University of New England

Beyond the Miracle Foal: A Study into the Persistent Effects of Gestational Immaturity in Horses

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Certification of Thesis

I hereby certify that the content of this thesis has not been and is not being submitted for any other degree to this or any other university. I also certify that all help received in preparing this thesis and all sources used have been duly acknowledged.


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Abstract

Breeding horses can be a financially and emotionally expensive undertaking, particularly when a foal is born prematurely, or full term but dysmature, showing signs normally associated with prematurity. In humans, a syndrome of gestational immaturity is now emerging, with associated long-term sequelae, including metabolic syndrome, growth abnormalities and behavioural problems. If a similar syndrome exists in the equine and can be characterised, opportunities for early identification of at-risk individuals emerge, and early intervention strategies can be developed. This thesis explores the persistent effects of gestational immaturity manifest as adrenocortical, orthopaedic and behavioural adaptation in the horse.

Basal diurnal cortisol levels do not differ from healthy, term controls, but when subjected to a low dose ACTH challenge, gestationally immature horses presented a depressed or elevated salivary cortisol response, suggesting bilateral adaptation of the adrenocortical response. This may be reflected in behavioural reactivity, but the outcomes from a startle test were inconclusive. A survey of horse owners indicated that gestationally immature horses tended to be more aggressive and active than controls, aggression being displayed mostly in families of Arabian horses. Case horses also tended to be more active, intolerant, and untrusting. Gestationally immature horses have restricted growth distal to the carpal and tarsal joints, and this results in a more ‘rectangular’ conformation in adulthood compared to controls. They also often present with angular limb deformities that adversely affect lying behaviour and recumbent rest. This, however, can be mitigated using analgesic therapy, suggesting chronic discomfort.

Based on these findings, it is reasonable to postulate that a syndrome of gestational immaturity may persist, both clinically and sub-clinically, in affected adult horses. Further work is required to fully characterise this syndrome and validate the outcomes in larger populations, thereby providing a foundation for interventions applicable in the equine breeding industry.

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Dedication

For the horses. For the ones who make it through in one piece, and for the ones who don’t, but who teach us instead.

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Chapter 1

INTRODUCTION

"Although all mammals experience variation in gestation length, is the syndrome of Pre Term Birth itself unique to humans?"(Phillips et al., 2015)

"There is emerging evidence linking preterm birth to cardiometabolic risk factors such as abdominal adiposity, insulin resistance and raised blood pressure, as well as to shorter stature, and internalizing problem behavior." (Finken, 2017)

Equine breeding can be an expensive undertaking. Uniquely among large domesticated mammals, the arrival of a single foal may be a long-planned event involving large stud and veterinary fees, and sometimes involving years of selective breeding and development of a business model. In many instances, loss of a neonatal foal may be financially and emotionally challenging to the breeders. Consequently, the arrival of a premature foal often presents both a veterinary emergency and the potential loss of financial investment. At the end of a process that can cost thousands of dollars, the outcome is all too frequently a horse that is undersized, prone to unsoundness, and unsuited for its intended athletic purpose.

Equine prematurity has been extensively researched within the field of perinatology. A body of research from Newmarket, UK, headed by P.D. Rossdale, comprehensively investigated the clinical and laboratory aspects of prematurity through a seminal series of papers in the Equine Veterinary Journal (Fowden,

1984, Pipkin et al., 1984, Rossdale et al., 1984a, Rossdale et al., 1984b, Silver et al., 1984, Webb et al., 1984).

In the growing foal, orthopaedic outcomes have been studied, mostly (but not exclusively) within broader research into Angular Limb Deformities (ALDs) (Levine, 2015, McIlwraith, 2003, Witte and Hunt, 2009). However, it is within the currently emerging, cross-species field of Developmental Origins of Health and Disease that the ongoing effects of premature and, to a lesser extent, dysmature birth in the adult horse is emerging as an important area for investigation (Fowden et al., 2013, Fowden et al., 2016, Jellyman, 2015, Valenzuela et al., 2016).

The present research hypothesises that a syndrome of development issues relating to gestational immaturity may be present in horses. To borrow from human literature, a premature birth can be defined as a combination of anatomical, physiological, biochemical, endocrinological, immunological, and clinical events. It has been described as a process in which some parturition pathways are activated, but others are not, presenting many aetiologies and being syndromic in its effects (Romero et al., 2006). As in human prematurity, some horses present some outcomes, while others present none.

This thesis proposes a syndromic effect of gestational immaturity in the horse evidenced by adrenocortical, orthopaedic and behavioural adaptations. These are addressed sequentially, with each area contributing to an emerging phenotype of the adult horse with a history of gestational immaturity. The thesis commences by defining gestational immaturity for the purposes of this research, with a chapter that includes a published review of studies into gestational length in horses. This

is followed by chapters concerned with the development of the Hypothalamic-Pituitary-Adrenal axis and adrenocortical dysregulation; incomplete ossification and its sequel of Angular Limb Deformity; growth retardation and conformational ratios of affected horses; the expressed behaviours of gestationally immature horses. Experimental chapters are presented in scientific journal format, preceded by a review of relevant scientific literature. The thesis concludes by postulating that as in human medicine, a syndrome relating to gestational immaturity persists in adult horses.

The author brings equine industry experience as an established musculoskeletal therapist to this research. Having continued to practice throughout its duration, she has located premature and dysmature cases and related controls from her professional network and introduced these to the studies. The limited availability of cases and related controls has presented a challenge, as has their distance from the author's base. Consequently, small groups of horses have been introduced periodically throughout the research, with most included in one or two studies, but none in all. Three horses were donated to the research and this contribution proved particularly valuable, with findings at necropsy adding to the understanding of pathologies associated with gestational immaturity.

Chapter 2

DEFINING GESTATIONAL IMMATURITY

"A mature neonate is one in which the organ systems are sufficiently developed at the time of delivery to undertake the transition from intra- to extra-uterine life without difficulty or assistance." (Rossdale, 1988)

"Many early studies were retrospective and focused on general populations of sick foals. More current prospective and retrospective studies are identifying differences in various prognostic indicators based on primary diagnostic categories, differences in management techniques, and changes in various indicators with time." (Wilkins, 2015)

2.1 Introduction

In horses, there is little consensus on a normal range of equine gestation and consequently the estimation of mean gestation length varies. The considerable number of variables involved creates difficulties for the researcher, in that chronological baselines for a 'normal' gestation are vaguely defined. In contrast, it is possible to closely define human prematurity as parturition at ≥ 21 d before the mean pregnancy duration of 266 d (Lester, 2011, Phillips et al., 2015), i.e. before the start of the 37th week of gestation. This makes identification of the neonate requiring intervention relatively straightforward, while a plethora of standard tests identifies those that are term but immature.

Parameters for equine full term and premature gestations vary in academic and popular texts (Rossdale, 1981, Rossdale, 2002, Madigan, 2014), while the gestational length given for equine prematurity of < 320 d is a generalised guideline. Many neonatal foals do not present as might be expected by their position on the gestational timeline: foals with apparently short gestations can present in excellent health and fully developed, whilst others that are full term can be dysmature and sick (Lester, 2011, Rossdale, 1976, Rossdale, 1997, Rossdale et al., 1984b,). Consequently, assessing dysmaturity can be a complex interpretive situation when signs are not obvious, especially given that not all foals are seen by veterinarians. For this reason, prematurity has typically been identified on a case by case basis.

2.2 The Aetiology of Prematurity

2.2.1 Causes

Prematurity in horses can be caused by a variety of factors. The most common is the presence of a placental abnormality, such as infection, oedema or placental separation (Lester, 2011, McIlwraith, 2003, Rossdale, 2002). Placental blood supply can also be compromised by colic or shock in the mare, a parasitic burden, or inappropriate administration of exogenous oxytocin or prostaglandins during pregnancy, for instance when intestinal colic is misinterpreted as ineffective labour (Lester, 2011). If twin foetuses are present, placental insufficiency also leads to early parturition and/or dysmaturity. The mare's general physical condition, including her nutritional status, can also affect foetal health through a

reduced delivery of nutrients. Mare diseases can also alter gestation length or compromise development (Lester, 2011, McIlwraith, 2003, Rossdale, 2002). Less common causes include thyroid abnormalities in the mare, and consumption of tall fescue grasses infected with endophytes (Cross, 1995). In Australia, another cause of compromised foals is Equine Amnionitis and Foetal Loss (EAFL), caused by the mare's ingestion of the spines of hairy caterpillars (*Ochrogaster lunifer*) (Todhunter et al., 2014).

2.2.2 Clinical Signs

The initial clinical indications that a premature foal is developmentally delayed at birth may include the following clinical and behavioural signs (Fig. 2.1). The foal is frequently small and low birthweight, appearing emaciated. It is weak and takes longer than 2 h to rise, with a depressed suckling reflex. Once standing, it may present hyperextension of the fetlock joints (due to laxity of the flexor tendons). It may have a silky coat over its back and hindquarters. The foal may have soft lips, and an orange-red tongue and buccal mucous membranes. The amniotic fluid or the foal's coat may be meconium stained, caused by the foal passing faecal material in utero due to foetal stress. It may exhibit a low body temperature and respiratory distress, with an increased heart rate (Madigan, 2014, Rossdale, 1976, Wilkins, 2015). Dysmature foals are usually emaciated and weak, and may also present some of the signs of prematurity, including hyperextension (Madigan, 2014).

Fig. 2.1 Premature foal presenting diminutive size, weakness, and hyperextension of the hind fetlocks (Image: used with owner's permission).

2.2.3 Laboratory Results

Laboratory findings at this stage include key differences to results for mature foals. Adrenocortical insufficiency is evident in a narrower neutrophil-lymphocyte ratio than in healthy term foals, in addition to higher lymphocyte counts, low plasma cortisol, and a depressed cortisol response to exogenous adrenocorticotropic hormone (ACTH). A compromised respiratory system and respiratory distress are evident in blood gas abnormalities, decreased partial pressure oxygen (PaO_2), increased partial pressure carbon dioxide ($PaCO_2$) and decreased venous pH. Other signs include depressed blood glucose 2 h post-parturition, and decreased absorption of colostral immunoglobulin (Madigan, 2014, Rossdale, 1976, Rossdale et al., 1984b, Silver et al., 1984,).

2.3 Researching Immaturity

Beyond the extremely premature foal born close to 300 d gestation, conception and birth dates offer little guidance as to the neonatal foal's presentation. Instead, only an individual set of physiological, physical and behavioural factors and developmental deficits affecting the individual may be observed and noted. This by necessity leads towards a definition of 'gestational immaturity' as a research category that encompasses both chronologically premature and full term dysmature foals.

In the following review paper, research into the variables affecting gestation length is considered. Research data are analysed to produce evidence of a further variable, that of localised climate, that renders conception and parturition dates of even less consequence in the consideration of a neonatal foal's maturity or lack thereof.

2.4 Article: Equine Gestation Length and Location: Is There More that the Research Could Be Telling Us?

This article was published in the *Australian Veterinary Journal* in December 2017 (Clothier et al., 2017).

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(To appear at the end of each thesis chapter submitted as an article/paper)

We, the Research ~~Master~~/PhD candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated in the *Statement of Originality*.

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We, the Research Master/PhD candidate and the candidate's Principal Supervisor, certify that the following text, figures and diagrams are the candidate's original work.

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Chapter 3

GESTATIONAL IMMATURITY AND ADAPTATION OF THE HYPOTHALAMIC-PITUITARY-ADRENAL AXIS

"Considering the time for fetal adrenocortical maturation, we should be surprised, not that extremely premature infants may have the ability to maintain homeostasis in response to postnatal stress, but that such infants can maintain homeostasis at all." (Watterberg, 2004)

3.1 The Hypothalamic-Pituitary-Adrenal (HPA) Axis

3.1.1 Functions of the HPA Axis

Temporary adaptation of the Hypothalamic-Pituitary-Adrenal (HPA) axis is known to be an outcome of prenatal stress leading to prematurity or gestational immaturity at birth in humans (Davis, 2010, Fernandez, 2009, Maniam, 2014, Soliman, 2004, Sullivan et al., 2008, Watterberg, 2004) and other species, including horses (Fowden et al., 2016, Hart, 2009, Hart and Barton, 2011, Ousey et al., 2004). Recently, growing interest in the perinatal origins of adult disease has stimulated research into the permanent dysregulation of the HPA axis in humans (Dalegrave et al., 2012, Maniam, 2014, Mcmillen, 2005, Sullivan et al., 2008, Winchester et al., 2016), pigs and sheep (Fowden et al., 2016, Jellyman,

2015, Moritz et al., 2011, Sloboda et al., 2007) and, to a lesser extent, horses (Jellyman, 2012a, Fowden et al., 2013, Jellyman et al., 2015).

The HPA axis maintains homeostasis of the body's organ, cellular and hormonal function through its regulation of systemic cortisol. When the central and peripheral nervous systems are activated by physical stressors such as injury, pain and disease, the hypothalamus responds by secreting the corticotropin-releasing hormone (CRH), which in turn stimulates the anterior pituitary gland to release adrenocorticotropic hormone (ACTH) into systemic circulation. ACTH then stimulates the adrenal gland into releasing cortisol from its cortex, the zona fasciculata. The zona fasciculata, which is the gland's middle and largest layer, accounting for roughly 75% of the gland's weight, is sited beneath the zona glomerosa and outside the zona reticularis (Hart and Barton, 2011). It is responsible for producing glucocorticoids on demand. These include cortisol, which it produces by converting cholesterol to pregnenolone, then progesterone, followed by 17-OH progesterone and finally cortisol (Barton, 2008, Dalegrave et al., 2012).

In circulation within the blood stream, cortisol circulates in three forms: free cortisol, protein-bound cortisol and cortisol metabolites. The protein-bound cortisol usually accounts for 90 - 95% of total cortisol, being bound to either corticosteroid binding globulin (CBG) or albumin, which transports the cortisol within the plasma. These proteins also function as a reservoir from which hormones can be released (Cizza, 2012). The free or circulating cortisol remains physiologically active, being available to enter cells via diffusion across the plasma membrane (Barton, 2008, Hart and Barton, 2011). Through passive

diffusion, free cortisol binds to glucocorticoid receptors within the cells, leading to systemic effects that are essential for the body's response to stressors. These include the maintenance of and restoration of normal blood pressure, maintenance of organ function, delivery of energy for tissue repair, inhibition of pro-inflammatory pathways, and diverting energy to defensive functions. (Barton, 2008).

Once free cortisol has exerted its range of physiological effects as a stress response to health and disease, its system effects reduce the physiologic stresses that initially activated the HPA axis. These changes, as well as cortisol itself, act via a negative feedback mechanism to downregulate HPA axis activity. The secretion of CRH by the hypothalamus and ACTH by the pituitary gland are downgraded, with the result that plasma cortisol concentrations are maintained at an appropriate level (Hart and Barton, 2011). When this does not occur, the HPA axis becomes temporarily or permanently dysregulated, with an inappropriately high or low level of circulating cortisol in relation to ACTH (Barton, 2008, Hart, 2009).

3.1.2. The HPA Axis and 'Readiness for Birth'

In horses, the HPA axis has little role to play during intrauterine life until around 300 days gestation when, in the healthy, full term foetus, maturation of the HPA axis and other endocrinological and physiological systems begins (Barton, 2008). At this stage, the HPA axis prepares to produce cortisol in response to stressors

prior to and during parturition, including, for example, maternal stress, placentitis, or malnutrition.

The HPA axis is also involved in maturation of the renin-angiotensin system and somatrophic axis, with the release of adipokines, leptin and the thyroid hormones (Fowden and Forhead, 2009, Fowden et al., 2012). In the same period, the sympatho-adrenal medullary axis matures and will respond to hypoxia during parturition with adrenal catecholamine release. The endocrine pancreas matures in preparation for the maintenance of plasma glucose and insulin levels via pancreatic hormone release. This maturation process continues in the few days after parturition, as the neonatal foal faces the early challenges of extra-uterine life (Fowden and Forhead, 2009, Fowden et al., 2012, Rossdale et al., 1984b).

In concert, these interconnected systems enable the neonatal foal's physiological systems to take over nutritional, respiratory and excretory functions from the placenta. Maturation continues in the first few days of life: following birth, further development of the pituitary and adrenal glands' tissue and function is triggered by a number of factors, including the delivery of arterial oxygen (partial pressure oxygen), temperature decrease, the start of enteral nutrition, locomotion, interactions with the dam, and environmental stimuli (Fowden et al., 2012).

These adaptations and challenges of equine gestation, parturition and the neonatal period that comprise perinatology were extensively researched by Rossdale (1988), who introduced the concept of equine 'readiness for birth' to describe the development of the endocrinological and physiological status of the foal (Rossdale, 1997, Rossdale and Silver, 1982). The absence of this status in the

premature foal was further researched in the 1980s by Rossdale, Fowden, Silver, Forehead et al, who published a seminal series of linked studies on prematurity in the Equine Veterinary Journal (Fowden et al., 1984, Pipkin et al., 1984, Rossdale et al., 1984a, Rossdale et al., 1984b, Silver et al., 1984, Webb et al., 1984).

These and subsequent studies have further defined haematological parameters and measurements of pancreatic B cell activity, adrenocortical and adreno-medullary function, and the renin-angiotensin system in evaluating the readiness, or lack of readiness, for birth and therefore gestational maturity of the neonatal foal (Rossdale et al., 1984b, Rossdale PD, 1991, Rossdale, 2003).

3.2 Maturation of the Equine HPA Axis

3.2.1 The Gestationally Mature Term Foal

In horses, maturation of the HPA axis in readiness for birth occurs later in gestation than in other species (Fowden, 1995). From around 300 d gestation to parturition in the full term foetus, the adrenal gland almost doubles in mass, increasing from around 60 to 100mg/kg BW (Fowden et al., 2012). This increase is primarily the zona fasciculata, or middle layer of the adrenal cortex; as it grows, the number of Golgi receptors increases exponentially, leading to increased ACTH sensitivity (Webb and Steven, 1981). In other species, including sheep, this adrenal growth has been associated with higher levels of steroidogenic

activity, including the increasing secretion of cortisol, the primary hormone produced during both preparation for parturition and parturition itself (Webb et al., 1984). Metabolic mechanisms that have previously protected the foetus from the effect of high cortisol concentration by converting bioactive cortisol to inactive cortisone in the placenta switch to favouring cortisol production (Davies Morel, 2008, Fowden et al., 2016).

Equine studies have reported an elevation of plasma cortisol in the last 3 - 5 days of gestation (Chavatte, 1997, Rossdale, 1997), 72 - 96 h prior to parturition (Silver M, 1994), 48 - 72 h before parturition (Silver, 1994), 24 - 48 h before parturition (Ousey, 1993), the final 5% of healthy gestations (Rossdale, 1973), or the final 1 - 2 % of healthy gestations (Fowden, 1995). Commensurately, Cortisol Binding Globulin (CBG) declines during the last days of gestation, enabling the increase of free cortisol (Ousey, 2011). This increasing concentration of plasma cortisol is associated with structural and functional changes in the lungs, liver, gastrointestinal tract and kidneys (Silver, 1990). It has been shown in other species that this rise in plasma cortisol stimulates the thyroid gland to produce bioactive T3 (triiodothyronine), which is essential for thermogenesis and respiratory function, including the reabsorption of lung liquid in the neonatal animal (Irvine, 1975, Nathanielsz, 2003).

Post-parturition, bioactive cortisol levels peak within the first 2 h, before declining to basal levels at around 24 h, and then stabilising for 7 - 14 d (Fowden et al., 2012). Another study reports a peak at 30 - 60 mins, with decline to basal at 6 - 12 h (Barton, 2008). Plasma ACTH peaks at 5 - 10 mins from birth (Hines, 1993), before decreasing to adult basal levels at around 8 h post-partum (Hart et

al., 2007, Wong et al., 2009), also reported as 6 - 12 h (Hines, 1993). CBG rises over the early months of life, as cortisol levels gradually decline to adult levels (Ousey, 2011). Compared with adult horses, which have a circulating cortisol fraction of approximately 10% of total cortisol, foals have free cortisol levels of around 53% of total, due to their lower protein-binding capacity; this reduces to 40% by 5 - 7 d of age (Hines, 1993). Although the percentage of free cortisol is higher in foals, total cortisol concentrations are lower (Irvine, 1987).

The HPA axis is believed to near maturity within the first 14 d, with adrenocortical responsiveness adequate to meet the stressful stimuli that increase during the first few weeks of life (Ousey, 1993, Ousey et al., 2004, Silver, 1994, Silver et al., 1984).

3.2.2. The Gestationally Immature Foal

There are a number of known causes of accelerated maturation of the HPA axis in the equine foetus, including hypoxemia, maternal malnutrition, placental or foetal infection, and the use of exogenous glucocorticoids such as dexamethasone and betamethasone in the pregnant mare (Fowden et al., 2016, Lester, 2011). The nature and duration of the stimulus can affect the rate of overall foetal maturation (Lester, 2011) and it is widely suggested that the rise in corticosteroids triggered by foetal stress can lead directly to early parturition (Davies Morel, 2008, Pashen and Allen, 1979a, Pashen and Allen, 1979b, Rossdale, 1992, Silver, 1994); foals born prematurely due to placentitis in the mare are known to present an earlier maturation of the HPA axis than mature term foals (Ousey et al., 1999) and this can be a positive factor for postnatal survival (Lester, 2011). The very short

window for HPA axis maturation in foals leads to more incidences when this system is dysregulated in equines compared with other species (Rossdale and Silver, 1982).

Hospitalised neonatal foals, including those born prematurely or presenting signs of gestational immaturity, provide an insight into the HPA axis that has failed to mature pre-parturition. At birth, premature foals born < 320 d differ from healthy term foals in having higher plasma ACTH and lower cortisol (Ousey et al., 2008, Rossdale et al., 1984a). Cortisol fails to rise during the first 2 h of life and remains < 33% that of healthy term foals (Rossdale et al., 1984a). Conversely, ACTH does continue to rise, and is 2 - 5-fold higher than in term foals during this period (Rossdale et al., 1984a, Silver et al., 1984). (There are exceptions: when prematurity has been caused by fescue grass toxicity, both low ACTH and low cortisol have been found in the foal (Ousey et al., 2008).

Inappropriately low cortisol and high ACTH peak levels indicate dysregulation of the HPA axis at adrenal level, although other components of the HPA axis may remain functionally unaffected (Silver et al., 1984). The nature of the foals' critical illnesses indicate the non-maturation of other endocrinological and physiological systems: for example, both low plasma cortisol and low T3 due to incomplete thyroid development are found in immature neonatal foals at immediate risk of respiratory illness (Silver, 1991, Wallace, 1996). When transient and related to critical illness in human infants, this condition is called Relative Adrenal Insufficiency (RAI) or critical illness related corticosteroid insufficiency (CIRCI), and is recognised as a threat to survival (Aucott et al., 2008, Fernandez, 2009, Hart, 2009).

Some hospitalized neonatal foals aged 1 - 7 d that were gestationally mature at birth have shown both high cortisol and high ACTH, demonstrating that neonatal mortality is not always associated with low plasma cortisol (Gold et al., 2007, Hart, 2009, Hurcombe, 2011, Panzani et al., 2009), and that some foals are able to mount an adrenocortical stress response to illness (Fowden et al., 2012). Hypoglycaemia and hypotension have been reported as stressors leading to increased plasma cortisol concentrations in sick foals aged 7 - 14 d (Silver, 1987), as have been umbilical cord occlusion, placental insufficiency, poor uterine perfusion, or deficiencies in maternal nutrition (Fowden et al., 2016). Meanwhile, dysmature foals, i.e. born > 320 d gestation but showing signs, behaviour and physiology associated with prematurity, including, but not limited to, weakness, diminutive size and weight, silky hair, domed foreheads, floppy ears, hyperextension of fetlocks (Madigan, 2014), can present plasma cortisol and ACTH levels intermediate to those of premature and gestationally mature term foals (Silver et al., 1984). Rossdale described such dysmaturity as a third or 'twilight' state of maturity (Rossdale et al., 1984a, Rossdale et al., 1984b).

Foals with minimal adrenocortical responses continue in what has been termed 'an ongoing foetal state' (Ousey et al., 2008). These are the least likely to survive (Hart, 2009), being ill equipped to counter the stresses of the early transition to extra-uterine life or disease (Panzani et al., 2009). Clinical conditions found in severely gestationally immature foals and associated with immature endocrinological and physiological systems, include neutropenia, high lymphocytes, hypoventilation and respiratory distress, low venous pH, depressed blood glucose, low colostral immunoglobulin and (IgG) absorption, with pneumonia, cardiovascular collapse, systemic sepsis and diarrhoea as further

complications (Lester, 2005, Lester, 2011, Madigan, 2014). Multiple stresses trigger further release of ACTH, yet the cortisol response is inadequate to maintain blood pressure, provide nutrients to tissues and control inflammatory responses (Hart, 2009); increased ACTH does, however, stimulate the sympatho-adrenal medullary system to secrete catecholamines and other neuropeptides, which have further cardiovascular and metabolic effects (Ousey et al., 2008). These increasingly insurmountable obstacles to survival leave the foal likely to succumb to the so-called 'second day syndrome' (Rossdale, 1993).

Affected foals that survive the first 24 - 48 h can continue to display adrenocortical dysfunction during the first 2 weeks. Surviving premature foals born at < 320 d have been found to have lower plasma cortisol at birth than mature term foals, and cortisol levels remain lower at 1 week of age than those of adult horses. This differs to mature term foals, which present high levels of cortisol during the post-partum period, gradually declining to the same level as adult horses at 1 week of age (Hines, 1993).

Again, there are exceptions. Foals born prematurely due to maternal placentitis have demonstrated an accelerated maturation of the HPA axis without apparent dysfunction, as have foals suffering intrauterine growth restriction (Ousey et al., 2004, Rossdale, 1991). The former require only low levels of intensive care in order to survive (Rossdale, 1991). This suggests that the cause of gestational immaturity and possibly its timing within the gestational timeframe may influence the extent of HPA axis maturation and the likelihood of temporary dysregulation in the neonatal foal.

A summary of the maturation of the HPA axis in the gestationally mature and immature foal is presented in Table 3.1.

