlation between sepsis score and p[AM], which may indicate a lack of true relationship between severity of sepsis and p[AM] in neonatal foals assuming that the conventional categorization using a weighted sepsis score and / or positive blood culture for sepsis in this study population was accurate.

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6. Publications in peer-reviewed journal related to the thesis

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