Table 3.1 HPA axis maturation and activity in late gestation and the perinatal period.

Perinatal Phase	HPA Axis Activity in the Foal	
	Gestationally mature, full term	**Premature or gestationally immature**
290 d to final 3 - 5 d of gestation	• Rapid growth of adrenal gland commences, as zona fasciculata cortical layer develops (Fowden et al., 2012) • Cortisol converts to cortisone in placenta (Ousey, 2004)	• Early activation of HPA axis due to maternal placentitis (Ousey et al., 2004)
Final 3 - 5 d of gestation	• Increased ACTH from anterior pituitary (Barton, 2008) • Adrenal cortices produce cortisol from cholesterol (Ousey, 2004) • Steep elevation in cortisol secreted by adrenal (Rossdale, 1973, Ousey, 1993, Silver, 1994, Fowden, 1995) • Cortisol stimulates organ maturation • Cortisol stimulates thyroid hormone production, including active T3 (Irvine CH, 1975)	• Parturition triggered by elevated cortisol (Davies Morel, 2008)
At parturition	• Plasma ACTH peaks within 5 - 10 mins from birth (Hines, 1993)	• Premature foals born < 320 d may have higher ACTH and lower cortisol (Rossdale et al., 1984a, Ousey et al., 2008) • Immature foals may have adequate ACTH and cortisol intermediate to that of premature/normal foals (Rossdale et al., 1984a, Ousey et al., 2008)

	Readiness for Birth	• At 30 mins, foals show higher basal ACTH concentrations than mature foals. (Barton, 2008) ***Reduced Readiness for Birth***
1 ≤ 4 h post-parturition	• High ACTH levels (Silver et al., 1984) , gradually decreasing to adult basal levels within 6 - 12 h (Hines, 1993, Wong et al., 2009, Hart et al., 2007) • Peak plasma cortisol at around 2 h of age (Silver et al., 1984) ***Viability***	• Plasma ACTH rises to 2 - 5 x normal levels (Rossdale et al., 1984a, Silver et al., 1984) • Plasma cortisol levels remain < 33% of normal (Rossdale et al., 1984a) • Sick foals, incl. premature show no significant difference in basal CC to controls. (Hart, 2009) • Sick foals incl. premature have high basal plasma ACTH:cortisol ratios. (Hart, 2009) • Sick foals incl. premature have low basal plasma ACTH. (Hart, 2009) ***Reduced Viability***
4 h ≤ 7 d post-parturition	• Plasma cortisol declines to basal level within 24 h • At 12 - 24 h, mean basal cortisol remains lower than in adult horses, although basal ACTH is higher. (Barton, 2008) ***Viable Foal***	• Sick foals incl. premature have higher basal plasma CC. (Hart, 2009) • Sick foals incl. premature have low basal plasma ACTH. (Hart, 2009) • Sick foals incl. premature have normal basal ACTH:cortisol ratios. (Hart, 2009) • Sick foals with very low plasma cortisol less likely to survive post 48 h (Lester, 2005, Lester, 2011) ***Reduced Viability / Non-Survival***

3.3. Assessing the Adrenocortical Response

3.3.1. The Use of Exogenous ACTH

The measurement of hormones is used to measure HPA axis activity through static or dynamic testing. Static testing of basal plasma (endogenous) ACTH and cortisol can lead to an assessment of hypo or hyper-adrenocorticism; dynamic testing and measurement of circulating hormones following administration of synthetic (exogenous) hormones provides information about adrenocortical responsiveness.

In foals, the administration of short-acting or long-acting synthetic ACTH stimulates the adrenal cortex to secrete cortisol, enabling a diagnosis of adrenocortical insufficiency / RAI (Madigan, 2014). Synthetic ACTH has been used to measure the adrenocortical responsiveness of foals, central to the assessment of maturity and health status of the neonatal foal (Fowden et al., 2012) and, via maternal administration, of the foetus. Different methods have been used by researchers and practitioners, which, as with human medical research, can make comparison of findings and establishment of diagnostic criteria problematic (Hart, 2009).

In the 1980s, Rossdale's group tested the sensitivity of the adrenal cortex in premature foals with long acting Depot or short acting ACTH / Tetracosactrin (ACTH 1 - 24; Synacthen^{TM,} Novartis or Ciba-Geigy) (Silver et al., 1984, Webb et al., 1984). Validated dosages included a single dose of 0.125 mg, and 3 doses

of 0.4, 0.2 and 0.2 mg, administered intramuscularly per animal (Rossdale PD, 1982).

In the 1990s, concerns were raised in human medical research regarding the effectiveness of high doses of synthetic ACTH in revealing subtler HPA axis dysregulation. Specifically, the standard dose of 250 µg, given intramuscularly or intravenously, was termed 'supraphysiological' in that being highly concentrated, it produced false positives, with cortisol responses well above normal ranges (Rasmuson et al., 1996). This also created clinical risk in cases of RAI associated with critically ill adults and premature neonatal infants (Watterberg, 2004, de Jong, 2006, Aucott, 2012). Subsequently, researchers established that 1 µg of synthetic ACTH was as effective as the standard dose of 250 µg in the identification of RAI in adults with septic shock (Kozyra, 2005) and premature babies (Soliman, 2004).

In equine veterinary medicine, discussion ensued over the ideal dosage of cosyntropin (a synthetic analogue of ACTH) required to evaluate adrenal response for conditions such as RAI in the neonatal foal (Wong et al., 2009). As in human medicine, supraphysiologic doses of 125 µg of ACTH used in the assessment of equine PPID were known to trigger levels of plasma cortisol up to 300-fold of basal, making the assessment of low adrenal responsiveness difficult (Barton, 2008).

Hart et al. (2007, 2009) validated the use of a lower dose of 1, 10, 100 and 250 µg of aqueous synthetic ACTH 1 - 24 (cosyntropin; Cortrosyn TM Amphastar Pharmaceuticals, Rancho Cucamonga, CA), administered intravenously, that was

sufficiently sensitive to register the subtler variations in adrenocortical response. This led to development of a more physiological, paired low dose/high dose ACTH administration (i.e. 10 µg and 100 µg, 30 mins apart) for use with the neonatal foal (Hart et al., 2009).

Research by Bousequet-Melou (2006) tested seven ACTH doses, ranging from 0.005 to 10 µg/kg BW administered intravenously to adult horses, and established that a dose of 0.1 µg/kg BW was sufficient to model the adrenocortical response (Bousquet-Mélou, 2006). Wong et al. (2009) also found that 0.1 µg/kg BW of cosyntropin was an appropriate dosage when tested in foals aged from birth to 12 weeks.

More recently, the ACTH stimulation test has been combined with the measurement of salivary cortisol concentration (SCC), which offers a less invasive method than venepuncture and plasma sample collection. Scheidegger et al. (2016) established the repeatability of a 1 µg/kg BW injected intravenously, with SCC measured as an indicator of adrenocortical response (Scheidegger et al., 2016).

3.3.2. Findings of HPA Axis Dysregulation in Foals

Hart et al. (2009) used the paired low dose / high dose cosyntropin (ACTH) test to assess RAI in hospitalised foals (Barton, 2008, Hart et al., 2007, Hart et al., 2009, Hart, 2009, Hart and Barton, 2011, Hart, 2012). No significant difference was found between the basal cortisol concentrations of hospitalized foals < 4 h of age and age-matched healthy controls.

Sick foals aged between 4 h and 7 d had significantly higher basal cortisol and ACTH concentrations than controls, although the basal ACTH:cortisol ratios were not significantly different (Hart, 2009). Forty-six percent of sick foals had subnormal basal cortisol concentration and 52% had an inadequate adrenocortical response to the high dose (100 µg) test. This adrenal insufficiency correlated with shock and multiple organ dysfunction syndrome (MODS), and in a subgroup of septic foals it also correlated with decreased survival. An inadequate adrenocortical response to the low but not the high dose correlated with an increased incidence of MODS, but not septic shock or survival. Additionally, 22% of sick foals presented negative delta cortisol values. With peak cortisol concentration reached either 30 or 90 mins after administration, minus the time 0 concentration, these foals' post-stimulation values dropped below baseline.

Hart et al. (2009) attributed the blunted responses to the ACTH tests amongst sick foals as being indicative of transient reversible adrenocortical dysfunction resulting in adrenocortical insufficiency during critical illness (Hart, 2009, Hart and Barton, 2011). Sick foals' low basal ACTH and lower basal ACTH:cortisol ratios than controls, indicated dysregulation at the hypothalamic or pituitary levels of the HPA axis, as well as at the adrenocortical level (Hart, 2009). Previous studies correlated low basal ACTH:cortisol ratios with survival and non-survival of septic and critically ill foals (Hurcombe SD, 2008), but this was not amongst the findings of Hart et al.

Correlations with both MODS and septicaemia, illnesses associated with gestational immaturity of physiological and endocrinological systems, suggest that some of the full term foals may have been dysmature at birth, i.e. full term

but showing clinical signs of prematurity. In the current study, one surviving twin was included in the group evaluated: although often born > 320 d, twins are frequently dysmature due to placental insufficiency and associated intrauterine growth restriction. (Lester, 2011) It is also possible that the concurrent low basal corticosteroid levels were a contributory factor to the illnesses. This highlights the challenges facing researchers in identifying and categorising foals in this intermediate, 'twilight' state of maturity (Rossdale et al., 1984a, Rossdale et al., 1984b). A summary of adrenocortical responses in gestationally mature and immature foals is presented in Table 3.2.

Table 3.2. Adrenocortical response in healthy term foals compared with gestationally immature foals, measured through elevations in plasma cortisol concentrations (CC) in response to administration of exogenous ACTH.

Perinatal Phase	Gestationally mature foal	Gestationally immature foal
290 d to final 3 - 5 d of gestation	• Before 290 d, foetal adrenal cortex has minimal to zero response. (Silver, 1994) • Post 290d, small but significant increase in CC. (Silver, 1994) • Final 15-20 d of gestation, adrenal becomes responsive to exogenous ACTH (Ousey, 2011)	
Final 3 - 5 d of gestation	• Foetal response highest in hours immediately preceding birth. (Silver, 1994)	
At parturition		• Pony foals born < 320 d present minimal cortisol response at birth, in similar range to a normal term late gestation foetus. (Rossdale et al., 1982, Silver, 1994)
1 - 4 h post-parturition		• A blunted response remained present for the 2 d post-parturition. (Silver et al., 1984)

4h - 7d post-parturition	• Respond normally to paired low dose/high dose stimulation. (Hines, 1993) • CC increased by 208% at 30 - 60 min post-stimulation. (Silver et al., 1984) • Maximal cortisol response on day of birth. (Silver et al., 1984) • In first week, normal cortisol response half that of health adults (Bousquet-Mélou, 2006, Hart et al., 2009) • Peak cortisol response decreased over first 5 d. (Silver et al., 1984, Ousey et al., 2004) • Aged > 30 h, cortisol response similar in stressed and non-stressed foals. (Hart, 2009)	• CC increased by only 28% at 30 - 60 min post-stimulation. (Silver et al., 1984) • Some sick foals incl. premature have negative delta cortisol (adjusted for baseline) response to low and/or high doses. (Hart, 2009)
7 d - 12 wks post-parturition	• Response higher at 3 months than in younger foals. (Wong et al., 2009) • In foals receiving ACTH or saline for 5 d post-parturition, basal cortisol higher in ACTH-treated than in saline-treated at 3 wks, but not at 13 wks. (Jellyman, 2012a) • In foals receiving ACTH or saline for 5 d post-parturition, response to ACTH at 3 wks is the same in both groups. (Jellyman, 2012a)	

3.3.3. Findings of Persistent HPA Axis Dysregulation

Interest in continuing HPA axis dysregulation as an aspect of developmental programming has grown with the emerging field of Developmental Origins of Disorders and Disease (DOHaD) (Fowden and Forhead, 2009, Lai and Huang,

2011, Mcmillen, 2005, Maniam, 2014, Sullivan et al., 2008). Early activation of the HPA axis has been associated with changes in the renal, cardiovascular, pulmonary, reproductive and metabolic systems as well as various endocrine axes (Harris, 2011, Moritz et al., 2011). It is also now recognised that intrauterine programming of the immature HPA axis can cause permanent changes to the adult physiological phenotype (Fowden, 2004, Mcmillen, 2005, Rossdale, 2002). In human prematurity, low birth weight and socioeconomic status have been identified as factors causing atypical HPA axis activity later in life (Maniam, 2014, Winchester et al., 2016).

Studies involving juveniles and adults in other species have reported permanent adaption of the HPA axis with adrenocortical dysregulation caused by stressors during gestation. Experimental prenatal stressors used for maternal administration besides synthetic ACTH have included Dexamethasone and Betamethasone. The effects of these synthetic hormones on the HPA axis of primates, sheep, pigs, guinea pigs and rats have varied (Haussmann, 2000, Moritz et al., 2011, Sloboda et al., 2002, Sloboda et al., 2007), with results affected by the glucocorticoid used, the dosage and number of doses, timing of administration within gestation, and timing of output tests (Waffarn, 2012). There is evidence of long term dysregulation of the HPA axis, with studies reporting higher levels of HPA activation in juvenile sheep and, more commonly, depressed HPA axis responses in adults of other species (Sloboda et al., 2002, Sloboda et al., 2007).

In horses, Jellyman et al. reported continued HPA axis dysregulation caused by exposure to exogenous ACTH during the neonatal period (Jellyman, 2012a, Jellyman, 2012b, Jellyman, 2013, Jellyman et al., 2015, Jellyman, 2015).

Neonatal pony foals were divided into two groups, with one group treated with long-acting ACTH and the other saline on days 1 to 5 of life, thereby triggering an elevation of plasma cortisol (Jellyman, 2012a). At 1 and 2 years of age, the HPA axis function of all ponies was tested with the administration of short-acting synthetic ACTH, with insulin administered to induce hypoglycaemia on alternate days. It was found that basal plasma cortisol, ACTH and glucose were similar for both groups, as was post-ACTH stimulation plasma cortisol. However, post-ACTH stimulation values were higher for ponies in the group given ACTH neonatally. This indicates that a permanent change occurred in the HPA axis at the pituitary-adrenal level, and that this was associated with neonatal stress caused by high plasma cortisol (Jellyman, 2012b, Jellyman et al., 2015).

While gestationally immature foals may not all experience neonatal stress, it is likely that many do, especially those that have associated neonatal illnesses or that are simply too weak to cope with the demands of the earliest days of life, during the maturation period of the endocrine axes. Intrauterine stress in mammals results in early HPA axis activation and/or elevation of plasma cortisol during the HPA axis maturation period, which can lead to longer term dysregulation at adrenal, pituitary or hypothalamic level, and permanent adaptation of the adult's physiological phenotype (Barton, 2008, Fowden et al., 2012, Fowden et al., 2013, Fowden et al., 2016, Hart and Barton, 2011). It is reasonable to presume that horses are similarly affected; the early research by Jellyman et al. indicates that permanent HPA axis dysregulation can be present in equines.

3.4 Assessing the HPA Axis for Dysregulation in Adult Horses with a History of Gestational Immaturity

3.4.1 Measuring the Cortisol Response to Exogenous ACTH

Synthetic corticosteroids are commonly used in the assessment of the HPA axis. Five tests are typically used to stimulate different levels of the HPA axis for the purposes of diagnosis, as follows.

1. Exogenous ACTH stimulation. The administration of short acting or long acting synthetic ACTH stimulates the adrenal gland to secrete cortisol for diagnostic purposes. In the past decade, this measurement has also been used for assessing stress responses from a welfare perspective (Liburt et al., 2013, Mormède et al., 2007, Scheidegger et al., 2016). In horses, exogenous ACTH was previously used in the assessment of secondary hyperadrenocorticism due to Pituitary Pars Intermedia Dysfunction (PPID), whereby an adenoma of the pituitary's middle lobe causes excessive ACTH release (van der Kolk JH, 1995, van der Kolk et al., 2001). Current practice uses combined TRH-Response and Dexamethasone-suppression tests to measure ACTH and Insulin, or the measurement of endogenous ACTH.) Low-level, synthetic ACTH continues to be used in the diagnosis of neonatal foals with HPA axis dysfunction, including RAI (Madigan, 2014).

2. The TRH-Response involves administration of thyrotropin-releasing hormone to stimulate release of ACTH from the pituitary. This is also used as a test for PPID, with elevated production for ACTH indicating positivity (Animal Health Diagnostic Center, C.U, 2018).

3. The Dexamethasone Suppression Test (DST) comprises administration of Dexamethasone, which reduces the output of corticotropin releasing hormone (CRH) from the hypothalamus; continued presence of basal plasma cortisol indicates hypersecretion of ACTH by the pituitary (Brown, 2012). This is also used for PPID diagnosis (Animal Health Diagnostic Center, C.U., 2018).

4. Betamethasone suppresses the HPA axis and is commonly used in humans where premature birth is a risk. Its usage has been associated with neonatal hyperadrenocorticism (Davis, 2010).

5. Insulin-induced hypoglycaemia. A bolus of insulin is administered to induce hypoglycaemia. Arterial blood samples are collected at 5 - 15 min intervals prior to and post administration for measurement of plasma glucose, ACTH and cortisol concentrations. Post-experiment, glucose levels are restored by glucose infusion (Jellyman et al., 2015). The cortisol response to insulin-induced hypoglycaemia is frequently considered the 'gold standard' test for adrenal insufficiency (Kozyra, 2005).

The method selected for the following study needed to meet certain constraints: namely, that it would be administered to privately owned horses during a single day visit, and would be performed by a non-veterinary researcher in a non-clinic environment. For these reasons, a low-dose ACTH stimulation test was selected. This also met horse owner requirements of being minimally invasive, involving just one intramuscular injection per horse.

3.5 Adrenocortical Dysregulation in Adult Horses as a Sequel to Gestational Immaturity at Birth

This study tested the hypothesis that adult horses that were gestationally immature at birth would demonstrate adrenal dysregulation through an elevated stress response, measurable in SCC, to the administration of a low-dose ACTH stimulation.

This manuscript has recently been submitted for journal publication and is included at the end of this chapter.

3.6 Other Effects

3.6.1 Thyroid Dysfunction

Associations between equine gestational immaturity and hypothyroidism have been established (Allen, 1994, McLaughlin, 1982,). Thyroid hormones stimulate bone development by stimulating the pituitary to produce growth hormone. Thyroid hormones also influence cartilage production and degeneration, and promote ossification in the juvenile animal (Saunders and Jezyk, 1991, Vivrette, 1984). Known as goitre, an enlarged thyroid gland can be caused by low or high dietary iodine; low circulating thyroid hormones lead to a compensatory rise in serum thyroid stimulating hormone (TSH). This, in turn, causes hypertrophy and hyperplasia of the thyroid follicular cells, with subsequent enlargement of the gland (Wong et al., 2003).

Congenital goitre and its endocrine effects can lead to flexural deformities of the forelimbs, common digital extensor tendon rupture, and immature carpal and tarsal bones (Allen, 1994, McLaughlin, 1982,). These foals have hypothyroidism and are born weak, with poor inclination to suckle, delayed reflexes and hypothermia (Coleman, 2017). A second form of hypothyroidism has been identified in western Canada, caused by an unknown toxicity or dietary deficiency; affected foals have a prolonged gestation with physical signs of prematurity, plus mandibular prognathia (McLaughlin, 1982).

McLaughlin and Doige (1982) compared the carpal and tarsal ossification of foals with congenital hyperplastic goitre to that of thyroidectomised foals and normal foals (McLaughlin, 1982). Controls presented rapid growth through the last few weeks of gestation, with less rapid growth from birth to 33 days, and the lowest level of ossification in the ulnar carpal bone. Foals with congenital hyperplastic goitre presented the lowest levels of ossification, particularly in the central and third tarsal bones, while thyroidectomised foals had a low level of ossification, but not as extreme. This appeared to confirm the extreme effect of prenatal hypothyroidism on skeletal development.

The further pathological study of records of 154 aborted, stillborn and dead neonatal foals with abnormal thyroid glands and normal placentas revealed thyroid gland lesions indicative of Thyroid Hyperplasia and Musculoskeletal Deformity (TH-MSD) (Allen, 1994). Although the cause of TH-MSD was considered to be environmental in these cases, an association between hypo- and hyperthyroidism with retarded skeletal development exists.

Despite this association, the prevalence of abnormal thyroid glands in gestationally insufficient foals is low. In a Canadian study of 2946 equine aborted foetuses, stillborn and dead neonatal foals, 8% of thyroid glands were found to be abnormal (Allen, 1994). Wong et al. (2003) reported that a neonatal dysmature foal presented with bilaterally enlarged thyroid glands, although at 5 h of age, radioimmunoassay showed thyroid hormone levels to be within normal range (Wong et al., 2003).

In the mature term foal, thyroid hormone levels are very high at birth, declining over the first few months of life (McLaughlin et al., 1986). Prenatal deficiencies may be obscured in the neonatal immature foal, as plasma thyroid hormone levels can appear normal (Wong et al., 2003). Nevertheless, it is possible to hypothesise that hypothyroidism associated with prematurity may have an effect on growth in the neonatal foal.

3.6.2 The Renin-Angiotensin-Aldosterone System

Gestationally immature foals with incomplete ossification of the carpal and tarsal cuboid bones require a greater calcium and phosphorous intake than can be met by the mare's milk, so need to be supplemented until ossification is complete (Vaala, 2011). However, it is possible that endocrinological issues may create persistent dysregulation in the maintenance of calcium and other electrolytes.

In human medical research, associations between premature birth and both osteopenia and osteoarthritis have been established. Research has found links between low birthweight and/or premature birth and hypertension, cardiovascular disease, insulin resistance and reduced bone mass in adulthood (Hussain, 2014, Shenoi, 2016, van de Lagemaat, 2012). Premature and small-for-gestational-age babies have lower bone mass in early infancy (van de Lagemaat, 2012), while prematurity has been found to be the greatest risk factor in cases of Juvenile Idiopathic Arthritis (Shenoi, 2016), while adults with a history of premature birth have been found to be at a higher risk of hip osteoarthritis (Hussain, 2014).

In horses, there is the possibility that in addition to HPA axis dysregulation, an incorrectly developed adrenal cortex can have a dysregulatory effect on the renin-angiotensin-aldosterone system, given its role in the production of aldosterone (Barton, 2008, Vaala, 2011). Aldosterone regulates parathyroid hormone (PTH), along with phosphorus, calcitriol, magnesium and acid-base status (Mendoza, 2017, Toribio, 2002,) and associates with skeletal integrity, striated muscle contraction, cell membrane stability, enzyme activation, hormone secretion and cell division (Mendoza, 2017). Gestationally immature foals may also have reduced kidney tubular function, leading to renal and circulatory issues (Ousey, 2011). Furthermore, dysregulated RAAS also associates with adverse cardiovascular conditions, including hypertension (Vaidya et al., 2015).

To the author's knowledge, research into links between equine prematurity or dysmaturity and osteoarthritis is scarce, with previous studies having focused on osteoarthritis of the carpals and tarsals arising mechanically as an effect of cuboid bone collapse or angular limb deviations. Otherwise, equine veterinary research has focused primarily on the effects upon calcium homeostasis of nutritional secondary hyperparathyroidism (Mendoza, 2017), renal disease, exercise (Aguilera-Tejero, 1998, Aguilera-Tejero, 2001, Vervuert et al., 2002), pregnancy (Berlin and Aroch, 2009, Martin, 1996), and environmental factors (Azarpeykan et al., 2016).

The complexities of diagnosing equine calcium dysregulation and identifying its causes are numerous, given the inclusion in the calcium 'homeostatic system' of calcium and phosphorous, magnesium, potassium and sodium, of several organs (parathyroid glands, kidney, intestines and bone) and hormones (PTH, calcitriol

and calcitonin). Identifying this condition and its causes in gestationally immature horses is beyond the scope of this research.

3.6.3 Equine Metabolic Syndrome

In common with neonates of other species, foals can experience abnormalities of glucose homeostasis (Hines, 2011). Insulin is present in the foetal plasma from around 150 d gestation, with the pancreatic β-cells responsive to changes in glucose throughout the last trimester (Fowden et al., 1984). During parturition, both insulin and glucose are lower than in adult horses, remaining slightly diminished during the first hours of life, before increasing during the first 2 d of life. Throughout this period, a positive correlation exists between insulin, glucose and β-cell response to changes in glucose concentration (Hines, 2011). Dysregulation can occur: premature and septic foals present lower insulin and glucose concentrations than full term, mature foals, and those with extremely low levels are unlikely to survive (Boston and Frank, 2013, Fowden et al., 1984, Frank and Tadros, 2014).

In humans, associations have been made between premature birth and type-2 diabetes mellitus in adulthood, believed to be associated with impaired glucose regulation from an early age. Prenatal stress due to extreme maternal anxiety has been linked to increased insulin secretion in the adolescent, this being a precursor to Insulin Resistance (IR) (Dancause, 2013). Neonatal stressors are also believed to be contributory factors, as these occur during what would otherwise be late stage foetal gestation from the premature infant (Kajantie, 2010).

A similar condition exists in horses. Marked by IR and severe pancreatic β-cell dysfunction, it has previously been associated with autoimmune conditions (Frank and Tadros, 2014, Johnson, 2012). Insulin dysregulation, comprising hyperinsulinaemia and IR, is a key component of Equine Metabolic Syndrome (EMS). This is a group of endocrine and metabolic abnormalities linked to obesity, pregnancy and PPID (also called equine Cushing's disease), all of which are risk factors for laminitis (Boston and Frank, 2013, Frank and Tadros, 2014).

Hyperinsulinaemia is caused by increased insulin secretion or delayed insulin clearance, and may be a cause or consequence of EMS. One explanation for hyperinsulinaemia is that insulin secretion increases due to decreased tissue insulin sensitivity; decreased hepatic insulin clearance is also associated with EMS. This has been termed compensated IR (Frank and Tadros, 2014).

Lower limits are not provided for insulin reference ranges; however, hypoinsulinaemia is a central component of diabetes mellitus. Type 1 diabetes mellitus is believed to be caused to immune mediated destruction of pancreatic β-cells, which then leads to inadequate insulin secretion. Type 2 develops when β-cell insulin production decreases following prolonged hyperinsulinaemia; this is termed pancreatic insufficiency or exhaustion, or uncompensated IR (Frank and Tadros, 2014).

Recent research suggests that low birthweight in foals can lead to persistently increased insulin sensitivity (Peugnet, 2014). When Saddlebred horse embryos were transferred into ponies, foals were born with intrauterine growth restriction (IUGR). By 6 months of age, their bodyweight was similar to that of Saddlebred

foal controls. However, affected foals presented increased fasting glucose, indicating increased glucose tolerance, and a tendency towards reduced insulin secretion after an intravenous glucose tolerance test, indicating increased insulin sensitivity. This finding does not align with an earlier study, which found that growth enhanced foals displayed adapted insulin secretion (Forhead, 2004).

Overexposure to cortisol in neonatal life, during a 'window of susceptibility', induced by exogenous adrenocorticotropic hormone (ACTH), altered the secretory responses of foals' pancreatic β-cells at 3 months of age (Jellyman, 2013). In contrast, Valenzuela et al. (2016) found that by 2 years of age, neonatal glucocorticoid overexposure induced by ACTH treatment had had no lasting effect on whole body glucose tolerance, insulin secretion or insulin sensitivity. It did, however, alter insulin receptor abundance in specific skeletal muscles.

Interactions between various dysregulated endocrine systems are not yet understood. However, some connections have been established. In mature horses, thyroid dysfunction has been associated with EMS (Himler, 2012), while foals subjected to neonatal glucocorticoid overexposure developed pituitary dysregulation (Jellyman, 2012a, Jellyman, 2012b, Jellyman, 2013).

3.6 Approaching a Phenotype – Endocrinology

HPA axis dysregulation is well-established in other species within the growing area of DOHaD, to which equine research is a relative newcomer.

Gestational stress causes a bilateral adaptation of the adrenocortical response, due to development issues affecting the adrenal cortex, which matures very late in gestation. When subjected to a low-dose ACTH challenge, horses with a history of gestational immaturity present a lower or a higher cortisol response than related controls. Horses with a history of extreme immaturity presented very high or low values in this research, while there was otherwise little difference in results of horses that were dysmature or slightly premature at birth. All gestationally immature horses were affected. It is likely that neonatal stress due to incomplete development and/or weakness is a co-contributor. Adrenocortical dysregulation appears to be ongoing. It is also subclinical, in that basal plasma cortisol levels are unaffected.

Gestational immaturity of the adrenal cortex might also cause dysregulation of the renin-angiotensin-aldosterone system through hyperaldosteronism, which is associated with renal issues, electrolyte imbalances and hypertension in humans. Other effects may be hypothyroidism due to iodine imbalance during gestation, and pituitary-adrenal dysregulation and reduced pancreatic β-cell function due to high cortisol secretion in the neonatal period.

This adaptation has the potential to affect horses' responses to stressors, including those relating to health, training and husbandry.

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STATEMENT OF AUTHORS' CONTRIBUTION

(To appear at the end of each thesis chapter submitted as an article/paper)

We, the Research ~~Master~~/PhD candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated in the *Statement of Originality*.

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Chapter 4

THE ORTHOPAEDICS OF GESTATIONAL IMMATURITY – THE APPENDICULAR SKELETON

"The chronological age of a horse is of limited value as a criterion for determining the stage of body maturation" (Luszczyński, 2011)

4.1 Introduction

Gestational immaturity due to prematurity (gestation < 320 d), dysmaturity secondary to placentitis, placental insufficiency, foetal infection, foetal stress (due to maternal stress caused by malnutrition, parasites, colic, shock) and maternal thyroid issues affecting foetal growth (McIlwraith et al., 2003) is associated with orthopaedic conditions that can limit future athletic soundness. In the affected foal, incomplete ossification of the carpal and tarsal bones is common; when present at birth, this can lead to angular limb deformities (ALDs) or degenerative joint disease (DJD) (Fretz, 1980, McLaughlin, 1981, Wong et al., 2003). It is possible that skeletal developmental issues caused by developmental delays and endocrinological dysregulation can cause additional problems.

4.2 Incomplete Ossification

In the mature, healthy, term foal, the carpal and tarsal bones ossify in last 2 - 3 months of gestation (Fretz, 1980, Auer, 1982, McIlwraith, 2003). Ossification starts within the bone and is radiographically visible from around day 300 of gestation (Auer and von Rechenberg, 2006, Sedrish, 1997). It proceeds outwards through the cuboid bone's cartilage template in centrifugal fashion, before becoming more rapid during the final two weeks of gestation. At birth, ossification is either already complete, with the periphery of the bone mature and surrounded by a thin layer of cartilage, with reasonably square edges, or is incomplete, with a slightly thicker layer of cartilage surrounding a smaller ossified bone with rounded edges. If not completed at birth, ossification is usually complete within the first 30 d of life (Auer, 1982, Fretz, 1980, McLaughlin, 1982, McIlwraith, 2003).

In the gestationally immature foal, parturition can take place before an appropriate level of ossification has occurred in these bones. This may be because the foal has been born prematurely at an earlier stage of ossification, or because the foal is dysmature, i.e. within normal full-term range, but with impeded or retarded bone development.

Cases have been reported wherein carpal and tarsal ossification was incomplete until 84 d of life (Wong et al., 2003); similarly, it has been suggested that ossification in a premature or twin foal can take several months (Sedrish, 1997). In such cases, the radiographic appearance is of a rounded centre of ossification,

surrounded by a thick cartilaginous template similar to the form to be assumed by the mature bone (Adams, 1988).

The most commonly affected bones are those that commence ossification closest to parturition, i.e. the third, fourth and ulnar carpals, and the second and third tarsal (McLaughlin, 1982, Wong et al., 2003). The ulnar is commonly the last carpal bone to become completely ossified (Wong et al., 2003), while the central and third tarsals are the last tarsal bones to ossify (McLaughlin, 1982, Adams, 1988, Sedrish, 1997). Comprising largely pliable, cartilaginous templates, these bones are vulnerable to load-bearing compression and can become misshapen, with the possibility of fracturing in severe cases (Fretz, 1980, Leitch, 1985, Lester, 2011, McIlwraith, 2003, Wong et al., 2003).

4.2.1 Assessing the Degree of Ossification

Chronological gestation length offers no reliable indication of degree of ossification. The variability of equine gestation makes calculating osteogenesis of the cuboid bones problematic (Soana et al., 1998). Ranges have been established, but even when exact gestation dates are recorded, it is impossible to know where the individual gestation was positioned within the range, due to the sheer number of variables involved. It is also unclear whether foals born prematurely due to maternal placentitis, which experience an accelerated maturation of the HPA axis in utero, are similarly affected (Hines, 2011, Ousey et al., 2004, Rossdale, 1991).

Variability is evident in the literature. One Quarter Horse foal had a normal gestation length of 335 d and no problems during delivery, but presented low birthweight with physical and clinical signs associated with prematurity, including incomplete ossification (Wong et al., 2003). In another study, the level of carpal bone ossification in foals aborted at 309 - 315 d gestation was reported as ranging from 23.3 - 44.4%, significantly lower than that of term foals 45.7 - 62.8% (McLaughlin, 1982). This study's numbers were small (premature n = 10, term n = 14) and the 'premature' foals were not spontaneously aborted, but were induced and then euthanised. Dutton et al. (1998) narrowed this figure to tarsal ossification percentages of around 35% for foals born at 309 - 315 d, compared with 60% for term mature foals (Dutton et al., 1998). Despite sample size limitations, the ranges do show the substantially higher percentage of cartilage in incompletely ossified cuboid bones (Fretz, 1980, Leitch, 1985).

Soana et al. (1998) assessed the morphological development of 140 foetuses of unknown gestation, which had been collected from slaughter houses. Criteria for gestational age estimation included crown-rump length, the length of the third metacarpal/metatarsal diaphysis, distance from occipital crest and tooth development. On this basis, a chronology for osteogenesis was given (Table 4.1) (Soana et al., 1998). Gestational ages were estimated to range from 70 - 340 d. Despite the large sample size, no foetuses could be identified as belonging to the period 280 - 310 d, which is unfortunate, given that this is a critical period for carpal and tarsal osteogenesis. Those researchers therefore went further in their estimations for the distal row of carpal bones and tarsals, basing their conclusions regarding the start of osteogenesis and the dimensions of ossification sites on the evidence of 300 d foetuses.

Table 4.1. Approximate day of osteogenesis of the carpal and tarsal bones (adapted from Soana et al., 1998). First carpal and first tarsal bones are excluded, due to the inconsistency of their presence in adult horses.

Ossification site	Approx. day of appearance	Month of appearance **
Accessory carpal	254	8
Radial carpal	274	9
Intermediate carpal	274 - 278	9
Third carpal	285 (280 - 310) *	10 - 11
Fourth and second carpals	295 (280 - 310) *	10 - 11
Ulnar carpal	310	11
Calcaneus	125	4
Astragalus site 1	220	7
Astragalus site 2	260	9
Central tarsal	285 (280 - 320) *	10 - 11
Third & fourth tarsals	295 (280 - 320) *	10 - 11
Second tarsal (sometimes fused to First tarsal)	320 (280 - 320) *	11

* Soana et al.'s hypothesised date of appearance within accompanying range.

** Column added by the author.

The findings of Soana et al. (1998) differed from those of previous studies, including Barone (1974) cited in Budras (2011), and Stashak (1990). The researchers noted discrepancies in the previously described methods for foetal age determination: for instance, crown-rump length failed to correlate with the length of the growing metaphyses. At best, a sequence and approximate timing of cuboid bone osteogenesis can be drawn from this study.

An earlier study by Adams and Poulos (1988) provides a more valuable skeletal ossification index (SOI) (Adams, 1988). This is a simple grading system based on the standardised radiographic assessment of the carpal and tarsal bones of neonatal foals. Fifty-two neonatal foals or foetuses were included. Of these, 24

were neonatal foals aged < 2 wks and 10 were premature at < 320 d. Gestational ages ranged from 297 - 374 d (328 ± 22 d). Bodyweights were 21 - 68 kg, with mean premature foal weight being 12 kg less than term foals. Based on two radiographic views of the carpus and tarsus (dorsopalmar / plantar and lateromedial), four grades of ossification were assigned according to the appearance of carpal and tarsal bones, development of the lateral styloid process of the radius, the malleoli of the tibia, and the proximal epiphyses of the third metacarpal and third metatarsal bones. The SOI allows for wide variation within these grades (Table 4.2).

Researchers found that although the SOI correlated with gestational age and bodyweight across both groups, this was not the case when the premature foals were tested separately. However, there was a correlation with bodyweight within the premature group, suggesting that body weight and bone development are more accurate measures of immaturity than gestational age. It was concluded that intrauterine growth retardation in all foals is a strong indicator for incomplete ossification. When considering gestational age, researchers adhered to < 320 d as the indicator for prematurity; however, 17 of the foals were born ≥ 310 d and of these, all but two were classified as grade 4, i.e. of normal skeletal development.

Table 4.2. Skeletal Ossification Index (SOI), adapted from Adams and Poulos (1988), based on dorsopalmar / plantar and lateromedial views of the carpus and tarsus.

Location	SO1	SO2	SO3	SO4
Carpal bones	Some carpals show no evidence of ossification	Each carpal shows some evidence of ossification	Carpals are small and rounded, joint spaces are wide	Carpals are cuboid like adults, joint spaces normal
Radius: Lateral styloid process	Absent	Small, just visible in latero-medial view	Distinct in both views	Well formed, Triangular
Third metacarpal: Proximal epiphysis	Absent	Open or closed	Closed	Closed
Tarsal bones	Some tarsals show no evidence of ossification	Each tarsal shows some evidence of ossification	Tarsals are small and rounded	Tarsals are cuboid like adults, joint spaces normal
Tibia: Malleoli	Absent	Small, just visible in latero-medial view	Distinct in both views	Well formed
Third metatarsal: Proximal epiphysis	Absent	Open or closed	Closed	Closed

It has been suggested that ultrasonography can be used in the diagnosis of incomplete ossification in neonatal foals, given that ultrasound waves are reflected from the surface of bones. This was tested in a study of 10 foals, 9 of which were term and one of which was born prematurely at 301 d (Ruohoniemi, 1993). Three foals had incomplete ossification, with the two foals graded SOI 1 being full term (329 d and 336 d gestation), again highlighting the difficulties in identifying ‘at risk’ foals when outward signs of dysmaturity are not obvious.

4.3 Acquired Limb Deformities

4.3.1 Carpal and Tarsal Collapse

Incomplete ossification can lead to collapse of the carpal and tarsal bones. In the forelimbs, carpal osteogenesis commences with the medial bones and progresses laterally. Combined with the narrowness of the neonatal foal's chest in the early weeks of life, this leads to additional loading on the laterally positioned ulnar and fourth carpals. The thick, soft cartilage layer of the insufficiently ossified lateral carpals can become crushed between the ulnar and fourth carpals, and to a lesser degree between the intermediate and third carpals. This leads to a crushing of the ulnar, intermediate, third and fourth carpals, which, lacking depth on the lateral aspect, give a sloped medio-lateral profile to the joint (Levine, 2015, McIlwraith, 2003). This predisposes the foal to carpal valgus (McLaughlin, 1981), which may or may not be concurrent.

In the hindlimb, the central area of the tarsal joint is vulnerable to collapse. The central and third tarsal are subject to the foal's bodyweight, transferred from the tibia to the third metatarsal via the talus, typically on a sagittal axis. Compressive forces on the central aspect of the third and central bones can lead to tarsal collapse (Fig. 4.1) (Jansson, 2005, McIlwraith, 2003). The third tarsal is most commonly affected and in immature foals with less severe incomplete ossification, it is often the only affected bone (Dutton et al., 1998, Hermans, 2008, Ruohoniemi, 1993, Sedrish, 1997). It is logical that the risk of tarsal collapse is

accentuated if inherited traits such as wide hock angles or conformational sickle hocks are also present in the foal.

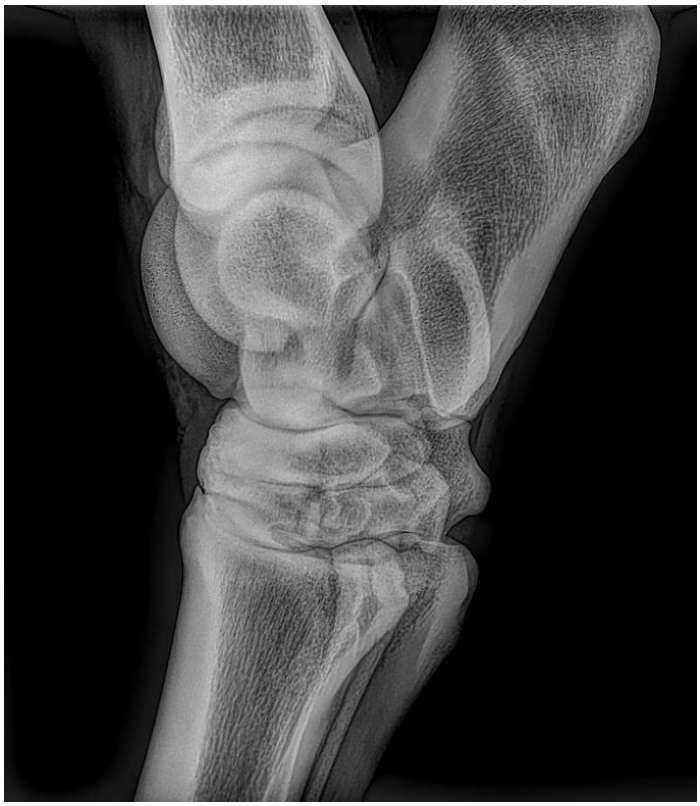

Fig. 4.1 Radiograph showing collapse and wedging of central and third tarsal bones (image: used with owner's permission).

Dutton et al. (1998) established further grading of malformed cuboid bones: radiographic lesions classified as type-1, mild collapse with < 30% collapse of dorsal aspect of affected bones) and type-II, severe collapse, > 30% collapse of affected bones, plus pinching or fragmentation of affected bones (Dutton et al., 1998) (Fig. 4.2). Other researchers have described a mediolateral effect, with bones that are narrow in the centre becoming wider again on the dorsolateral

aspect (Kane, 2000a). This may be associated with a tarsal valgus that is present in weak foals that are not yet stable when standing.

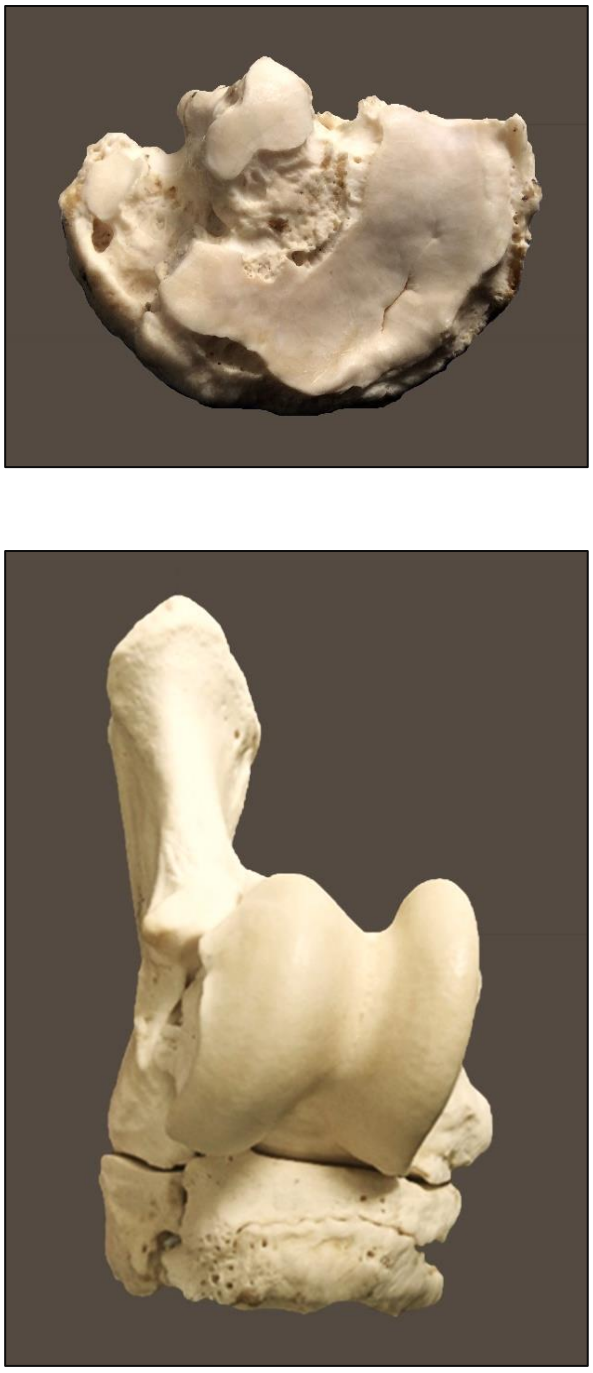

Fig. 4.2 Skeletal samples showing collapse and advanced osteoarthritis of central and third tarsals in an 8 yo mare (images: author's own).

Other associated musculoskeletal signs that may also be present, contributing to instability of the loaded carpus or tarsus, include subluxation of the intercarpal and carpal-metacarpal joints (McLaughlin, 1981).

Wedged tarsals have been reported in 1.2% of individuals affected by incomplete ossification. As an acquired angular limb deformity, and one that is not always externally visible, the lesion is associated with reduced likelihood of athletic success in racehorses (Haywood et al., 2018, Kane, 2000b, Kane, 2003). In the horse affected by both incomplete ossification and collapsed tarsals, the hindlimbs have an appearance that is both sickle and cow-hocked (Fig. 4.3) (McIlwraith, 2003).

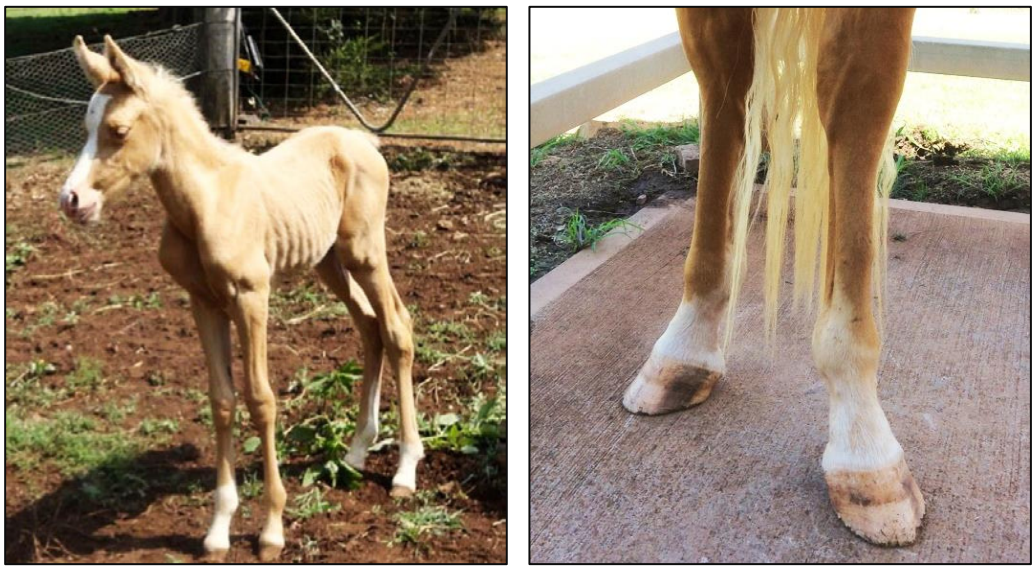

Fig. 4.3 Tarsal collapse and valgus in (left) a dysmature foal and (right) the same horse at 4 yo, showing the combined cow-hocked and sickle-hocked appearance caused by the concomitant pathologies (McIlwraith, 2003). (Left image: used with owner permission. Right: author's own)

With veterinary assessment of incomplete ossification, treatment can involve applying support bandages or sleeve splints to the limbs from the distal third metacarpal to proximal radius, or distal third metatarsal to proximal tibia (Auer and von Rechenberg, 2006, Witte and Hunt, 2009, Wong et al., 2003) with remedial hoof trimming (Baxter, 2011a), until sufficient ossification has occurred for correct axial loading. In less extreme cases, restricted movement is recommended during the early days of life. Daily round pen or small paddock exercise for 12 hours in non-severe cases has been recommended, while for foals with more markedly immature cuboidal bones, stall or box confinement with short daily walks alongside the mare has been found effective (Santschi et al., 2018).

Podiotherapy in the form of shoeing or trimming with prosthetic heel extensions can support the joints and reduce inappropriate loading of the carpals and tarsals (Baxter, 2011a, Levine, 2015). This is especially important for the hindlimbs, where the tendon laxity is also on the sagittal plane, further adding to inappropriate loading of the cuboid bones. Maintaining the longitudinal axis of the limbs requires muscular effort, whereas immature foals are often weak. The resulting abnormal flexion can lead to further compression of the cartilage (Dutton et al., 1998).

4.3.2 Carpal and Tarsal Valgus

There is a strong association between incomplete ossification and Angular Limb Deformities (ALDs) (McIlwraith, 2003). The most common congenital ALD in all foals, whether mature or immature at birth, is carpal valgus, in which the long

axis of the forelimb distal to the carpus deviates from the midline (Fig. 4.4). This is usually mild and self-resolving: the effect of a narrow chest and some external lower limb rotation lessens as the foal grows in its early weeks (Levine, 2015). A valgus of < 5° at birth and < 3° at one month is therefore considered normal (Stoneham, 2011).

Congenital tarsal valgus is an outward deviation below the tarsal joint. This is also common and usually resolves as the foal widens through the body. Carpal varus, the medial deviation of the lower limb, is less common, but has been reported in cases of immaturity, usually as an acquired ALD developing in response to a valgus deformity in the hindlimbs (Hermans, 2008). Tarsal varus is considered extremely rare (Baxter, 2007).

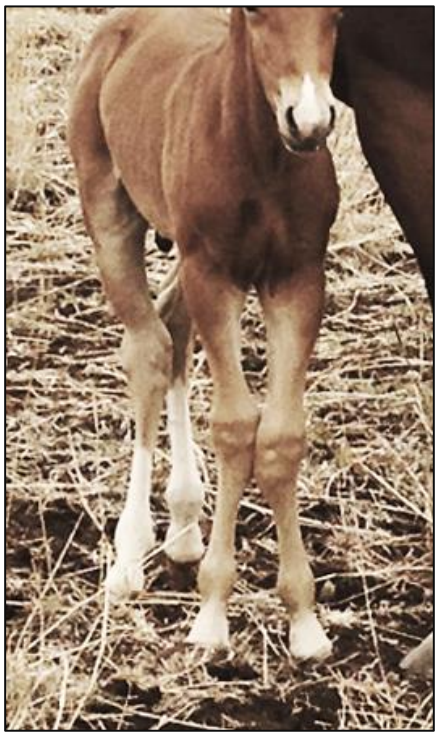

Fig. 4.4 Foal with history of dysmaturity, presenting carpal and tarsal valgus, with rotational deformities (image: author's own).

ALDs relating to incomplete ossification are acquired and the aetiology is clear. With incomplete ossification of the carpals and tarsals, loading asymmetries can both lead to a valgus deformity and perpetuate it due to a compounding interaction between the pathologies (Fretz, 1980, Leitch, 1985). This may be accentuated if the foal also has inherited conformational traits such as offset carpus (bench knees) or rotational deformities of the lower limb (Jansson, 2005), which further contribute to the developmental pattern.

Further complications are congenital deformities such as hyperextension (i.e. laxity) of the flexor tendons, periarticular laxity, with other developmental deformities such as fetlock (metatarsophalangeal or metacarpophalangeal) valgus and varus also developing. Flexural deformities are also known, but are less common than laxity of the tendons (Lester, 2011). These can lead to greater medio-lateral instability, plus increased dorsiflexion of the metacarpophalangeal or metarsophalangeal joints, and the distal interphalangeal joints. More common in the hindlimbs, hyperextension of the flexor tendons is presented more frequently by immature than term mature foals (Lescun, 2011).

Severe cases that show early signs of ALDs or a broken tarsal axis are usually visually identifiable. Additionally, a hindlimb ‘bunny hop’ gait anomaly may be observable (Fretz, 1980), and tarsal bone collapse may lead to visible swelling of the tibiotarsal joint (McIlwraith, 1987). However, many go unnoticed, especially as many foals have a small degree of carpal or tarsal deformity in the early weeks of life, due to weakness when standing (Levine, 2015); owners of foals hospitalised at 10 months for tarsal valgus had clearly missed earlier signs of deformity (Dutton et al., 1998). Unidentified, these deviations can progress to

more severe deformities (Dutton et al., 1998, Hermans, 2008). If the limbs are straight, confinement and rest are commonly recommended until ossification has taken place, usually within a 4-wk period; initial diagnosis and confirmation of ossification is achieved via radiographs every other week. To treat valgus deformity or poor tarsal ossification, splints, casts or bandages are recommended, as these maintain alignment and protect bones from inappropriate loading. The cast starts above the fetlock, to avoid complications involving excessively weakened flexor and extensor tendons. It is recognised that some weakness will occur, but this is rectifiable through exercise once ossification has taken place (Levine, 2015). Commercially produced braces help to reduce this risk (Auer, 1999).

4.3.3 Treatment Outcomes

While treatment outcomes for carpal and tarsal collapse with associated ALDs are variable, it is agreed that the prognosis for soundness improves if the issues are identified and addressed as early as possible. In cases where the valgus is more severe, i.e. > 12°, surgical intervention may be performed. Otherwise, mild-to-moderate angles may be left to self-correct in the young horse, with the recognition that that non-severe angulation does not adversely affect either soundness or riding performance in riding horses (Holmström, 2013).

Wong et al. (2003) report that conservative treatment was successful in the case of the dysmature Quarter Horse foal. With movement and load bearing restricted in a clinical setting, the ALD responded slowly but positively to physical therapy,

bandages and splints (Wong et al., 2003). Four out of 6 foals treated this way in another study responded favourably (Dutton et al., 1998).

Dutton et al. (1998) reported on the progress of 22 foals, including 11 premature (< 320 d). 16 individuals had tarsal valgus. Severity was associated with the outcome: 4 of 6 foals with type-I (< 30°) incomplete ossification performed as intended, but only 3 of 16 foals with type-II were able to achieve athletic soundness (Dutton et al., 1998). Of the 13 foals for which follow-up information was available, 6 had type-II lesions and did not reach intended athletic function (racing, western cutting and showing) and were being used as pleasure horses. With 4 horses, this was associated with lameness or ALDs, while with 2, it was due to stunted growth (Dutton et al., 1998).

Despite apparent success of conservative treatment in the early stages, the deformity may worsen over the first 1 - 2 years (Fig. 4.5). Worsening of limb issues can occur during the periods of most rapid weight gain (Lester, 2011). For foals born in spring and early summer, this can be towards the end of the first 5 months, as the rapid scaling up of muscle mass concludes before levelling off over winter, and around 11 months, as spring pastures once more become available (Brown-Douglas, 2011).

Surgical intervention appears to have limited success in cases where ALDs are associated with incomplete ossification and carpal or tarsal collapse. Several studies report on cases where hemi-circumferential periosteal transection and stripping in an attempt to correct a valgus deformity has failed over the longer term (Dutton et al., 1998, Dutton et al., 1999, Hermans, 2008, Santschi et al.,

2018). Dutton et al. (1998) reported that although 10 out of 22 hospitalised foals underwent the procedure, lasting correction was only achieved in 2 foals. Only 1 of these was still able to perform as intended. Of the remaining 8 foals, 5 were unable to perform as intended and 2 were euthanised, while 1 was lost to follow-up (Dutton et al., 1998).

Fig. 4.5 Excessive epiphyseal growth and osteoarthritis in the radii of a horse born as dysmature twin. The left forelimb (right of image) presented a severe carpal varus, which is a less common ALD sequel. (image: author's own).

Of 13 foals with collapsed tarsals included in a retrospective study of 27 surviving premature and twin foals at the University of California, Davis, 3 foals developed tarsal valgus (Hermans, 2008). Follow up information was obtained for 11 horses: 6 were smaller in size than anticipated, and 7 were unable to perform their intended use. Of 2 that underwent periosteal stripping as foals, one still had a tarsal valgus as an adult. Interestingly, although 13 foals had carpal valgus at admission, no wedged carpals were reported.

Haywood et al. (2018) investigated whether associations exist between incomplete ossification, gestation length, and racing performance in Thoroughbreds (Haywood et al., 2018). In this retrospective study, veterinary and studbook records for 115 foals < 90 d old with incomplete ossification of the tarsal bones were obtained. Early radiographs of the foals' tarsal bones were graded using a modification of the SOI; under the Neonatal Skeletal Ossification Index (NSOI), foals with complete ossification were assigned to a fifth category (Table 4.3). Race records for the same foals at 2 and 3 years old were obtained, along with those of maternal siblings. It was found that NSOI 1 - 2 foals were usually premature, here defined as < 325 d. Foals graded 2 - 3 were significantly less likely to race than their maternal siblings, while those that did earned around U.S.$ 30,000 less in winnings. Even foals graded 4 earned around U.S.$ 14,000 less than maternal siblings.

Although limitations in the above study include the age range of foals radiographed (0 - 71 d), foals with gestational ages < 300 d appeared within the grades 1 - 3. These were outliers, for median and mean gestation ages correlated with increasing levels of ossification. What is striking about the findings,

however, is that a large number of foals included were full-term; all foals graded 3 - 4 were within the 325 - 355 d 95% confidence limit for term parturition (Rossdale, 1976, Haywood et al., 2018). As it was not clear why foals were radiographed at 90 d, researchers could not exclude other health issues. Variations in management practice were also impossible to ascertain.

In these studies, many of the foals presenting for incomplete ossification had been admitted for reasons of unrelated conditions, or only once ALDs had become severe and therefore noticeable. The SOI grading scale is useful in assessing the extent of ossification in foals, while the grading of tarsal collapse provides indicators for sequalae of incomplete ossification, including osteoarthritis (Adams, 1988, Dutton et al., 1998).

Table 4.3. Modified neonatal skeletal ossification index (NSOI) for tarsal bones used by Haywood et al. (2017), based on the earlier Skeletal Ossification Index (SOI) devised by Adams and Poulos (1988). Radiographically normal foals are here assigned to a new fifth category, NSO5.

Location	NSO1	NSO2	NSO3	NSO4	NSO5
Tarsal bones	Some tarsals show no evidence of ossification.	Each tarsal shows some evidence of ossification.	Tarsals are ossified, but small and rounded. Joint spaces appear widened.	Tarsals are normal size, but slightly rounded. Joint spaces normal.	Tarsals are cuboid, with adult shape. Joint spaces normal.
Tibia Malleoli	Absent.	Absent or barely visible.	Distinct in both views.	Well formed.	Well formed.
Third metatarsal Proximal epiphysis	Absent.	Present and open.	Closed.	Closed.	Closed.

4.4 Secondary Lesions

4.4.1 Juvenile Arthritis

It has been suggested that weight bearing on incompletely ossified bones can cause microfractures of the hyaline cartilage surface, leading to early onset or juvenile arthritis of the distal tarsals (Degenerative Joint Disease; DJD) (Sedrish, 1997, Baxter, 2011c). For example, in the retrospective study by Hermans et al. (2008), 2 foals previously graded with SOII lesions in the tarsals went on to develop bilateral DJD of the distal intertarsal joint and the tarsometatarsal joint in the first 2 years of life. One horse also developed DJD in the fetlock and pasterns (distal interphalangeal joints / pasterns) of the forelimbs (Hermans, 2008).

Routine carpal and tarsal radiographs of 100 full term (317 – 379 d), neonatal Thoroughbreds aged ≤ 7 d at 5 Kentucky breeding farms were examined by Santschi et al. (2018). Of 45 foals identified as lacking ossification, 28 were affected at the carpus and tarsus, 5 at the carpus only, and 12 at the tarsus only. Further radiographs were taken of 96 foals at around 186 d (approx. 6 months) of age. Thirty-four foals presented normal cuboid bones, 45 were classed as 'blemished' (i.e. in the carpals, fragments or ulnar carpal bone lucencies; in the tarsals, large dorsal osteophytes on the central or third tarsal, with no osteoarthritis present), and 13 had mild radiograph abnormalities (small tarsal osteophytes or minor shape change of the central or third tarsal). Four others had moderate radiographic abnormalities (subchondral lucencies in the carpus, tarsal and carpal osteoarthritis).

These findings are similar to those of the earlier study by Dutton et al. (1998). Upon admission, 5 foals aged 3 - 10 months old, with type-II lesions, i.e. < 30% tarsal collapse of both central and third tarsal bones, had radiographic evidence of DJD. Three foals aged 3 - 30 d, also with affected central and third tarsals, developed DJD within 4 months of initial examination. In these instances, degenerative change involved loss of joint space and development of osteophytes. Furthermore, of the 13 foals for which longer term follow-up information was available, 8 presented tarsal DJD. All of these had presented bilateral incomplete ossification and type-2 lesions of both the central and third tarsal bones upon hospital admission. Only 1 of these 8 foals was able to perform as intended. These foals were aged just 2 - 3 y at the time of follow-up, raising the possibility of a far higher incidence by 6 - 8 y.

By the time osteoarthritis relating to incomplete ossification is diagnosed, it is usually advanced, the distal tarsals showing an undulating intertarsal joint surface and severe osteophyte formation (Baxter, 2011a). This has been the author's experience with case studies included in this research (Fig. 4.6).

The literature offers equally varied viewpoints on how important the association between incomplete ossification and osteoarthritis is in the foal aged < 1 year. One view is that while tarsal collapse may lead to DJD, this will not be an issue once the central and third tarsal bones fuse (McIlwraith, 1987). An opposing view is that DJD at such a young age presents a poor prognosis of unsoundness and failure to achieve athletic potential (Sedrish, 1997, Baxter, 2011c). A further view is that if foals are treated early, prior to excessive cartilage damage, a healthy limb axis may be retained and the possibility of DJD as a sequel is minimised (Baxter,

2011b, Fretz, 1980). On the other hand, carpal collapse is considered to have a far more guarded prognosis (Baxter, 2011b). The osteoarthritic end stage is viewed as being the development of exostoses, which at necropsy are always accompanied by disrupted cartilage of variable thickness, disrupted growth plates, increased loading on joints, and ALDs (Baxter, 2011c).

Clearly, there are difficulties involved in conducting longitudinal studies, in that horses are sold with incomplete records and contact with owners is lost. This is less the case in Thoroughbred racing, where studbook and racing association records, plus weanling and yearling radiographs offer comprehensive data until the horse leaves the industry. Longitudinal studies into Thoroughbred horses in the racing industry are now emerging and providing more information on the long-term implications of tarsal collapse in horses.

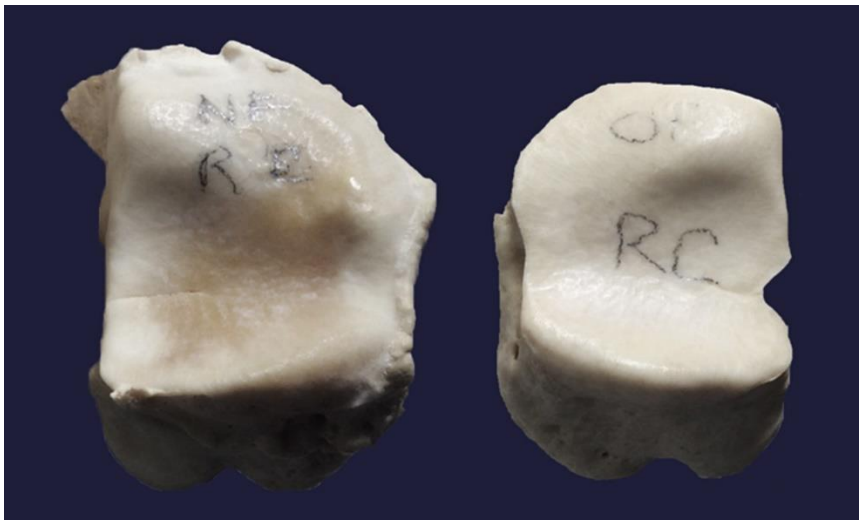

Fig. 4.6 Radial carpal bones from the forelimbs of a horse born a dysmature twin. Both bones show collapse and lesions, with the left radial carpal also showing enlargement due to advanced proliferative osteoarthritis (image: author's own).

4.4.2 Carpal and Tarsal Fractures

Issues affecting the carpal and tarsal cuboidal bones can lead to chronic lameness and decreased athletic ability in Thoroughbreds (Haywood et al., 2018). Abnormally shaped third tarsal bones are associated with slab fractures, a significant cause of lameness in Thoroughbred racehorses (Baird and Pilsworth, 2001, Winberg, 1999).

The radiographs of 10 affected horses and 10 unaffected Thoroughbred racehorses in Newmarket, UK, were examined and an association between a wedge-shaped conformation of the third tarsal and the occurrence of a slab fracture was confirmed (Baird and Pilsworth, 2001). However, the nature of this causal link could not be confirmed, because although wedged tarsals were highly represented in this group, slab fractures of this bone also occur in horses without wedging. Contributory factors can include abnormal concentration of forces at the joint surface due to the conformational change; the development of sclerosis and a propensity to sagittal fractures; and excessive loading at the apex of the wedged zone leading to bony change.

As the last carpal bone to ossify, the ulnar carpal is a common site for evidence of subchondral lucencies, with one study of Thoroughbred racehorses reporting a prevalence of 20 - 22% (Jackson, 2009, Kane, 2003). In the study of incomplete ossification in Thoroughbred foals, Santschi et al. (2018) did not observe any ulnar carpal bone lucencies in neonatal radiographs from 100 foals, yet these were observable in 18% of individuals at 6 months of age. This radiographic anomaly

is viewed by many to represent an avulsion of the lateral palmar intercarpal ligament on the axial surface of the bone (Beinlich, 2005). There is discussion as to the significance of these anomalies, which once identified usually heal with conservative treatment.

4.4.3 Osteochondrosis

Further problems in foals with carpal valgus relating to incomplete ossification have been reported. The development of osteochondrosis in foals is known to derive from a combination of genetic, nutritional, environmental and biomechanical causes. In the immature foal, incomplete ossification renders cartilage canals running across the ossification front vulnerable to injury during the postnatal period, with nutritional factors and rates of growth also contributory factors (Auer, 1982, McLaughlin, 1981). Early osteochondrosis lesions on articular surfaces may either heal, or contribute to the later development of osteochondritis dissecans lesions (Fig. 4.7) (Semevolos, 2017).

There is evidence that shearing forces in the incompletely ossified carpals of immature foals have developed Osteochondritis dissecans (OCD) lesions. In one study, 9 forelimbs from 6 immature foals were examined. In 6 of these limbs, the articular surface of the third carpal bone presented a clinical OCD lesion, with plaque-like elevations in the cartilage also present (McLaughlin, 1981).

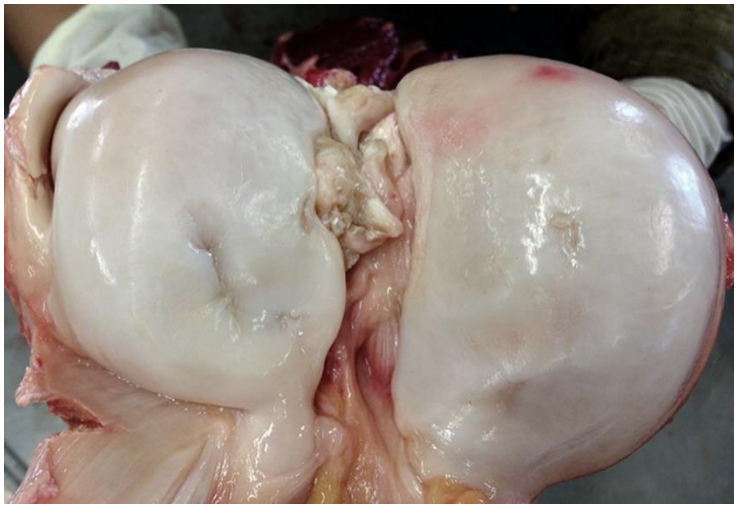

Fig. 4.7 Lesions in the lateral and medial condyles of the right femur of a mare with a history of extreme dysmaturity (image: author's own).

4.4.4 Navicular Lesions

In cases where the distal tarsal bones are incompletely ossified, the navicular may be similarly affected. The osteogenesis date given for the navicular bone is around 325 d, commensurate with that of the distal tarsal bones (Adams, 1988). The absence of navicular bones was reported in one case of extreme dysmaturity (Wong et al., 2003), while incomplete ossification of the navicular bone was also radiographically detected, along with SOI grade 1 carpii and a valgus deformity of the left metacarpophalangeal joint (McIlwraith, 2003). Meanwhile, the longer term effects of incomplete ossification of the navicular have been discussed in case studies of adult horses presenting bipartite or tripartite navicular lesions (van der Zaag, 2016).

In mature, term foals, the navicular at birth has an oval radiographic outline, and develops radiographically visible articulating contours throughout the first 11 months of life (Dik, 2001), although it has also been reported that ossification completes at around 18 months (Butler, 1993). In the distal limbs, the correct balance between tension and compression is essential for continued and orderly bone development and growth, with each growth plate only responding within a certain range (Baxter, 2011c). It has been noted that the navicular's shape may be determined at birth, and that its shape may in turn influence the biomechanical forces applied to it, heightening the risk of navicular syndrome (Dyson, 2003). An effect of breed on the navicular's shape has also been established (Dyson, 2003).

Severely immature foals commonly present flexural hyperextension, which tends to affect the hindlimbs more than the forelimbs. The forelimbs tend to be less affected by hyperextension, yet are more heavily weighted than the hindlimbs. The combined effects of inappropriate loading due to carpal or tarsal collapse higher in the limb, leading to additional pressures due to tension through the suspensory ligaments, and/or poor distal limb angles due to hyperextension could lead to lesions within the bone. The hyperextension may be resolved in the first few weeks of life, yet the effects of tarsal collapse continue and can only worsen with later weight gain and commencement of work (Dutton et al., 1998).

Presently, no association has been identified between incomplete ossification and the pathogenesis of navicular syndrome later in life. However, long term longitudinal research into the ongoing development of gestationally immature foals has been limited and typically ceases at 18 to 36 months of age (Dutton et

al., 1998, Hermans, 2008, Wong et al., 2003); problems with forelimb navicular syndrome tend to arise with commencement of ridden work at a later stage (Dik, 2001). However, in the hindlimb, is possible that inappropriate loading due to tarsal collapse may lead to ongoing issues with negative plantar angles of the third phalanx. This is an area where research might reveal a further contributory factor in a complex pathology.

4.5 Using Movement Sensors to Assess Lying Time in Horses With and Without Angular Limb Deformities

Studies into outcomes for gestationally immature foals show that angular limb deviations often persist into adulthood, with surgical intervention failing to prevent ongoing development of the pathology. In this study, the lying behaviour of 4 unridden paddock horses with a history of gestational immaturity and a range of ALDs and 4 controls was measured with movement sensors.

This paper was published by the *Journal of Equine Veterinary Science* in April 2019 and is included at the end of this chapter.

4.6 Towards a Phenotype – The Appendicular Skeleton

Gestational immaturity is strongly associated with incomplete ossification. If undiagnosed, incomplete ossification can lead to collapse ('wedging') of the cuboidal bones of the carpus and tarsus, with the latter being more common. External signs can be minimal and, if no clinical care is administered, the pathology often escapes notice, whereupon continued inappropriate loading can lead to the development of ALDs. Tarsal and carpal valgus are the ALDs most commonly seen and without early intervention, these are often persistent. Even with intervention, surgical approaches are of limited effectiveness in the presence of carpal or tarsal collapse (Fig. 4.8).

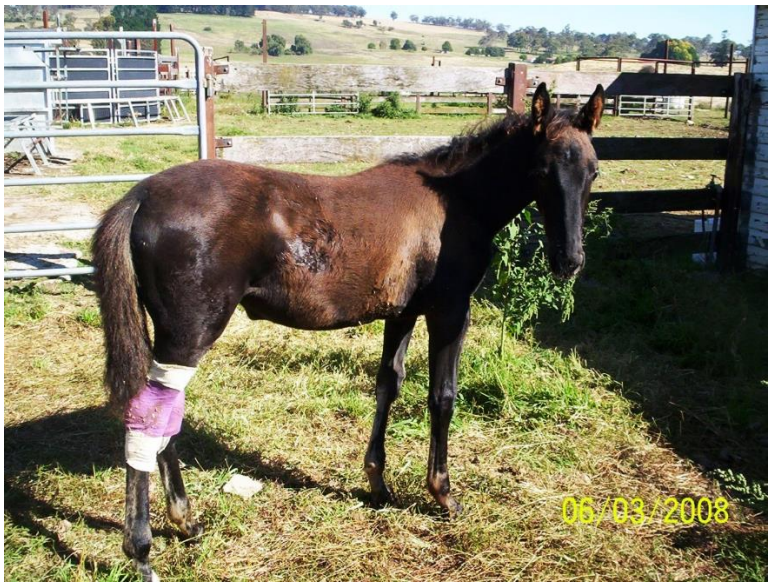

Fig. 4.8 The more dysmature of twins after periosteal stripping for tarsal valgus at 4 mo. The intervention was unsuccessful in this case: as an adult, the horse presented severe bilateral tarsal valgus. (Image: used with owner's permission.)

The sequelae to immaturity, incomplete ossification and carpal or tarsal collapse include juvenile arthritis of the distal tarsal joints and fracturing of the ulnar carpal and third tarsal bones. Further, if the distal tarsals lack ossification, ossification of the navicular bone may be absent, as this has its osteogenesis at or after 325 d of gestation. Collapse or ALDs may then have a continued effect, for the navicular does not complete ossification for several months.

ALDs with all, some or even none of these concomitant factors may continue to present problems for the mature horse with a history of gestational immaturity. It is the author's observation that problems can worsen during the various growth phases leading up to 3 years of age, particularly in the case of heavier breeds that present rapid growth and weight gain, thereby intensifying the lateral deviation from the appendicular axis. Lighter breeds, which are frequently narrower through the shoulders and ribcage at maturity, appear to be less subject to the extreme manifestations of ALDs, although this is clearly a subjective viewpoint.

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STATEMENT OF AUTHORS' CONTRIBUTION

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We, the Research ~~Master~~/PhD candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated in the *Statement of Originality*.

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STATEMENT OF ORIGINALITY

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We, the Research Master/PhD candidate and the candidate's Principal Supervisor, certify that the following text, figures and diagrams are the candidate's original work.

Type of work	Page number/s
Article in press with Journal of Equine	Pages 103-120
Veterinary Science:	
Using movement sensors to assess lying	
time in horses with and without angular	
limb deformities	

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15th January 2019

Date

Chapter 5

THE ORTHOPAEDICS OF GESTATIONAL IMMATURITY - GROWTH RETARDATION

"Most premature or dysmature foals will be smaller than their peers for the first 12 - 18 months of life, but this physical difference becomes less noticeable after that time. Surviving premature Thoroughbred foals are less likely to be successful athletes than their siblings." (Lester, 2011)

5.1 Introduction

Premature or dysmature foals frequently mature at lower than anticipated height (Dutton et al., 1998, Wong et al., 2003). Although this sign of retarded growth is mentioned in follow-up studies of foals presenting incomplete ossification, (Dutton et al., 1998, Hermans, 2008), little research exists as to the exact mechanisms in terms of skeletal development.

Skeletal growth occurs through endochondral ossification in the discoid growth plates at the end of appendicular long bones (Baxter, 2011c). At birth, the long bones of the distal limb are around 68% of their adult length (Stoneham, 2011), with the distal plates of second and third phalanxes already closed (Table 5.1).

Table 5.1. Approximate radiographically visible closure of metaphyseal growth plates and completion of ossification of cuboid and sesamoid bones in juvenile horses with normal gestational history.

Forelimb (Anatomical)	**Closure range**	**Hindlimb (Anatomical)**	**Closure range**
Radius (Radius)	Distal: 20 - 24 mo *	Tibia	Distal: 17 - 24 mo *
Knee (Carpus)	≤ 33 d **	Hock (Tarsus)	≤ 33 d ***
Cannon bone (Third metacarpal)	Proximal: At birth Distal: 6 mo *	Shannon bone (Third metatarsal)	Proximal: At birth Distal: 6 mo *
Distal sesamoids	3 - 4 mo	Distal sesamoids	3 - 4 mo
Long pastern (First phalanx, P1)	Proximal: 12 mo *	Long pastern (First phalanx, P1)	Proximal: 12 mo *
Short pastern (Second phalanx, P2)	Proximal: 8-12 mo *	Short pastern (Second phalanx, P2)	Proximal: 8 - 12 mo *
Pedal bone (Third phalanx, P3)	Birth	Pedal bone (Third phalanx, P3)	Birth
Navicular bone	≤ 12 mo	Navicular bone	≤ 12 mo

* Budras et al. (2009) ** Dik et al. (2001) *** McLaughlin & Doige (1982)

After birth, endochondral ossification continues in the metaphyseal cartilage at one end of the diaphysis, as well as radially from the secondary ossification centre of the epiphysis. The extension of ossification from the diaphysis into the metaphyseal cartilage stimulates further growth in the latter, enabling longitudinal growth. This continues until the bone reaches its maximal length, whereupon the

cartilage ceases proliferation and becomes fully involved in the diaphyseal ossification. As this process of ossification completes, the metaphyseal growth plate closes, with the diaphysis and epiphysis fusing together (Baxter, 2011, Le Gros, 1958, Sisson and Grossman, 1975). Obliteration of the metaphyseal growth plates occurs within specific time ranges from birth; in horses, the radiographic timing of growth plate closures tends to occur within specific 4 - 5 month ranges (Table 5.1).

Growth rates are rapid throughout the first 140 - 150 d (5 months), during which period the foal quadruples its bodyweight. After this point the growth curve levels off, with the foal standing at around 80% of its mature height (Brown-Douglas, 2011, Kocher and Burton Staniar, 2013, Stoneham, 2011). Third metacarpal and metatarsal growth has been reported to plateau at around 140 d of age (Thompson and Smith, 1994). This is the stage at which nutritionally available forage declines in the winter months for foals born in spring or early summer in temperate climates (Brown-Douglas, 2011). Skeletal maturity of the limbs is usually assessed as the point when closure of the distal radius occurs at around 20 - 24 months, with some variation associated with breed and sex (Hintz et al., 1979, Luszczyński, 2011).

Premature foals may have undergone less growth than their mature, term counterparts during these early months. This is particularly the case for sick immature foals, given that their resting metabolic rates are approximately 75% of their healthy age-matched counterparts (Vaala, 2011). Additionally, they may be developmentally delayed due to their lack of readiness for birth.

This is recognised in human medical practice, wherein ages of premature babies are sometimes adjusted to facilitate assessment of developmental deficits (D'Agostino, 2013, Wilson, 2004). The following formula is used:

adjusted age = chronological age - (‘normal’ gestation - actual gestation).

When applied to a foal aged 140 d (5 mo) that was born at 305 d gestation, and utilising mean gestation length, this would yield the adjusted foal age:

140 - [336 - 305] = 109 d.

The extreme variability of equine gestation precludes use of such a formula in practice, as in some areas of human assessment (Wilson, 2004). However, for research purposes, this correction provides a basic indicator of the extent of developmental delay or deficiency in chronological terms. It follows that any deficit is proportionately greater in the earlier months of life.

This has implications for appendicular growth, if it is accepted that metaphyseal growth plate closures occur at chronologically determined stages, and at a temporal distance from parturition that is unchanged by the foal’s immature status. In the following study (Chapter 5.2), the effect of developmental delays on the overall proportions of horses with a history of gestational immaturity is investigated.

5.2 Study: Growth Retardation Associated with Gestational Immaturity

5.2.1 Introduction

Foals that were immature at birth can present a range of orthopaedic issues during the perinatal and juvenile (weanling to 2 y) phases, including incomplete ossification of the cuboid bones of the carpal and tarsal joints, carpal and tarsal collapse and associated ALDs, osteoarthritis, and osteochondrosis (Auer, 1982, Baxter, 2011b, Haywood et al., 2018, McLaughlin, 1981, McIlwraith, 2003). While these issues have been investigated in the neonate, little research has been conducted into the immature foal's physical phenotype at maturity. This is partially due to the difficulties involved in completing equine longitudinal studies, given that although relatively long-lived compared with many domestic and livestock animal species, horses are frequently subject to changing ownership.

Given the potential severity of pathologies associated with gestational immaturity, it is surprising that anatomical variations have not been prioritised. Research into growth rates in the general horse population has typically been concerned with breeding practices, training and exercise physiology, and nutrition (Anderson, 2004, Bridges, 2016, Hintz et al., 1979, Luszczyński, 2011). Skeletal development is often estimated on the basis of time from birth to growth plate closure in the distal radius, based on radiographic images of the forelimbs (Anderson et al., 1993, Luszczyński, 2011).

Proportionality between lower limb bone length at birth and height at maturity is well known in the equine breeding community, with the third metacarpal length of the neonatal foal commonly taken as an indicator of eventual height at the withers. This popularly held belief probably owes much to the fact that the distal third metacarpal metaphyseal plate closes at around 6 - 8 months of age (Strand, 2007, Budras, 2011), after which only limited growth occurs below the carpus, in the first and second phalanges until around 12 m. In Thoroughbred horses, growth from weaning to 3 yo has been reported as accounting for only 5 - 7% of total growth; conversely, wither height is amongst measures known to increase significantly throughout both first and second years (Anderson, 2004). A moderate to strong correlation between long bone length and height at the withers at all ages and development stages indicates that horses remain proportional within their age groups (Anderson, 2004).

It is possible that the timing of physeal closures in the distal limb of the gestationally immature foal undermine this conclusion. In the full term, mature foal, the proximal metacarpus and metatarsus physes are closed before birth, thereby providing a supporting 'platform' during the final ossification of the newly loaded carpal and tarsal cuboid bones. However, in the gestationally immature foal with SOI grade 2, this growth plate is either fully or partially open, while in SOI grade 1, it is absent altogether (Adams, 1988).

These growth plates are not always recognised: one major lameness text states that the third metacarpal and third metatarsal, along with the first and second phalanges, have just one growth plate (Baxter, 2011c). This is presumably due to the lack of epiphyseal activity in plates that have usually closed prior to

parturition. In their ossification indices, Adams and Poulos (1988) and Haywood et al. (2018) certainly provide clearly labelled, radiographic documentation of the developing third metacarpal and third metatarsal epiphyses.

This indicates that growth of the third metacarpal and third metatarsal is incomplete at parturition. It can be speculated that the pressure caused by load bearing once the neonatal foal rises to its feet will precipitate its growth plate closure, despite the bone's immature length.

Systems for assessing conformational proportions and angles have been developed to inform breeding selection and to analyse biomechanical relationships between performance and conformation variables (Holmström, 1990, Holmström and Philipsson, 1993, Holmström, 2013). These have been widely adopted in research and industry, with various bespoke programs developed (Senna, 2015). However, these frequently necessitate palpation of bony landmarks by the researcher and/or application of adhesive markers directly to the horse. Such an approach was impractical in this study, as not all horses could be visited by the researcher.

In this study, the proportions of external measurements of horses with a history of prematurity or dysmaturity were compared with those of unaffected, related horses. Measurements were combined into indices to evaluate metric properties of conformation. Indices were analysed by one-way ANOVA before a Principal Components Analysis was applied to identify the proportions of maximum variation between gestationally immature and unaffected control horses.

5.2.2 Materials and Methods

5.2.2.1 Study Design

Small groups comprising gestationally immature horses and related controls were recruited. Lateral conformation photographs of each horse were taken by the researcher or, in instances where distance precluded a visit, by the horses' owners. All owners who contributed photographs had been provided with written instructions regarding correct positioning of the horse and photographer, lighting and camera angle. Horses were standing as square as possible, or in lieu of square, with limbs nearest the camera squared. Each family of horses was photographed by the same person, with the exception of the two individual horses. One image was selected per horse; if an image was low resolution, it was excluded.

Each animal was characterised with measurements to evaluate metric properties of conformation, adapted from Komosa et al. (2009). Of the original 24 measurements, five were later eliminated due to the difficulties involved in their application across the full image set (Fig. 5.1). Using the software, ImageJ (Rasband, 1997 - 2018), 19 measurements were then made from photographs. Measurements in pixels were then combined in a spreadsheet to create 22 indices reflecting proportional anatomical relationships, also adapted from Komosa et al. (2009) (Appendix 2). If a measurement could not be effectively made due to photographic artefacts (e.g. horse's tail obscured the talus/point of hock), no value was recorded.

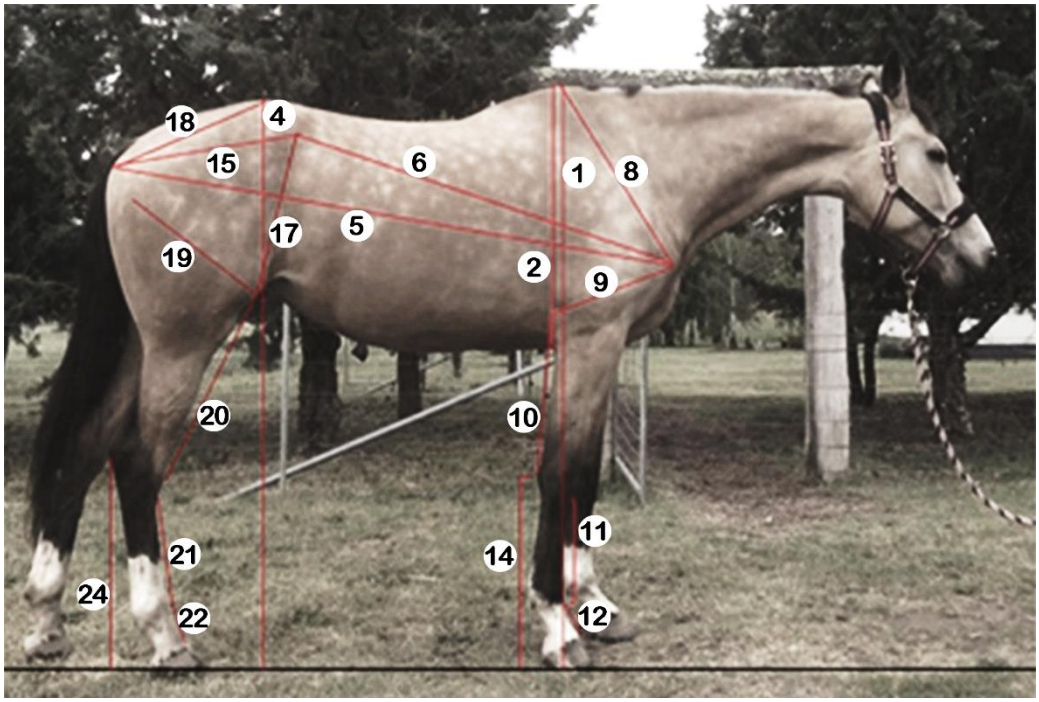

Fig. 5.1 Anatomical measurements adapted from Komosa et al. (2009) were applied to lateral images of the horses. Some measurements were eliminated due to impracticality, leading to omissions in the numbering sequence.

5.2.2.2 Animals

The horses included in this study (n = 47) comprised 19 animals with a history of gestational immaturity and 28 controls. The immature cases comprised horses with a history of prematurity (< 320 d gestation, n = 9) or dysmaturity (mature, full term gestation, showing physical signs of prematurity, n = 10). Physical characteristics associated with dysmaturity included foals being small for gestational age, abnormally weak, or presenting hyperextension of flexor tendons in the forelimbs, hindlimbs or both (Madigan, 2014, Rossdale et al., 1984b). Horses with histories of either IUGR or neonatal maladjustment syndrome without any of the listed physical signs were not included.

A number of breeders were interviewed and the case horses were selected on the basis of foaling records. Case histories were recorded through interviews with breeder-owners. The related controls had no known issues, with reported gestational histories within 335 - 345 d. Where possible, cases were matched with closely related controls of similar age; 16 family groups were represented. As 2 dysmature cases had no related controls available, the individuals, a purebred Arabian and a purebred Australian Stock Horse, were carefully matched with controls sharing breed and type, age and sex. Breeds represented overall were Arabian, Australian Warmblood, Irish Draught, Australian Pony, Waler, Shire, Standardbred, and first crosses thereof. Ages ranged from 3 - 16 y.

5.2.2.3 Data Analysis

Statistical methods employed were one-way ANOVA, Linear Mixed Effects model, and Principal Components Analysis (PCA), using the software, R Studio (R Core Team, 2015a).

One-way ANOVAs were applied to the measurement indices for effect of gestation group. Principal Components Analysis (PCA) was applied to the index values to establish the most variable morphological ranges. PCA belongs to the group of multivariate analysis methods, with the basic concept being to use a small number of ‘hidden’ factors, or components, in relation to an extensive set of primary variables. Using this method, it was possible to assess the morphological distance between case horses and controls.

The next stage of the analysis was to select the key features related to a given component and further assess these to establish effect of gestational immaturity by one-way ANOVA. The distribution of the residuals in the model was tested for normality using the Shapiro-Wilk Test. $P < 0.05$ was considered significant and $0.1 > P > 0.05$ was considered to indicate a tendency towards statistical significance. Values are presented as means ± S.D.

All 47 horses were included in the analysis of basic measurements (one-way ANOVA) and the PCA. When varimax rotation was applied to the components, 3 horses with index values missing due to photographic artefacts (1 dysmature, 2 controls) were excluded from the analysis. Gestational effect within the primary Principal Component is therefore based on n = 44 horses.

5.2.3 Results

Analysis of index values by one-way ANOVA showed a significant effect of gestational immaturity on 4 anatomical relationships (Table 5.2).

An effect was also suggested for index 8 (thoracolumbar length vs. wither height), index 10 (femur vs. height at croup) and index 19 (height of hock from ground vs thoracolumbar length, but residuals could not be transformed to satisfy a normal distribution.

Table 5.2. Anatomical indices that are affected by gestational immaturity. Measurements were combined to create indices which were then used to assess anatomical proportionality (Komosa, 2009). One-way ANOVAs were performed on the index values to analyse the effect of gestational immaturity.

Index	Measurement relationship	Effect of gestational immaturity (P =)
11	**Index of Cannon:** Proximal third metatarsal to metacarpophalangeal joint (hind cannon length) vs. Height at croup	2.14e-05
13	**Index of Foot:** Height of calcaneal from ground (hock height) vs. Height at croup	0.0001
18	**Index of Foot-Trunk (Greater)** Height of calcaneal from ground (hock height) vs. Thoracolumbar-pelvic length (full body length)	0.0004
17	**Index of Hand** Height of accessory carpal from ground (knee height) vs. Thoracolumbar length (trunk)	0.041
9	**Index of Femur:** Greater trochanter to patella (femur length) vs. Height at croup	0.09
1	**Index of Scapula:** Major tubercle of humerus (point of shoulder) to wither via scapula spine vs. Height at withers	0.09

In the PCA of index values, the first two principal components account for 43.78% of the total conformational variation of the horses' external features (Table 5.3). The four dominant components of both PC1 (31.17%) and PC2 (12.61%) tend to separate horses with a rectangular conformation, i.e. body length > height at the withers, from those that are more square, i.e. body length = height at the withers (Fig. 5.2). The shape change along PC1 from negative to positive is dominated by a reduction in the length of the distal limbs in combination with

a lengthening of the thoracic body length in relation to height at the withers. The shape change along PC2 from negative to positive is dominated by a lengthening of the distal limbs in relation to proximal limbs and height at withers.

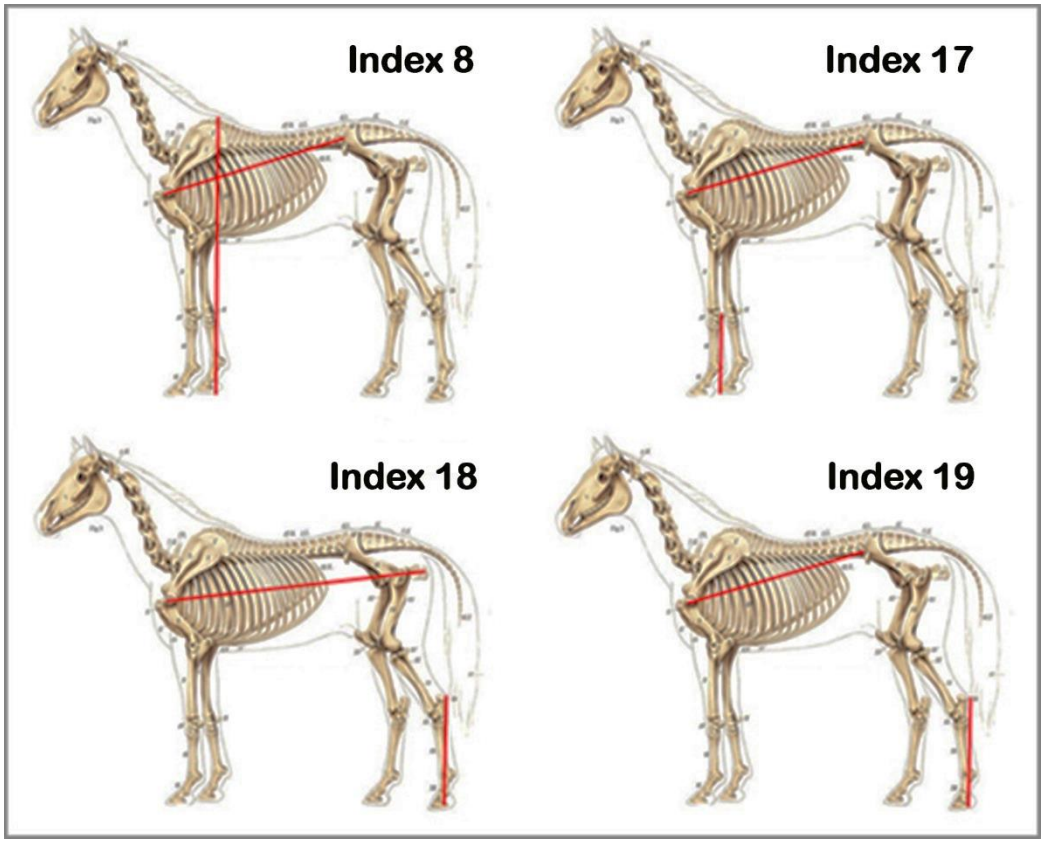

Fig. 5.2 Four indices created from anatomical measurements showed a significant effect of gestational immaturity and comprised the dominant components of PC1 (P = 0.001).

Table 5.3. The relationships of individual indices within PC 1 - 2, after varimax rotation (n = 44).

		PC1	PC2
	Std. dev.	2.6363	1.6769
	Prop of var.	31.17%	12.61%
	Culm. Prop.	31.17%	43.78%
Index	**Measurement relationships**		
3	Olecranon (point of elbow) to Accessory carpal *vs*. Height at withers		0.313
4	Accessory carpal height from ground *vs*. Height at withers		-0.309
8	Major tubercle of humerus (point of shoulder) to Tuber coxae (point of hip) *vs*. Height at withers	0.310	
15	Olecranon (point of elbow) to Accessory carpal *vs*. Accessory carpal height from ground		0.471
17	Accessory carpal height from ground *vs*. Major tubercle of humerus (point of shoulder) to Tuber coxae (point of hip)	-0.318	
18	Height of Calcaneal (point of hock) from ground *vs*. Major tubercle of humerus (point of shoulder) to Tuber ischii (point of buttock)	-0.345	
19	Height of Calcaneal (point of hock) from ground *vs*. Major tubercle of humerus (point of shoulder) to Tuber coxae (point of hip)	-0.329	
20	Tuber coxae (point of hip) to Tuber ischii (point of buttock) *vs*. Major tubercle of humerus (point of shoulder) to Tuber coxae (point of hip)		-0.307

When PC1 is rotated to analyse different gestational groups, a significant effect of immature gestation is evident (P = 0.001). Further analysis shows the higher significance of the premature group compared with dysmature groups (P = 0.009,

P = 0.012, n = 44). Mean values for the four dominant indices all show that the immature groups have significantly lower distal limb to body length relationships than controls (Table 5.4). There was no effect of gestational immaturity in PC 2.

Table 5.4. Mean index values ± SD for premature, dysmature and control groups within the four dominant indices of Principal Component 1.

Index	Measurement ratio	Premature	Dysmature	Controls
18	Index of Foot-Trunk (Greater)	34.75 ± 2.09	35.78 ± 1.41	38.29 ± 2.97
19	Index of Foot-Trunk (Shorter)	48.48 ± 3.37	50.69 ± 2.22	54.14 ± 4.5
17	Index of Hand-Trunk (Shorter)	3.95E-03 ± 2.34E-04	3.91E-03 ± 2.34E-04	4.14E-03 ± 3.69E-04
8	Index of Trunk (Shorter)	73.78 ± 2.55	73.59 ± 2.76	71.30 ± 4.07

5.2.4 Discussion

This study reveals significant variation in some external anatomical relationships of horses with a history of gestational immaturity. The dominant PC1 indicated that within this study, some horses with a history of gestational immaturity are proportionately longer in the body for their height, with shorter distal limbs for their body length (Fig. 5.3). These results do not align with a commonly held perception that growth retarded, neonatal foals either ‘catch up’ with postnatal growth, or grow as more diminutive but proportionately similar versions of their unaffected siblings.

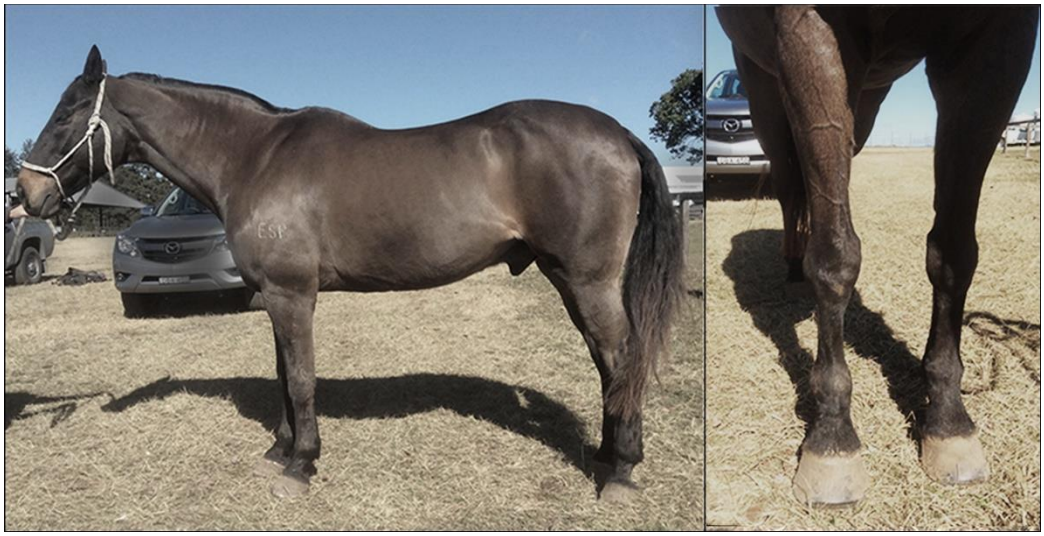

Fig. 5.3 Twenty-year-old Hanoverian gelding, born at 298 d gestation, presenting short third metacarpals, with carpal deformity associated with rotation at the proximal epiphyses (image: author's own).

Horses that mature at less than anticipated height are included in previous studies and it is informative to consider corresponding developmental issues in these individuals. For example, the Quarter Horse foal assessed by Wong et al. (2003) presented as an extreme SOI grade 1 when radiographed at 2 days of age, was described as being 'subjectively short in stature with stunted long bone growth' at day 84 of life (Wong et al., 2003). Dutton et al. (1998) report that 2 horses that presented Type-II lesions (> 30% collapse) as foals < 10 months old that were not being used for intended purpose due to stunted growth. Data are unavailable to confirm the association, but these findings suggest that a correlation between poor carpal and tarsal ossification at birth and retarded growth may possibly be true.

In the extremely immature foal, metaphyseal growth plates that should be fully closed before parturition may still be open (Adams, 1988). SOI grade 2 foals may

have a partially or fully open proximal third metacarpal metaphyseal growth plate; SOI grade 1 foals, being the least developed, also lack any radiographically detectable epiphyseal ossification. It is entirely possible that inappropriate loading of the endochondral cartilage could precipitate this growth plate closure at a point before optimal longitudinal growth, normally a prenatal affair, has been achieved.

Furthermore, ossification requires the availability of calcium and phosphate ions, cobalt and collagen, appropriate pH and the enzyme alkaline phosphatase (Savage, 1998). For immature foals that are endocrinologically challenged, obtaining the required pre- and postnatal nutrition for optimal growth may be a challenge, leading to poorer quality ossification. Meanwhile, early cessation of metaphyseal growth can occur as a result of excessive pressure (Baxter, 2011). There is opportunity for this to occur once an immature foal with inadequate bone development is standing and mobile, and particularly once it starts to gain weight more rapidly as a 1 - 2 yo.

Critical questions remain about the early rate of growth in the gestationally immature foal. There is no indication that closure is delayed to allow for necessary development to take place in the growing foal and, likewise, there is no indication that the foal's growth accelerates to meet the normal development landmarks of unaffected counterparts. Considered in terms of weeks, developmental retardation is proportionately greater during the earliest months of life, and therefore potentially impacts the third metacarpals and third metatarsals most. The distal physes of these bones close at around 6 months of age (Budras, 2011, Rooney, 1975). Even in immature foals graded SOI 3 - 4, if the individual is diminutively sized, these bones will cease longitudinal growth at a relatively early stage in the

foal's growth. For SOI grade 1 - 2 foals, this is of further consequence, as the bone's growth is potentially arrested at both metaphyseal plates.

This has ramifications: many conformational analyses of horses hold that short-limbed horses and those with sickle hocks have shortened strides (Baxter, 2011d, Ross, 2003,), that relatively long backs are more prone to weakness (Holmström, 2013, Ross, 2003), and also that long backed horses may have trouble engaging the hindlimbs (Ross, 2003). It is also held that in the balanced horse, wither height should approximate the horse's body length from point of shoulder to point of buttock (Fig. 5.4) (Ross, 2003); a horse with a longer body than its height often experiences difficulty in synchronisation and coordination of movement (Baxter, 2011d). It is recognised, however, that wither height is not related to stride length (Holmström, 2013).

Historical literature from the 19th and early 20th century does, however, place value on a long radius and short forelimb cannon (Holmström, 2013). Meanwhile, more recent research into sports horses shows that these principles do not necessarily hold true in relation to successful performance: Royal Dutch Warmblood stallions and racing Thoroughbreds tend to be more rectangular in build, while short third metacarpals in Thoroughbreds are considered to be a strength when combined with a long radius and short humerus to enable maximal stride length (Holmström, 2013, Ross, 2003).

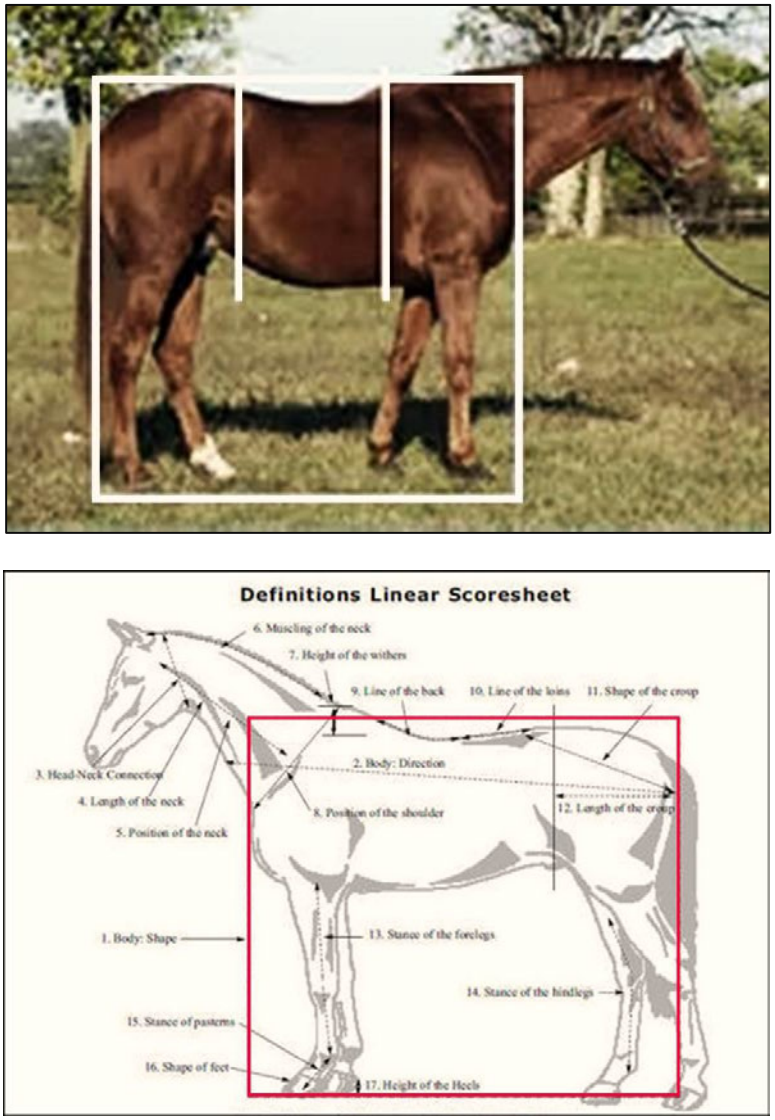

Fig. 5.4 Square vs. rectangular conformation. (Top) A square conformation with equal thirds for forequarters, back and hindquarters is often held to be ideal (Winkel, 2009). (Bottom) Sports horse societies may recommend a more rectangular frame to allow for greater athleticism (KWPN-NA, 2019).

These standards are applied, naturally, on the performance outcomes of horses in good musculoskeletal health; short cannons are only considered to be a conformational strength when also accompanied by a straight or near straight forelimb axis (i.e. valgus no greater than $\leq 3°$) (Gaughan, 2011) and in the absence of carpal or tarsal collapse.

For gestationally immature horses that fail to mature at less than anticipated height, it appears that skeletal health may be compromised. There will be additional differences in horses with longer backs and proportionately shorter distal limbs. At present, the musculoskeletal and biomechanical effects of this non-proportionality are only partially understood.

5.2.5 Conclusion

Horses with a history of gestational immaturity may present significantly different anatomical proportions to unaffected related horses, with a shorter wither height to thoracolumbar body length ratio, and shorter third metacarpals and third metatarsals to thoracolumbar body length ratios. These conformational attributes are often associated with a shorter stride length and a higher risk of back injuries.

5.3 Study: Skull Morphology

5.3.1 Introduction

In premature foals, a domed head is a characteristic used as an identifier of the condition (Austin, 2013, Lester, 2011, Madigan, 2014). This feature is effectively the cranium of the foetus in late gestation, which is proportionately larger than in extra-uterine life to accommodate the rapidly growing brain.

It is interesting that in human medical research, certain skull growth patterns have been identified in cases of extreme prematurity. These include a disproportionately large head (Ranke, 2014), and accelerated 'catch up' growth in the postnatal period, despite poor overall growth (Cockerill, 2005, Cooke, 2008). Both patterns have been associated with impaired neurological, developmental, and cognitive outcomes (Ranke, 2014).

The author's subjective assessment of the skulls of horses with a history of gestational immaturity suggests that the cranium of these individuals could be smaller than average, appearing more 'teardrop' shaped, being both narrower and shallower, particularly in the caudal region. It can be hypothesised that as with other growth stages occurring during the perinatal period of a normal disruption, premature birth might also lead to disruption of growth processes of the skull.

The cranium is formed primarily by the two convex parietal bones, which form the greater part of the roof of the cranial vault and unify centrally in the sagittal or parietal crest (Fig. 5.5). Rostral to these are the frontal bones, which join the parietals at the coronal or parieto-frontal suture. The frontal bones form the flatter area of forehead in the neurocranium (Rooney, 1975). In the growing foal, the central part of the parietal bones lose their convexity as the sagittal sutures start to fuse, and there is no reason to suspect gestationally immature foals are any different.

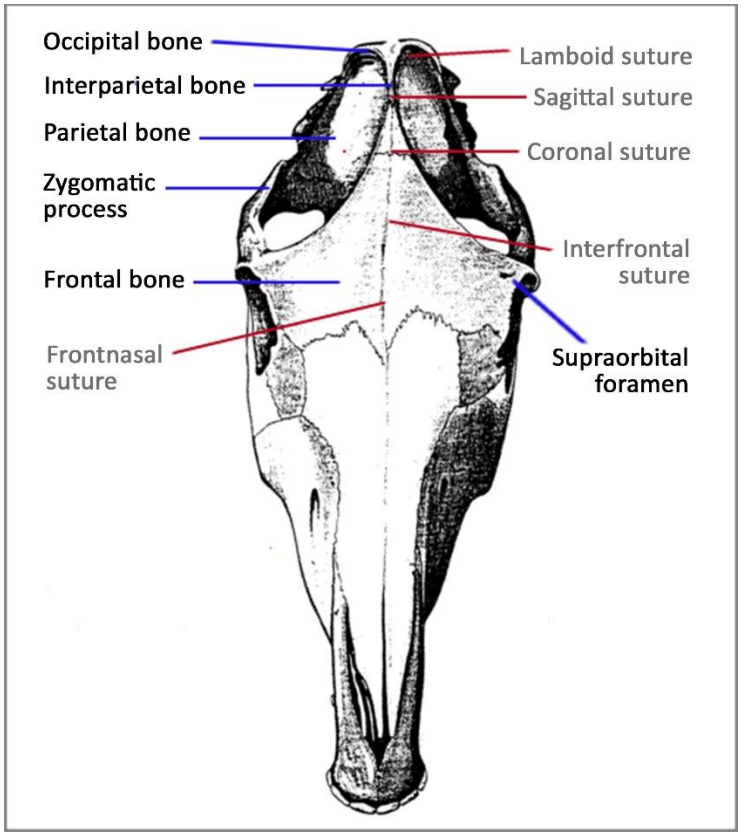

Fig. 5.5 Skull morphology: the bones and sutures of the dorsal neurocranium.

Growth in the membranous bones of the skull differs from the osteochondral growth of the appendicular long bones. During mid to late gestation, the cranial bone fronts achieve their approximate dimensions and either overlap to create the transverse sutures, or abut one another to create the midline sutures. External signals arising from genetic and epigenetic factors trigger bone formation at the sutures, which retain their soft tissue composition throughout normal growth periods (Libby, 2017).

Closure times for the sutures of the dorsal cranium in horses are not well established (Dixon, 2014), although the nasofrontal suture is fused within the first

month of life (Mair, 2015) and is obliterated by 12 m (Butler, 1993). Timing of the fusion of the parietal and parieto-occipital sutures is not known, although these are obliterated by 4 - 5 y (Butler, 1993). However, sutures of the ventral cranium and occiput start to fuse radiographically soon after birth and are obliterated within the early months of life; a fontanelle between the upper frontal bones is radiographically closed by 3 - 4 m (Butler, 1993).

This ossification process can be disrupted by biomechanical or biochemical factors that lead to increased cell differentiation. As a consequence, too little bone growth can lead to open sutures, while too much or accelerated growth can lead to ossification and premature suture closure (craniosynostosis). When a suture ossifies, perpendicular growth is halted, although longitudinal growth continues (Opperman, 2000). Certainly, the sagittal suture is most commonly affected in human craniosynostosis, along with the coronal and the lamboid sutures of the occipital bone; premature fusion leads to an elongated skull shape (Sharma, 2013).

5.3.2 Materials and Methods

In a small preliminary study, the skulls of the two research cases, an 8 yo Warmblood mare born the smaller of twins and a 5 yo Australian Stock Horse gelding that was dysmature, were assessed against 10 skulls of horses with known and unknown gestational history.

Each skull was marked with adhesive tape at two measurement points of the cranium: at the level of the median auditory meatus, and at the widest point adjacent to the base of the zygomatic arch. The maxillofacial skull was placed flat and photographed from a tripod-mounted camera directly above the centre of the nasofrontal suture. Linear measurements for these points and two additional area measurements for the cranium were made with the software ImageJ (Rasband, 1997 - 2018). Area measurements comprised the cranium itself and the neurocranium. The latter was a representative area within a rectangle bounded caudally by the nuchal crest of the occiput, rostrally by the supraorbital foramen, and laterally by the centre of the zygomatic arch (Fig. 5.6).

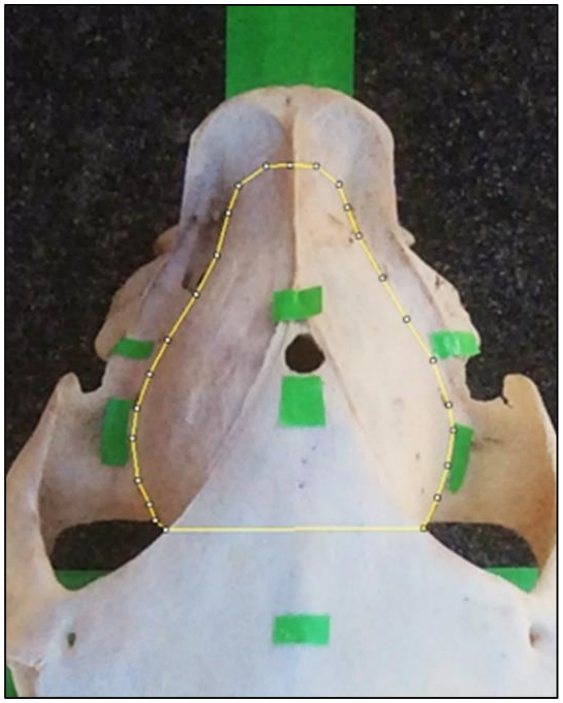

Fig. 5.6 Image of a skull of a mare born as a dysmature twin prepared for area measurement in ImageJ (Rasband, 1997 - 2018). Note: this skull is asymmetric. This horse had severe Angular Limb Deformities in four limbs (bilateral tarsal valgus, carpal valgus and carpal varus). Asymmetric development was evident in all limb bones and the cervical vertebrae, indicating that the deformation of the skull arose from musculoskeletal and biomechanical asymmetry.

Index values for each horse were created from these values: a linear index (maximum parietal width*100 / parietal width at the level of the meatus) and an area index (cranial area*100 / neurocranium area). These were analysed by one-way ANOVA. Post-hoc power analysis and sample size calculations were carried out using a Z-test in Microsoft Excel, comparing case and control for all non-significant outcomes in response variable by phase.

5.3.3 Results

Results (Table 5.5) indicated an effect of gestational immaturity that tended towards significance (linear index P = 0.06, area index P = 0.07). Z-test power analysis indicated that 205 skulls would be required to achieve statistical significance at an α-level of 0.05 and 90% power.

Table 5.5. Cranial morphology of horses with a history of immaturity. Mean values ± SD are shown for linear index (parietal width) and area index (cranial area proportions). Images were measured with ImageJ (Rasband, 1997 - 2018).

Group	Linear index (width of cranium)	Area index (area of cranium)
Immature (n = 2)	104.88 ± 37.43	24.34 ± 8.73
Term (n = 10)	119.38 ± 5.61	27.57 ± 2.10
P =	0.06	0.07

5.3.4 Discussion

It is possible that morphometric modelling from CT scans might yield stronger results through the creation of cranial 'endocasts', by which volumetric measurement of the cranial vault space could be achieved. It is also possible that a breed effect is present. A previous study has shown a breed effect in skull morphology between Thoroughbreds, Arabians and Standardbreds, with significant differences found in the cranial length, but not the width (Evans and McGreevy, 2006). The small numbers in the present study preclude statistical findings on the effect of breed. Further research is unlikely, given the numbers required and the difficulty in tracing and obtaining samples from horses with the relevant gestational history.

5.3.5 Conclusion

It cannot presently be determined whether horses with a history of gestational immaturity are affected by premature suture closure leading to a reduced cranial volume.

5.4 Towards a Phenotype – Growth Retardation

As in humans born prematurely, some horses with a history of gestational immaturity experience adaptations of growth patterns and fail to reach anticipated height. The deficiency appears to be in the distal limb, which is short in relation to the body length, rendering them squarer in conformation as opposed to rectangular. It is possible that this is due to the developmental delays that are present from birth, which have a proportionately greater effect in the first 6 - 8 months of life, in the period prior to the closure of the distal third metacarpal and third metatarsal metaphyseal growth plates. Affected horses do not appear to have compensatory, accelerated growth rates during this period.

The absence of accelerated growth may be pronounced in foals graded SOI 1 - 2 at birth. In the most affected SOI 1 cases, which are least developed, the proximal epiphysis of the third metacarpal has yet to start ossifying. In others, ossification has commenced, yet the growth plate is still open. These foals are also likely to exhibit Grade II lesions, i.e. carpal or tarsal collapse. Such cases have been reported as failing to fulfil their athletic potential. The cumulative biomechanical and performance effects of an adapted limb to body ratio are unknown. It is possible that the skull may also be affected, due to premature closure of sutures proximal to the cranium.

Chapter 6

EXPRESSIONS OF GESTATIONAL IMMATURITY

"We have argued that obstetrical disorders are really syndromes, the features of "The Great Obstetrical Syndromes" are the following: 1) multiple aetiology; 2) long preclinical stage; 3) frequent fetal involvement; 4) clinical manifestations which are often adaptive in nature; and 5) predisposition to a particular syndrome is influenced by gene-environment interaction and/or complex gene-gene interactions involving maternal and/or fetal genotypes"(Gotsch, 2009)

"More knowledge is always needed, and nowhere is this more evident than when considering another species' ontology; those interested in bettering equines' lives, whether scientists or practitioners, must therefore begin to ask questions to advance from what we know (whether or not this is the result of scientific investigation)."(Kiley-Worthington, 2011)

6.1 Introduction

In considering the persistent effects of equine gestational immaturity, it is apparent that there is no single phenotype, with individuals being affected by a wide array of variables. With equine research into ongoing effects being limited, researcher observation and anecdotal evidence from owner-breeders provides important indicators as to associated behavioural traits that appear to lead to

heightened reactivity, behavioural issues and an elevated sensitivity to touch. These characteristics are already well reported in human literature (Arpi, 2013, Chorna et al., 2014, Eryigit-Madzwamuse et al., 2015, Dusing et al., 2016, Haley et al., 2006, Hornman, 2016, Raju et al., 2017, Ranke, 2014).

With certain endocrinological adaptations and musculoskeletal irregularities established, there are likely to be observable behavioural expressions associated with developmental issues linked to gestational immaturity. For example, persistent dysregulation of the HPA axis, manifest through an elevated or blunted adrenocortical response to stressors, may cause different expressions in the affected horse when faced with an unpredictable environment that includes the pressures of handling, riding and training, or health issues and pathologies. Should development issues affecting the adrenal gland also affect other endocrinological systems, including the renin-angiotensin-aldosterone system, then the effect of calcium dysregulation may also be evident in behavioural changes due to anxiety, depression or pain, as well as soundness issues (Kritchevsky, 2011, Toribio, 2002). It is important to identify the expression of these conditions if further stress is to be avoided.

In this research, observation and anecdotal evidence underpinned a number of studies. Corresponding methodologies were developed, with the aim of identifying indicators of this hypothetical phenotype as follows.

- A method for assessing the adrenocortical response in a variety of situations was required, which could then be used to measure the responses of privately owned horses in non-clinical environments. The

circadian SCC cortisol profiles were measured in a pilot test, which also confirmed the presence of herd behaviour through the use of movement sensors (Chapter 6.2).

- A method for measuring reactivity through the use of a startle stimulus test and the measurement of SCC was piloted, and then conducted in field studies. This study was also filmed (Chapter 6.3).
- The horses' sensitivity to pressure stimuli were measured through the use of algometry (Chapter 6.4).
- The subjective views of owner-breeders on their horses' behavioural traits were collected via a questionnaire and analysed (Chapter 6.5).

6.2 Study: A Pilot Method for Measuring Basal Salivary Cortisol Levels of Horses at Pasture

6.2.1 Introduction

This pilot study examined a methodology for assessing stress responses in paddock-kept horses using SCC. This simple and easily administered method to assess stress levels in horses is widely used for physiological stress testing in horses, including those in competition (Peeters et al., 2013), training (Smiet et al., 2014), travel (Clark et al., 1993, Fazio, 2008, Shanahan, 2003), weaning (Moons et al., 2005), and horses at colic risk (Leal et al., 2011). However, limitations such as circadian and basal levels, delayed secretion of cortisol into saliva, and sources

of negative and positive arousal, which can also elevate cortisol secretion, must be taken into account.

In common with other mammalian species, horses have circadian rhythms in cortisol release, with a peak occurring in the morning, typically between 8am and 10am (Peeters et al., 2011), and the lowest point in the evening from 8 pm - 10 pm, with oscillations every 20 - 180 min throughout the 24-h period (Bohák et al., 2013, Cordero et al., 2012, Evans, 1977, Johnson and Malinowski, 1986, Lebelt, 1996). Cortisol levels are also higher in spring than in autumn (Cordero et al., 2012). Variation in cortisol concentrations, not just between companion horses but for a single horse on subsequent days, has also been reported (Evans, 1977, Hughes et al., 2010, Kędzierski et al., 2013).

Compared with serum cortisol testing, saliva sample collection is non-invasive, less arousing than venipuncture, and easier to undertake in the field (Bigert et al., 2005, Bohák et al., 2013, Peeters et al., 2011, Schmidt et al., 2010). Unlike serum, in which most cortisol is bound to proteins, including cortisol-binding globulin (CBG), saliva contains the smaller percentage of free, unbound cortisol, meaning concentrations are lower. However, the post-stress event increase in unbound cortisol in saliva is about 10 times that of serum (Peeters et al., 2011, van der Kolk et al., 2001), making SCC a clear indicator for stress responses.

However, collecting and measuring SCC is subject to limitations, and failure to address these can compromise results. The circadian effect, combined with variability between individuals (Evans, 1977, Harewood and McGowan, 2005, Hoffsis, 1970b), means that the sample period needs to be carefully selected, with

samples collected frequently in order to accurately identify any post-stimulus elevation (Hart, 2012, Hart and Barton, 2011,). Meanwhile, the slow rate of secretion of cortisol into saliva from plasma means the sampling period should be long enough for peak elevation and return to baseline to be identifiable.

While no relationship between sex and basal SCC has been reported (Peeters et al., 2013), age has been found to affect basal profiles in other species, possibly due to physiological changes in HPA axis responsiveness (Peeters et al., 2013). Studies involving horses should therefore be matched in age group, environment and handling (Hart, 2012). For this reason, many studies have used same-breed, stabled horses (Bohák et al., 2013), although the stable environment presents a risk that the positive arousal caused by routine feeding and exercise might influence the values measured (Peeters et al., 2013), and as such, the data may not be directly relevant to pastured horses.

In developing a methodology for a field experiment assessing stress responses in paddock-kept horses, we opted to utilise SCC as an indicator, as it seemed to be the best way to overcome potentially confounding influences associated with the circadian rhythm, environment and management. It was hypothesised that with unrestricted movement and constant access to feed, paddock horses might have different basal cortisol profiles to those of stabled horses used in previous studies, which are subject to positive arousal due to daily feed and exercise routines (Hart, 2012). Therefore, the aim was to first establish a 24-h basal profile for two paddock-kept horses by mapping the peaks, troughs and amplitude of their circadian profiles, while monitoring their activity levels to assist with

interpretation of values. These profiles would enable a more accurate interpretation of SCC values across the later stress stimulus tests.

As this was an observational pilot, the correlation of the two horses' SCC values was completed with R Studio computing software (R Core Team, 2015b), but no further analysis was undertaken.

6.2.2 Materials and Methods

6.2.2.1 Animals

The subjects were Horse 1, a 14.1 hh, 4 yo Arabian gelding, and Horse 2, a 14.2 hh, 7 yo Australian domestically-bred brumby gelding. Horse 1 was born prematurely at 305 d. Neither horse had ever been stabled or ridden. The pair had lived in a small herd of 4 horses for over 12 months and for the most recent 4 months in a 0.7 ha fenced paddock, with mixed-pasture grass. As saliva samples with a pH ≤ 3.5 or ≥ 9.0 can artificially raise or lower SCC values, a grass sample was macerated and tested with a benchtop pH meter (TPS Pty Ltd, Brendale, Queensland), giving a 6.25 pH value. The protocol and conduct of the experiment was approved by the CSIRO Armidale Animal Ethics Committee under the NSW Animal Research Act, 1985 (Animal Research Authority 14/02).

6.2.2.2 Movement Sensors

Prior to the sample collection period, movement sensors were fitted to the horses to confirm synchrony of behaviour. IceQube movement sensors (IceRobotics, Edinburgh), as previously described (Chapter 4.5), are triaxial accelerometers that continuously monitor and record recumbent rest (lying time), number of steps taken, standing time (i.e. all non-recumbent time), as well as the number of recumbent rest periods and their duration.

These were fitted over an exercise bandage on the horses' near hind legs, where the horses were considered less likely to interfere with them by rubbing. The horses were observed regularly to ensure that sensors remained in place. Data were collected for the 24 h period from 08:00 - 07:59.

6.2.2.3 Sample Collection

Horses were habituated to sample collection prior to the study's commencement (Fig. 6.1). The saliva samples for the 24 h profiles were collected on 4 separate days in early spring (temperature range -1.8°C to 13.1°C) and late spring (11.6°C to 30.9 °C). Starting at 8am, samples were collected at 90 min intervals until 8am the following morning. Records were kept of each sample's colour, which was attributable to feed in the mouth, and the horse's activity at the collection point. Weather conditions, including high winds and storms, were noted.

Fig. 6.1 Saliva samples were collected by swabbing the horse's mouth with a Salivette held with allis tissue forceps. (Image: author's own)

Swabs were used for saliva collection (Cortisol Salivette®, Starstedt, Nümbrecht) (Peeters et al., 2011, Wood et al., 1998). The same three handlers collected samples throughout, using allis tissue forceps to administer the swab. Positive reinforcement was given in the form of a thin slice of carrot, weighing < 2 gm, to negate the possibility of the horses becoming averse to the repeated sample collection. Neither horse sought out the reward, indicating it was not a source of anticipatory arousal that might affect SCC levels.

Samples were chilled within 10 min of collection and frozen within 24 h. On the day of assay, they were centrifuged at 3000 rpm for 2 min to ensure removal of all feed debris, and aliquoted into duplicate samples. They were then assayed in duplicate (Salimetrics© Salivary Cortisol Enzyme Immuno-Assay Kit (Shanahan, 2003, Harmon, 2007), State College, PA).

6.2.2.4 Analysis

IceQube data were downloaded manually via the IceRobotics device reader and software to a PC. The data were then exported to MS Excel 2013 for collation into daily and phase totals, and further analysis. Correlation of the horses' movement sensor and SCC values were calculated in R Studio computing software. SCC values were plotted with SigmaPlot software and a cosinor analysis was undertaken using CircWave software (Hut, 2013).

6.2.3 Results

The movement sensor results confirmed similarity in the timing and number of steps taken across the 24 h period (Table 6.1). Lying time was dissimilar; this was researched further in the study into lying time amongst horses with and without ALDs (Chapter 4.5).

Table 6.1 Movement behaviours of two paddock horses over a 24-h period.

	Lying Time	**Standing Time**	**Steps Taken**
Horse 1	22.6 m	23 h 37 m	5,464
Horse 2	113.9 m	22 h 6 m	5,299

The two horses presented similar movement patterns across the full 24 h period tested (Fig. 6.2).

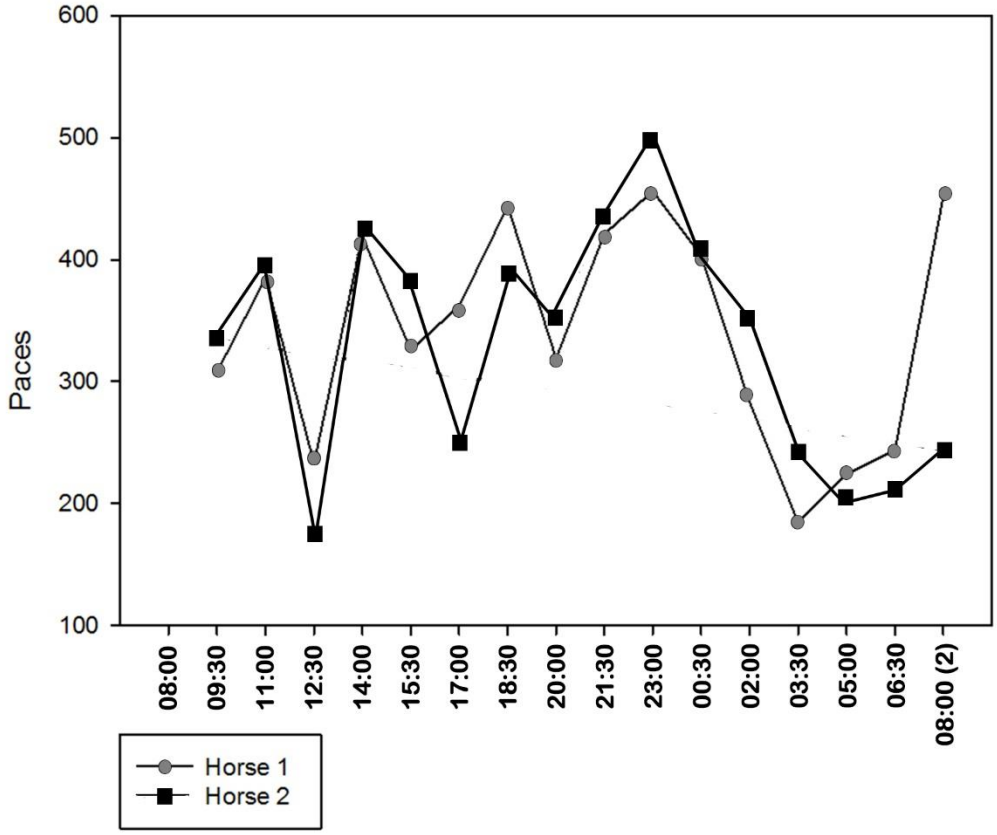

Fig. 6.2 Total steps taken by two paddock horses during the 24-h pilot period.

The amplitude of the horses' SCC elevations was larger in late spring than early spring (Table 6.2). The highest mean SCC values occurred at 8 am, with levels falling throughout the morning. Lowest mean SCC values occurred at 15:30, 18:30, 23:00, and 00:30 h.

Table 6.2 Basal circadian SCC range for 2 paddocked horses over 24-h periods in early and late spring. Measurements are given in nmol/L.

	Horse 1	**Horse 2**
Season	**Basal range 24 hr**	**Basal range 24 hr**
Early Spring	0.47 - 2.59	0.28 - 2.32
Late Spring	0.52 - 3.67	0.74 - 3.56

Pearson's correlation coefficients for two day's SCC values were positive, being moderate to strong (day 4, r = 0.66), moderate (day 1, r = 0.5), weak (day 3, r = 0.29) and negligible (day 4).

A cosinor analysis confirmed a circadian component for SCC ($P < 0.001$). MESOR was 1.73 nmol/L, amplitude was 0.55 nmol/L, and acrophase was 08:00 h (Fig. 6.3).

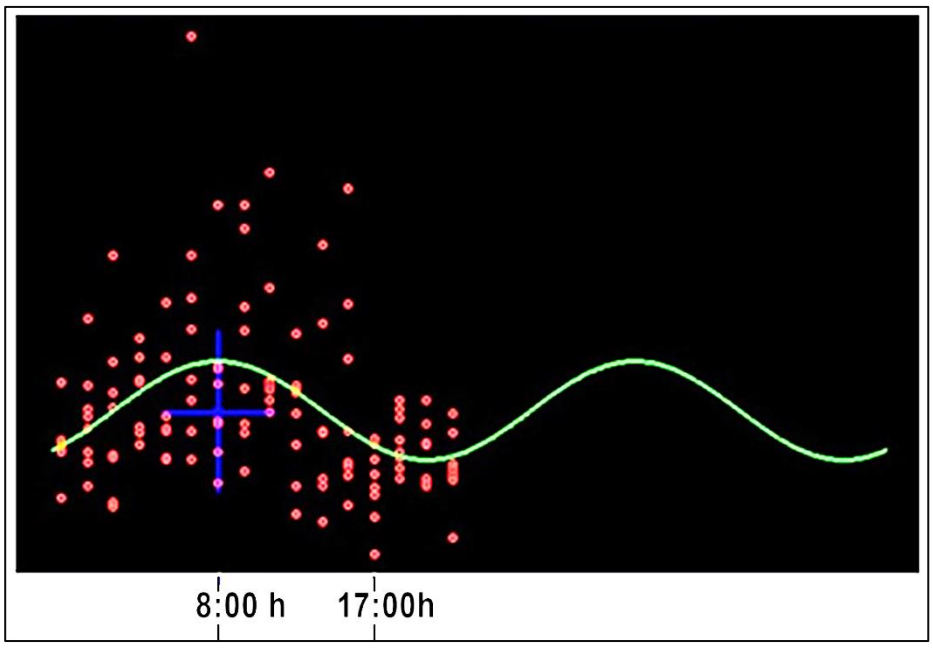

Fig. 6.3 A sinusoidal curve fitted to the data ($P < 0.001$) shows the diurnal rhythm of SCC of two paddock horses, with acrophase at 08:00 h.

6.2.4 Discussion

This pilot provided the first indication that horses with a history of gestational immaturity, and with associated ALDs, might present lower lying time values than unaffected controls. Given the small numbers in this pilot, it was not possible to reach conclusions regards 24 h time budget allocations. However, this was measured in a study into lying time amongst horses with ALDs (Chapter 4.5).

The horses in this study presented a circadian rhythm of salivary cortisol secretion, with the basal range corresponding to stabled horses (Lebelt, 1996, van der Kolk et al., 2001, Peeters et al., 2011, Nagel et al., 2012, Young et al., 2012, Kędzierski et al., 2013, Peeters et al., 2013,). Mean SCC values of the present

study aligned with those of Bohak et al. (2013) to confirm a similar circadian rhythm. In both paddocked and stabled horses, early morning peaks and evening lows are observed. Both horses showed a rhythmic profile with oscillations, peaks and troughs (Fig. 6.4).

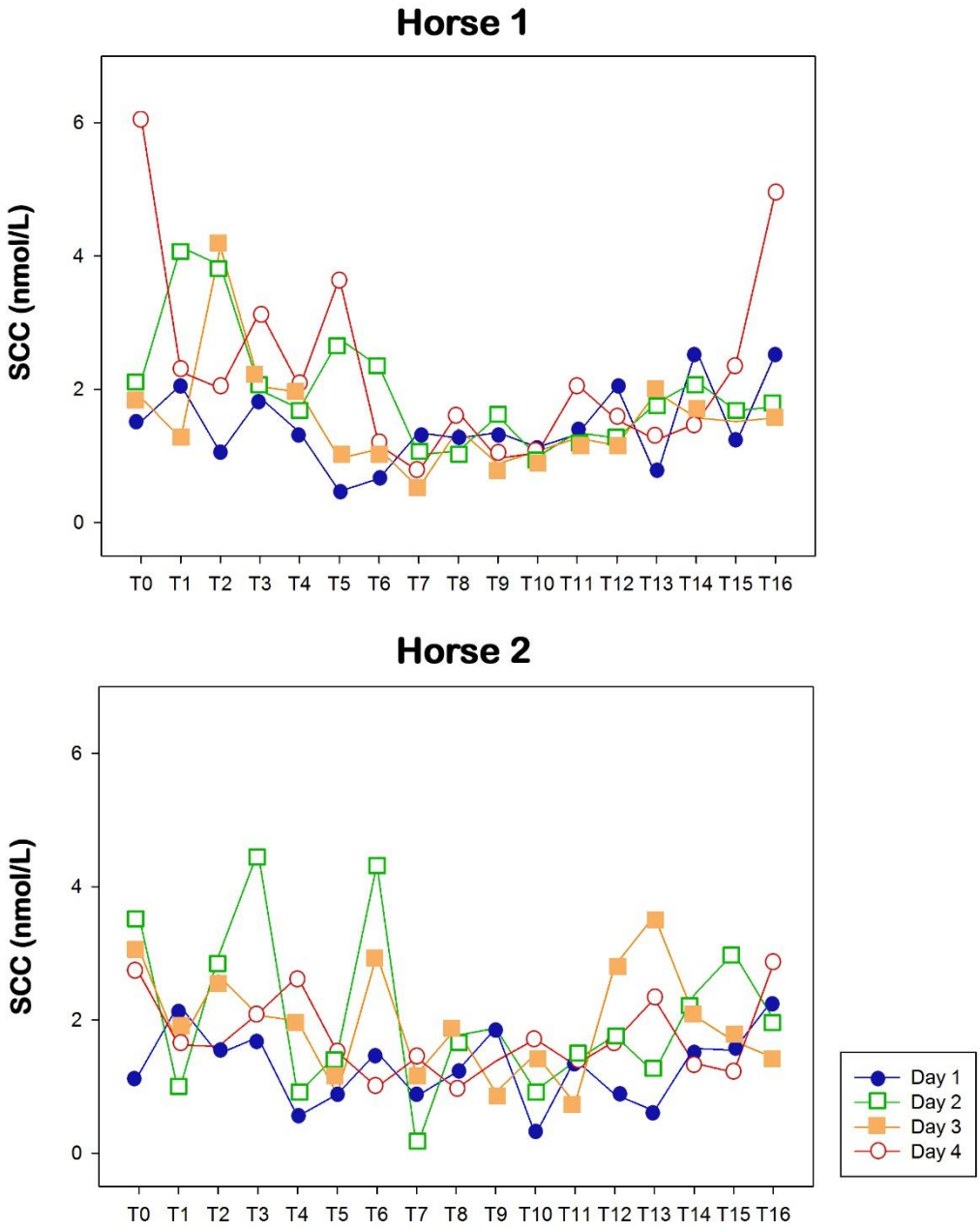

Fig. 6.4 SCC values for two horses over 4 x 24 h sampling periods in early and late spring.

It was previously unclear whether feed present in the horse's mouth would have a detrimental effect on saliva samples collected for cortisol assay. Hay fed to stabled horses is largely absorbent, but fresh grass in the paddock adds moisture to the saliva. The ELISA assay kit manufacturer advises that human subjects should not be sampled within 60 min of a major meal, and only then after rinsing the mouth 10 mins before collection, as high or low pH levels can affect the assay (Salimetrics, 2014, Shirtcliff et al., 2001). In the present study, pastured horses were often grazing at sample collection time, but no problems were experienced with the assay. This suggests that the forage available, which had neutral pH levels, did not adversely affect assay performance.

This methodology was piloted for future use in field trials involving paddock horses. Prior knowledge of the basal profiles for paddock horses facilitates interpretation of SCC values, whether these are elevated due to a stressor event or otherwise. An over-reliance on daily mean ranges, without reference to basal values for time of day, increases the chance that such variations in individuals may be overlooked.

6.2.5 Conclusion

This pilot suggests that it may be possible to profile circadian rhythms of SCC in paddocked horses through the measurement of SCC. Further study with more animals would be required to confirm this.

6.2.6 Towards a Phenotype: Circadian Cortisol Rhythm

In this pilot study, the circadian profile of two horses' SCC was measured, with results suggesting that the measurement of SCC may be a method that can be used effectively with horses living in paddocks. In this research, the method was used effectively in horses in a range of settings, including open paddocks, during low-dose ACTH stimulations (Chapter 3.5) and startle stimulus tests (Chapter 6.3).

This study provided further information beyond the objectives: Horse 1, the 4 yo Arabian gelding, was born prematurely at 305 d gestation. Despite this, the horse's basal SCC levels were not significantly different to those of Horse 2, who had a normal gestational history. This aligns with previous studies showing that basal serum cortisol levels are normal in affected children (Brummelte et al., 2015, Kaseva, 2014). Further investigation is beyond the scope of the present research.

6.3 Study: Do Horses with a History of Gestational Immaturity Present an Elevated Response to a Stress Stimulus?

6.3.1 Introduction

Alongside performance ability and appearance, horses' temperaments are of concern to horse owners and breeders. Behavioural traits such as fearfulness have direct implications for manageability and safety, both in riding and handling (Lansade et al., 2004, Christensen JW, 2006). A high level of fearfulness and reactivity has major implications for the horse-human relationship.

Anecdotal evidence from the owner-breeders of gestationally immature foals, especially those with experience of raising and training affected horses and their dams, full or half-siblings, suggests that affected horses can be more reactive. Observed traits range from these horses being variously 'different' to train, slower to learn, more reactive to negative stimuli, and more challenging to handle. The author's own experience has been that such horses can be more reactive during physical therapy sessions and general handling, responding nervously and with less predictability than unaffected horses.

Various tests are used in an attempt to assess behavioural changes in horses relating to stress, with physiological measures including heart rate, heart rate variability and plasma cortisol concentrations often combined with more

subjective measures, such as postural and behavioural ethograms (Dalla Costa et al., 2014, McCall et al., 2006, Wagner, 2010, Young et al., 2012). Reactivity is often tested through the level of a fear response to a stressor, including flight speed. This type of reaction can be measured with a timer, with associated physiological responses measurable through heart rate and heart rate variability (Noble et al., 2013).

In this study, it was hypothesised that horses with a history of premature or dysmature birth would show a heightened response to a startle stimulus, and that this would be evidenced through a post-stimulus cortisol elevation. A method was required that would not be affected by the challenges associated with field tests: a diversity of locations, with different amenities and layouts at properties, and owners with different views towards invasive methods. Additionally, the researcher was only able to access each group of horses for one day, due to travel distances. It was essential, therefore, to pilot a method before undertaking the study itself. The method selected was the measurement of SCC, as utilised in the previous pilot study (section 6.2).

6.3.2 Materials and Methods

6.3.2.1 Pilot

Two horses were included in the pilot for the stress stimulus trial: a 4 yo Arabian gelding, born prematurely at 305 d gestation, and a 7 yo domestically bred Australian brumby with no known gestational issues. The pilot took place over four separate weeks in September, October, and November, i.e. spring in the

Southern hemisphere. Climatic conditions did not vary between two subsequent test days. The protocol and conduct of the experiment was approved by the CSIRO Armidale Animal Ethics Committee under the NSW Animal Research Act, 1985 (Animal Research Authority 15/02). Ten days prior to its start, the horses were given access to *ad libitum* Rhodes grass hay, and this was continued throughout the test period. The paddock contained a 22 m round yard and a fenced enclosure, and the horses were habituated to being placed individually in both areas, with additional supplies of hay, for short periods in the mornings (Fig 6.5). All tests were filmed.

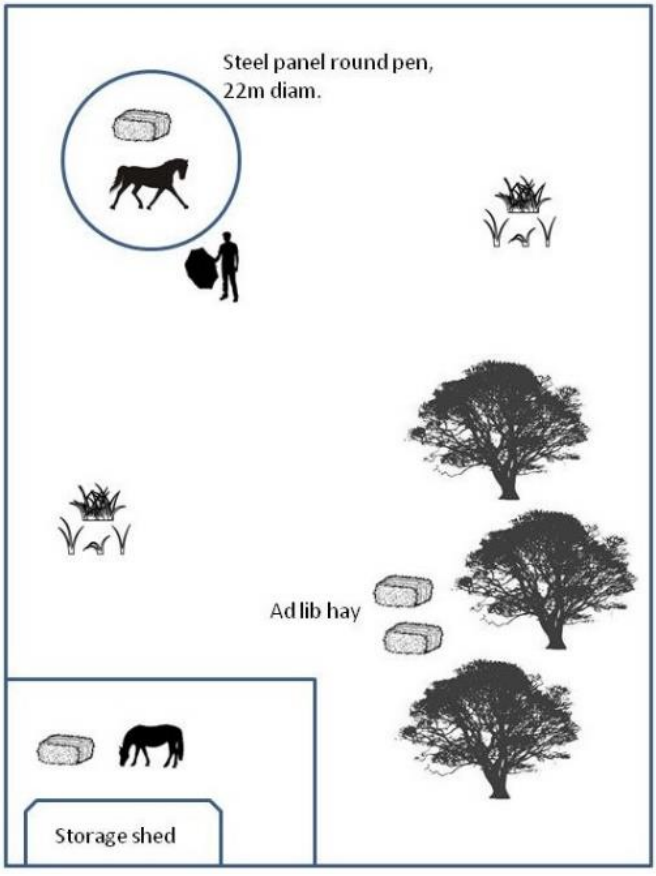

Fig. 6.5 Design and dimensions of the arrangement used to measure stress responses in horses in the pilot test.

Each trial week, stress stimulus tests took place on 2 days, with each horse being tested on a separate day. The stimulus was administered at 11 am, as basal levels descended from the early morning peak identified during the circadian profiling pilot. Shortly before 11 am, the subject horse was placed in the round yard and the second horse was placed in the fenced enclosure. A fresh source of hay was available in both areas.

Saliva samples were collected at 30 min intervals from 10.30 am to 12.30 pm. At 11am, a technician unknown to the horses approached the round pen entrance, and delivered the stress stimulus for 6 - 8 s (Fig. 6.6). Four different stimuli were used in consecutive weeks in the following order: a sun umbrella (visual), a white plastic feed bag (visual + aural), a shredded plastic feed bag on a pole (visual + aural), and a wooden soccer rattle (aural). The umbrella was rapidly opened and closed, the feed bags were shaken, and the soccer rattle was spun. The 11am saliva sample was collected immediately after the stimulus, and both horses were then released back into the main paddock for subsequent samples.

In the early tests, the horses' heart rates were measured before and after the stimulus administration. As it would not be possible to familiarise field test horses with a heart rate monitor affixed to a surcingle, a portable monitor was used instead. This was a Polar Equine Healthcheck Heart Rate Monitor (Polar Electro Oy, Kempele, Finland), the use of which involves placing a 'handlebar' transmitter against the horse's ribcage. While easy to use, the monitor's effective use necessitated wetting the horse's coat with water or a lubricant, and it quickly transpired that this caused arousal in one of the horses. As it would not be possible

to habituate the horses to this process in field tests, the heart rate monitor was not taken forward in the methods.

Fig. 6.6 In week 3, an aural + visual stimulus (feed bag on a pole) was administered for 6 - 8 s by a technician unknown to the horse.

6.3.2.2 Field Tests

A series of field tests using the above stress stimulus methodology was conducted in the Northern Tablelands and Coffs Coast, NSW and in the Lockyer Valley, QLD in April, June, July and August. Owner-breeders had been recruited to the study from the author's industry network, or had responded to a request for participants via a social media post. Telephone interviews (CSIRO Human Research Ethics Committee Research Authority LR11/2014) were conducted to

introduce the study and these were followed up with written information outlining the methods, including nature of the stimulus to be used. Swabs for saliva collection (Cortisol Salivette®, Starstedt, Nümbrecht) (Wood et al., 1998, Peeters et al., 2011) were also sent, so that horses could be habituated to their use.

The study involved 7 groups of horses, which included 9 cases with a history of prematurity (300 - 319 d gestation) and dysmaturity. Eleven controls were related to the case horses in their groups. Breeds included Arabian, Warmblood, Shire, Waler, domestically bred Brumby, and Standardbred, aged 3 - 14 yo. All cases were bred on the properties where they were tested and had been managed by the same owner-breeder throughout. Within the groups, all horses were ridden, or all were unstarted under saddle, thus ensuring commonality of experience. All horses were accustomed to one another, and were familiar with the round yard or fenced enclosure where the test was to take place. *Ad libitum* hay and/or pasture was freely available during the test periods.

There were some unavoidable changes to the methods used in the field tests. The wooden soccer rattle, an aural stimulus, had proved the most effective stimulus in the pilot test. In two field tests, this had to be adapted, as it transpired that some horses were habituated to loud noise. In these instances, the stimulus was changed to the shredded plastic feed bag on a pole (aural + visual). Further, while all tests took place in the morning, in some locations the timing had to be adjusted to fit with daily routines on that property. Due to the remote locations, a technician was not available, so the researcher had to administer the stress stimulus. Timing of sample intervals was unchanged, and all tests were filmed.

6.3.2.3 Data Analysis

Field test horses with a history of prematurity and dysmaturity were analysed as a single group, referred to as 'gestationally immature'. For each individual, total SCC production (deltaAUC) was generated by first calculating the difference between baseline and recorded value for each time point, and then using these to calculate the integrated area under the curve.

DeltaAUC was calculated from baseline SCC value to T = 90 min post-stimulus. These values were used to characterise the groups' responses to the stress stimulus test used. Subsequently, the distribution of the case horse values were compared to the distribution of the control horse values.

Relationships between gestational history and DeltaAUC or Peak SCC were assessed using one-way ANOVA in a statistical computing program R Studio (R Core Team, 2015b), with Horse nested within Family, fitting the fixed effects of Group (gestationally immature or control), Sex, Breed, Month of Test and Nature of Stimulus (aural or aural + visual).

Field test videos were audited and a simple ethogram of behaviours, adapted from previous studies, was compiled (Young et al., 2012). Observed behaviours included head height and ear position, direction of attention, grazing, and movement. This was used to score each individual's video for baseline (-2 min to stimulus), stress response, and recovery behaviours to 2 min post stimulus (Appendix 3). An elevated or long lasting response received a high score. Total scores for the three phases were produced for each horse, with an additional

latency score for time taken to return to baseline behaviours post-stimulus. Area under the curve was then calculated from each horse's four values, using the previously described method.

The distribution of the residuals in the models were tested for normality using the Shapiro-Wilk Test. $P < 0.05$ was considered significant and $0.1 > P > 0.05$ was considered to indicate a tendency towards statistical significance. Pilot test (n = 2) results were not included in the statistical analysis.

6.3.3 Results

One group of 2 horses was removed from the statistical analysis due to an outlying peak value from the control horse (41.95 nmol/L).

In the Field Tests, case horses produced higher mean peak and mean Delta AUC values than controls. However, these were not significantly different. Post-hoc power analysis and sample size calculations using a Z-test indicated that at least 50 horses in each group would be required to show differences between Cases and Controls in terms of DeltaAUC and Peak SCC at an α-level of 0.05 and 95 % Power. All mean values were lower than those recorded for the Pilot test (Table 6.3). There was a significant effect of test location for two properties ($P = 0.02$, 0.001), plus a non-significant effect of sex ($P = 0.08$) and age group (8 - 12 y, $P = 0.08$). There was no significant effect of Breed, Month of test or Nature of stimulus.

Analysis of the video data showed no effect of gestational immaturity on stress responses. There was a significant effect of Breed for Arabians (P = 0.02) and Waler (P = 0.024), the former showing higher values and the latter lower values. There was also a significant effect of aural + visual stimulus (P = 0.03).

There was no correlation between SCC Delta AUC and video AUC.

Table 6.3 Basal and post-stress stimulus salivary cortisol concentration values for Pilot and Field Tests. Measurements are nmol/L, values are means ± SD.

	Basal mean	**Peak mean**	**Peak range**	**DeltaAUC mean**	**DeltaAUC range**
Pilot Test (n = 2)	1.99 ± 0.84	3.88 ± 1.01	3.7 - 5.43	N.A.	N.A.
Field Test: Cases (n = 9)	1.51 ± 1.45	2.24 ± 1.07	1.49 - 4.8	2.01 ± 4.15	-2.62 - 10.68
Field Test: Controls (n = 11)	1.15 ± 0.65	1.62 ± 0.5	0.77 - 2.26	0 ± 0.79	-1.18 - 1.19

6.3.4 Discussion

This study did not show a significant effect of a history of gestational immaturity in adult horses' response to a stress stimulus measurable through SCC, or in the observed behavioural responses. In the pilot test, peak SCC elevations were above basal levels, with the highest values in each group presented by the horse with a history of prematurity. This did not happen in the field tests: while a number of Case horses did present high values than controls, mean peak values were close to basal values recorded both in this study and in the literature (Kędzierski et al., 2013, Peeters et al., 2011, van der Kolk and Wensing, 2000, Young et al., 2012). Behaviour scores for videos showed some breed variation.

This method of physiological assessment does not appear to have been appropriate for the stress stimulus test. A suitable alternative may be the measurement of salivary Alpha-Amylase, a digestive enzyme produced in the saliva glands that has been found to be an effective biomarker for the sympathetic nervous system in mammals (Behringer et al., 2012, Fuentes et al., 2011, Petrullo et al., 2016). Laboratory assays have been validated for horses (Fuentes-Rubio et al., 2015), however, the currently available commercial kits lack sufficient range for the low concentrations in equine saliva samples. An alternative means of measuring heart rate might have enabled this to be measured physiologically (Bachman, 2017, Noble et al., 2013), however, this particular method of data capture proved problematic, as discussed above (5.3.1).

Variables were difficult to manage in this study. Prior consultation with owners included discussion of the horses' experience and general temperament, the physical environment for the test, and the need to familiarise horses with the saliva collection process. Despite this, some conditions varied more than anticipated. One test was abandoned because the case horse escaped the owner and then could not be caught. In another, horses not involved in the study were adjacent to the test area, providing distraction. On another property, horses that had been declared sensitive to noise were in fact living close to an air base. This group was therefore fully habituated to low flying jets.

The remaining option would be to change the nature of the stressor to one that exerts a more sustained effect. Examples in previous studies include imposing 2.5 h isolation, without visual or aural contact with fellow herd members (Bachman, 2017). However, this is less likely to be acceptable to owners, while creating safety concerns for the horses. It would also involve logistical requirements that are harder to meet, given that many Australian properties do not have suitable stables or stalls.

Ultimately, there is also the question of what is actually being tested in this type of behavioural study. The specific causes of a reactive response are mixed, being attributable various fear (response to the stimulus), underlying anxiety (low level fear about stressors in general), response to stimuli with specific associations (past experiences), social stress (individual is taken away from a herd for the test), or a mixture thereof (Forkman, 2007).

6.3.5 Conclusions

This study did not show an effect of gestational immaturity on adult horses' responses to stress stimuli through the measurement of elevations in SCC.

6.3.6 Towards a Phenotype – Reactivity

Conducted in the first year of this research, this study contributed to a stronger understanding of the challenges involved in researching a diverse group of horses at different locations.

The research findings described in Chapter 3 also add to the explanations for an observed heightened reactivity in affected horses. With a dysregulated adrenocortical response, horses may take longer to return to pre-stressor homeostasis, which would hypothetically delay their learning experiences. Difficulties in learning about environmental stressors might heighten general anxiety, and thereby readiness to react.

If present, maldevelopment of the adrenal gland may lead to dysregulated adrenalin release from the adrenal medulla as well as cortisol from the cortex. A maldeveloped adrenal cortex might lead to dysregulation of the renin-angiotensin-aldosterone system, meaning that affected horses may experience an electrolyte imbalance. With the added possibility of renal tubule dysfunction heightening calcium dysregulation, this can lead to muscular excitability and higher tension levels (Chapter 3.6.2).

Furthermore, the presence of musculoskeletal pain in any horse can heighten tension and lead to increased reactivity. The study into ALDs using movement sensors (Chapter 4.5) confirmed that levels of REM sleep may be depleted, thus providing another possible source of tension. With gestationally immature horses, there may also be the presence of further appendicular joint issues (Chapter 4.4), which may be associated with low-grade chronic pain, again adding to anxiety levels.

It therefore became necessary to further refine the research hypotheses, as greater clarity was needed in what the horses are actually being tested for, as well as which measures to actually include in the studies. Subsequently, the use of an exogenous ACTH stimulation was explored as a method for investigating a dysregulated adrenocortical response, with some limitations affecting the present study avoided (Chapter 3.5).

Subsequent to the ACTH study described, the results of the present study were revisited. A scatter plot indicated that in comparison to their control counterparts, case horses presented a wider range of values (Fig. 6.7). However, in light of the small sample size and the large number of confounding variables in the above study, it was decided not to proceed with further statistical analysis. Further research is warranted to understand if the biphasic nature of adrenal dysfunction identified in Chapter 3.5 may be reflected in behavioural tests such as these.

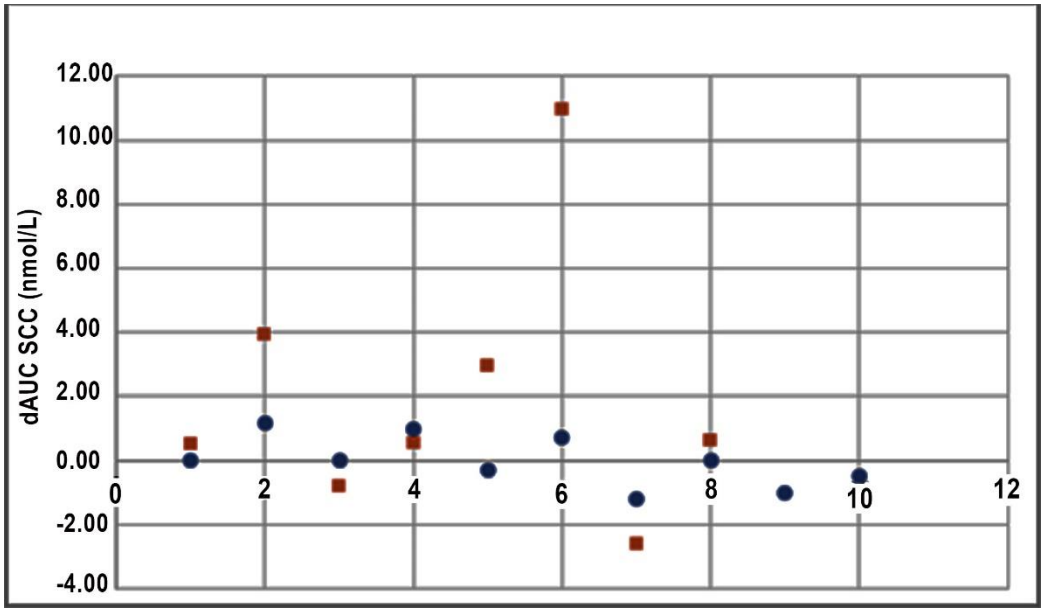

Fig. 6.7 Post-stimulus deltaAUC (dAUC) values for horses in the reactivity study. Horses with a history of gestational immaturity (red square) show a wider range of values than controls (blue circle)

6.4 Study: Pressure Sensitivity

6.4.1 Introduction

Anecdotal evidence from owner-breeders suggests that some horses with a history of gestational immaturity are intolerant of grooming or general handling, such as rugging and hoofcare, and therefore require extra caution during daily management routines. This is supported by the author's subjective observations that cases have difficulty settling during hands-on therapy, reacting strongly against procedures that usually induce relaxation in the subjects.

This adverse response to touch may be due to nociceptive pain, i.e. that which is evoked by a noxious stimulus. In humans, the intensive, tissue-damaging treatment experienced by premature babies in the neonatal phase, when the central nervous system's pain pathways are still in development, can lead to long term changes in pain sensitivity that affect them in later life, with chronic pain conditions reported (Fitzgerald, 2009). There is further evidence that the infant's normal ability to determine benign touch from noxious stimuli can be impaired, leading to an amplified noxious neuronal effect (Slater et al., 2010).

One method of measuring nociceptive responses in humans and animals is through pressure algometry, which uses a calibrated mechanical instrument to attempt to quantify pain thresholds within musculoskeletal structures (Haussler, 2007, Potter, 2006). Algometry provides an objective assessment of heightened localised pain, including peripheral sensitisation due to noxious chemical or mechanical stimuli. In equine research, it has most commonly been used to measure pain in the back muscles (De Heus et al., 2010, Haussler and Erb, 2006b, Pongratz, 2017, Sukovaty, 2017, Varcoe-Cocks, 2006). An unacceptable increase in algometric pressure leads to avoidance actions such as turning of the ears, looking at the operator, moving away, or suddenly lifting a limb (Pongratz, 2017). Some studies include responses including muscle fasciculations, twitching of the *Cutaneous trunci* muscle, or a shifting movement in joints (De Heus et al., 2010).

In this pilot, pressure algometry was used to determine Mechanical Nociceptive Thresholds (MNTs), the minimum pressure required to induce an avoidance response in a group of Arabian horses with a history of gestational immaturity and related controls.

6.4.2 Materials and Methods

Four family groups of Arabians were tested (n = 12). Each group included 1 horse with a history of gestational immaturity and 2 controls. All horses were accustomed to having their limbs handled and hooves checked by the owner-breeder, as well as professionals such as hoofcare practitioners or veterinarians. No horses were identified as having current unsoundness or pre-existing veterinary issues. The protocol and conduct of the experiment was approved by the CSIRO Armidale Animal Ethics Committee under the NSW Animal Research Act, 1985 (Animal Research Authority 16/02).

A digital pressure algometer manufactured by Wagner Instruments, CT, USA (Haussler and Erb, 2006b, Pongratz, 2017, Sukovaty, 2017) with a 1cm tip and a calibrated range of 25 kg/cm^2 was used to determine Mechanical Nociceptive Thresholds (MNTs), the minimum pressure required to induce an avoidance response (Fig. 6.8).

Fig. 6.8 Digital pressure algometer with 1cm tip by Wagner Instruments.

Pressure was applied to the forelimbs at 4 bilaterally symmetrical sites adapted from locations previously identified by Haussler et al. (2007) (Table 6.4, Fig. 6.9). Pressure was increased slowly for 2 - 5 secs, without sudden increases (Haussler, 2007, Pongratz, 2017). A single operator carried out all tests. Horses were checked for dermatological lesions prior to starting the procedure, and the sites did not coincide with myofascial points associated with soft tissue tension or trauma.

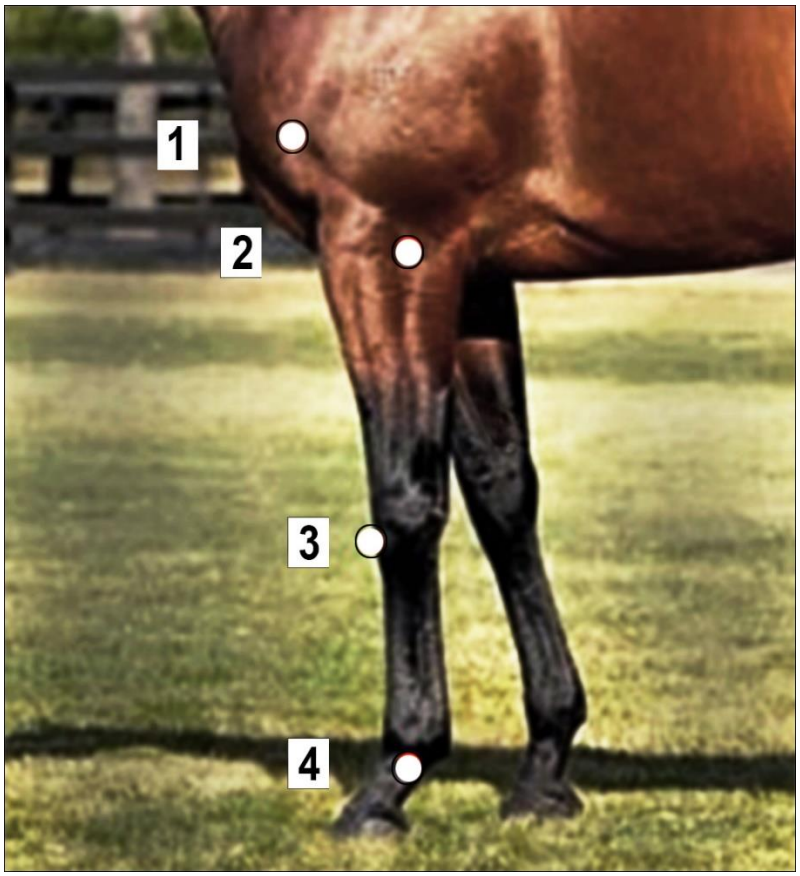

Fig. 6.9 Points on the thoracic limbs tested for algometric pressure sensitivity, adapted from those used by Haussler et al. (2007). These are (1) point of shoulder, caudal to greater tubercle of the humerus, (2) elbow, caudal to lateral tuberosity of the radius, (3) knee, dorsal aspect of ulnar carpal bone, and (4) pastern, lateral aspect of first phalanx.

Table 6.4 The locations of algometry application points on horses' forelimbs.

Point	Location	Code	
		Left	Right
Point of Shoulder	Caudal to greater tubercle of the Humerus and attachment of Infraspinatus muscle, and over Teres minor muscle.	LP1	RP1
Elbow	Caudal to lateral tuberosity of the Radius, between Extensor carpi radialis muscle and Common digital extensor muscle.	LP2	RP2
Knee	Dorsal aspect of Ulnar carpal bone.	LP3	RP3
Pastern	Lateral aspect of first phalanx, caudal to distal branch of suspensory ligament.	LP4	RP4

Avoidance was determined as the exact moment at which the horse shifted weight to the contralateral forelimb to reduce the pressure from the algometer tip. At this point, the researcher did not follow the avoidance movement with the algometer, but remained still and recorded the algometric pressure for that point in time.

As the objective was to measure minimum pressure level at which avoidance occurred, only values < 2 kg/cm^2 were included in the analysis. Values exceeding this level were therefore recorded as 2 kg/cm^2. Only one measurement was recorded for each point, as this served as an indicator of sensitivity, whereas

repeated actions risked inducing adaptation (tolerance of sequentially increasing pressure) or sensitisation (heightened response to sequentially lower pressure) (Haussler, 2007).

Data were analysed in the computing software, R Studio (R Core Team, 2015b). Bilateral points were tested for correlation coefficient (Pearson test) and for effect of gestation group, age and test location. The distribution of the residuals in the models was tested for normality using the Shapiro-Wilk Test. $P < 0.05$ was considered significant and $0.1 > P > 0.05$ was considered to indicate a tendency towards statistical significance.

6.4.3 Results

There was a significant effect of gestational immaturity in cases' response to the left pastern ($P = 0.04$, LP4, Table 6.4). This was not reflected in RP4. There was no significant effect on any other points and nor was there any effect of age, sex or test on any of the points (Table 6.5).

There was a moderate positive bilateral correlation between LP1 - RP1 ($r = 0.52$), LP2 - RP2 ($r = 0.53$), and LP3 - RP3 ($r = 0.59$). However, LP4 - RP4 had a weak positive correlation ($r = 0.23$). There was no effect of age or test location on LP1.

Table 6.5 Responses of Arabian horses with history of gestational immaturity (n = 4) and controls (n = 8) to pressure algometry on the thoracic limbs. Values are kg/cm^2 .

	Gestationally Immature		**Control**	
	Left	Right	Left	Right
Point 1 - Shoulder	0.65 ± 0.65	0.88 ± 0.43	1.14 ± 0.68	1.12 ± 0.53
Point 2 - Elbow	0.85 ± 0.5	0.97 ± 0.35	0.96 ± 0.58	0.85 ± 0.34
Point 3 – Knee	1.33 ± 0.86	1.39 ± 0.83	1.54 ± 0.6	1.49 ± 0.59
Point 4 - Pastern	0.91 ± 0.73 *	1.12 ± 0.85	**1.81 ± 0.62 ***	1.51 ± 0.56

* significant effect of gestation group (P = 0.04).

6.4.4 Discussion

In this study, Arabian horses with a history of gestational immaturity responded to a significantly lower algometric pressure applied to LP4, the left pastern, than controls. It is unclear why a difference existed between the left and right forelimbs for this measurement, as values for Points 1 - 3 all showed bilateral consistency.

It is impossible to address this unilaterality without considering forelimb dominance, asymmetric forelimb loading and hoof imbalance, which is beyond the scope of this research.

In the present study, horses were tested during summer and it is possible that the horses were used to moving their feet in response to the presence of flies. However, the lack of a bilateral correlation does not support this as a sole cause. In daily management, handling of horses tends to commence on the horse's left, as did the bilateral procedures of this study. However, this was the same for all horses.

The effect of Angular Limb Deformities must be considered. Of the 5 case horses in this study, 4 had some degree of fetlock valgus on the left forelimb. While this does not indicate pain, examination of conformation photographs taken prior to the tests shows that these horses were leaning into the right forelimb (Fig. 6.10). In these instances, the right hoof is also shorter, being more worn through loading than the left hoof. Asymmetric loading could account for greater freedom to move the left foot suddenly. Conversely, the remaining case horse can be seen loading onto the left forelimb; this case horse had a higher value for its left pastern than the right, which supports this interpretation. Despite this, it is apparent that the control horses are not all loading evenly, so this explanation cannot be viewed as complete.

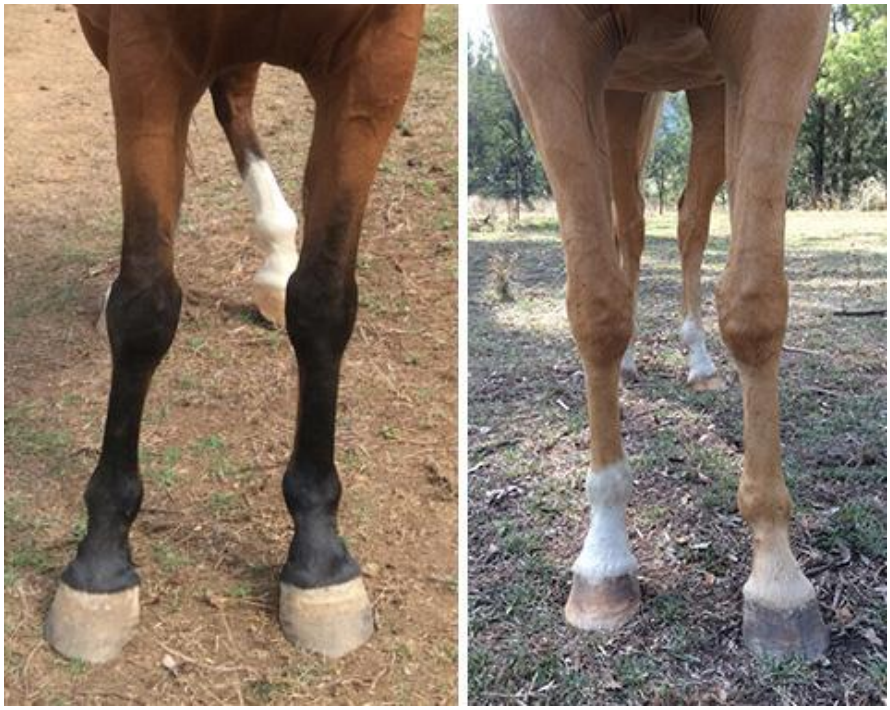

Fig. 6.10 Two Arabian mares with a history of gestational immaturity included in the algometric pressure study are shown (left) loading onto the left forelimb, and (right) loading onto the right forelimb. Both mares have a mild carpal valgus of the unloaded limb.

The values in this study are low: 'normal' range MNTs for the pastern have been established at around 27 kg/cm^2 (Haussler, 2007). However, these related to a point lower on the long pastern bone; it is possible that pressure closer to the joint is more likely to induce an avoidance reaction, such as lifting the limb or shifting weight away from the algometer.

Unlike the present study, which involved Arabians, a breed renowned for sensitivity and reactivity, the earlier study involved Tennessee Walking Horses (Haussler, 2008). This breed is subject to damaging soring practices that are devised to create elevated leg actions, a practice that could cause desensitisation

due to scar tissue; It has been reported previously that tissue thickness can raise the pressure threshold and that tissue is thicker over bony structures (Pongratz, 2017). Equally, it could cause short term pain, hyperalgesia and/or lower MNTs.

In algometry, low values can indicate either a lack of tolerance to pressure related pain, or a heightened pain sensitivity (hyperalgesia) due to chronic pain. Pain might occur locally to a pathology or at a focal site peripheral to the issue, particularly in muscle tissue (Haussler and Erb, 2006b). The present study aimed to avoid such locations, popularly known as trigger points, but it is impossible to rule out referred pain as a factor.

Intra-operator variability is a limitation with studies involving algometry (Haussler et al., 2008) and cannot be ruled out in the present study, particularly as the researcher was not blinded to the horses' gestational status or the algometer readings. A validation study would require multiple operators blinded to the horses' identities and algometry readings, yet this is beyond the resources available to this research.

6.4.5 Conclusion

Horses with a history of gestational immaturity may respond differently to algometric pressure applied to the pastern, although whether this is due to more sensitive, localised neuronal responses to noxious stimuli or to a general intolerance of handling is not known. Due to this and the fact that intra-operator variability may be present, it was decided not to continue with the approach.

6.4.6 Towards a Phenotype: Pressure Sensitivity

In this pilot study, Arabian horses with a history of gestational immaturity demonstrated a greater sensitivity to algometric pressure than control horses. The difficulty remains that while a different response is present, it is unclear what exactly is being measured. It may be a physiological experience of pain, or it may be a lack of tolerance to being handled. One is underpinned by endocrinology, while the other is a behaviour that may or may not be influenced by endocrinological systems (Chapter 6.5).

Another factor may be the presence of joint issues in the affected limb, although it is not always possible to attribute these to gestational deficiencies: spinal and hindquarter pathologies can also affect forelimb loading. Musculoskeletal issues that affect the horse's whole body can affect algometry readings at localised sites, due to lowered MNTs. It is only possible to confirm that a response is present, unless other assessment methods are also used to confirm a physiological or behavioural response.

6.5 Study: Behavioural Traits

6.5.1 Introduction

There is considerable discussion regarding which aspects of an individual horse's phenotypically expressed temperament are genetically determined and which are shaped by previous and present environments (Hausberger, 2004, Lansade, 2008, König von Borstel, 2013, Roberts, 2016). Studies have attempted to assess the effect of breed (Lloyd et al., 2008, Morris et al., 2002a, Staiger, 2016), sire and dam genetics (Wolff et al., 1997). Components of personality have been studied, including extraversion (Morris et al., 2002), emotionality (Le Scolan et al., 1997, McCall et al., 2006), reactivity (Lansade et al., 2008, McCall et al., 2006, Wolff et al., 1997) and anxiety (Momozawa, 2003). Others have attempted to assess the effect of personality on aspects training and performance (Visser et al., 2001, Visser et al., 2003, Wolframm, 2010). From a welfare perspective, understanding the equine personality variations is valuable in ensuring horses and riders are suited and that the horse is managed appropriately (König von Borstel, 2013). For horses affected by gestational immaturity, this is especially the case, should related differences persist.

In this research, owner-breeders of case study horses have provided anecdotal evidence that suggests some horses with a history of gestational immaturity present temperaments that differ to closely related horses, despite sharing genetics and environments and work experiences, i.e. lifelong management by the same owner/breeder, and being started or unstarted under saddle. It was hypothesised

that cases would have express behaviour that tended towards anxiety and intolerance, while being less socially adapted than their related counterparts.

A questionnaire was developed to quantify aspects of behaviour. This needed to be short, as earlier experience had shown that while owner-breeders are interested in this research, alternative demands on their time tend to preclude time-consuming contributions.

6.5.2 Materials and Methods

A questionnaire was produced using 10 pairs of adjectival descriptors adapted from previous equine studies (Appendix 4) (Lloyd, 2007a). These were behavioural traits that aligned loosely with the Five-factor model used in human studies, which in animal studies comprises the categories of *Curiosity*, *Conscientiousness*, *Extraversion*, *Agreeableness* and *Neuroticism* (Goldberg, 1990, König von Borstel, 2013, Morris et al., 2002b) (Table 6.6). Descriptors were organised in opposing pairs on a 7-point interval scale, with a brief explanation beneath each for further clarity.

Owner-breeders of a horse with a history of gestational immaturity, who also owned one or more closely related horses (i.e. full or part-siblings, dam) were invited to take part. Breeds represented were Arabian, Andalusian, Quarter Horse, Australian Stock Horse x Clydesdale, Irish Draught, Shire, Warmbloods and Warmblood X. Ages ranged from 3 - 16 yo. All owners had contributed data on their horses or participated in other studies within this research. Ten owner-

breeders of 31 horses, including 14 cases and 17 controls, participated. The protocol and conduct of the experiment was approved by the CSIRO Human Research Ethics Committee (Research Authority LR11/2014).

Table 6.6 Ten pairs of behavioural traits included in the questionnaire.

Five-factor Model	**Behavioural Traits**
Curiosity	Anxious vs. Curious
Conscientiousness	Fast learner vs. Slow learner
Extraversion	Submissive vs. Dominant Active vs. Inactive
Agreeableness	Agreeable vs. Aggressive Cooperative vs. Uncooperative Tolerant vs. Intolerant
Neuroticism	Untrusting vs. Trusting Excitable vs. Stoic Insecure vs. Confident

The questionnaire was sent out in the body of an email, with accompanying instructions. One questionnaire was included per horse, with the horses' names already entered. Respondents clicked reply and then completed the questionnaires in the reply email by entering an X on each of the ten scales per horse. Responses were scored, with traits supporting the hypothesis receiving high scores and their paired traits receiving low scores.

Principal Components Analysis (PCA) was applied to the values to establish the most variable personality traits across the 10 groups of horses. One-way

ANOVAs were applied to all responses to test for effect of gestation group and other variables, and a Welch *t*-test was used to compare cases versus controls when data could not be transformed to satisfy a normal distribution, or where significant first-order interactions precluded comparison between groups. The distribution of the residuals in the model was tested for normality using the Shapiro-Wilk Test. $P < 0.05$ was considered significant and $0.1 > P > 0.05$ was considered to indicate a tendency towards statistical significance. Values are presented as means ± S.D.

6.5.3 Results

Gestationally immature cases were significantly more *Aggressive* (Q.1, $P = 0.025$) and more *Active* ($P = 0.045$) than controls. When data for Q.1 were fitted in a Linear mixed-effects model by REML, a significant interaction between gestation group and family was evident (group $P = 0.002$; family $P = 0.02$; group: family $P = 0.0118$), representing families of Arabians. There was also an effect of sex ($P = 0.07$), representing mares, but without a significant interaction with gestation group. There was an interaction between the group and breed for Q.7 (*Intolerant vs. Tolerant*; group $P = 0.001$, breed $P = 0.048$, group:breed $P = 0.032$).

Data for Q.6 could not be transformed to satisfy a normal distribution, so were analysed using Welch's t-test. The gestationally immature group were significantly more *Intolerant* (Q.7, $P = 0.005$), *Aggressive* (Q.1, $P = 0.05$) and *Untrusting* (Q.6, $P = 0.03$) than controls.

In the PCA of traits, the first two principal components account for 45.17% of the total variation of the horses' personality traits (Table 6.7). The four dominant components of PC1 (32.16%) tend to separate horses on the basis of nervous behaviour, with the shape change from negative to positive dominated by a shift from a confident and curious behavioural type to an anxious and excitable type. PC2 (13%) tends to separate horses on the basis of their activity levels and interactions with others, with the shape change from negative to positive dominated by a shift from inactive and agreeable to active and aggressive.

Table 6.7 The dominant relationships of behavioural traits within PC 1 - 4, after varimax rotation (n = 41).

		PC1	PC2	PC3	PC4
	Std. dev.	2.8	1.78	1.48	1.41
	Prop of var.	32.16%	13.01%	8.96%	8.15%
	Culm. Prop.	32.16%	45.17%	54.13%	62.3%
Behavioural Traits					
Q.1	Aggressive* vs. Agreeable		0.48	0.38	- 0.43
Q.2	Anxious* vs. Curious	0.46		- 0.36	
Q.3	Active* vs. Inactive		0.62		
Q.4	Submissive vs. Dominant*				
Q.5	Cooperative vs. Uncooperative*			0.57	
Q.6	Untrusting* vs. Trusting	0.33			
Q.7	Intolerant* vs. Tolerant	0.35			
Q.8	Excitable* vs. Stoic				
Q.9	Fast learner vs. Slow learner*			0.5	
Q.10	Insecure* vs. Confident	0.49			
	Effect of gestational immaturity on PC values	N.S.	P = 0.048	N.S.	P = 0.028

* positive values within the PCs.

6.5.4 Discussion

Analysis of the questionnaire responses suggest that gestational immaturity is associated with certain behaviours, with these horses being less secure and less relaxed than related horses, and in some instances aggressive. These behaviours are not usually viewed as positive in horses that work closely with humans, as they can render a horse potentially unsafe to handle or ride.

The breed that differed from others in *Aggressive vs Agreeable* was the Arabian, with two family groups represented. Nevertheless, gestation group remained significant. The interaction suggests that the trait was most visible in this breed, which is known to be highly reactive and expressive. In an earlier study, Thoroughbred horses, which display a similar range of behaviours due to their Arabian breed ancestry (Lloyd, 2008, Sackman, 2019) displayed higher scores in components relating to *Dominance, Anxiousness, Excitability, Sociability* and *Inquisitiveness* (Lloyd, 2008). In contrast, in the same study, Irish Draught Horses displayed low scores in these components. In the present study, larger numbers might have revealed a clearer distinction between behaviours typically associated by the equine industry with 'hot' breeds such as the Arabian and 'cool' breeds such as the Shire, Waler and Irish Draught.

With a higher number of responses, a greater statistical power might have provided clearer outputs. Alongside low numbers, a limitation of the study was the number of breeds represented (n = 8). Breed is a determinant of inherited traits in horses and breed-typical personalities are established in the literature as well

as in the anecdotal evidence of popular equine literature (Lloyd, 2008). Therefore, the high number of breeds in the present study ensures greater variability, yet this was unavoidable. Unsurprisingly, a significant breed interaction was identified in the study.

Nevertheless, the existence of an effect of gestational immaturity suggests that endocrinological factors are also involved. Behaviours can be linked to neurotransmitter pathways, and in human studies, an overactive central nor-adrenergic system leads to anxiety, irritability and emotional instability, with an amplification of stress responses (Yamamoto, 2013). It is possible to speculate that adrenocortical dysregulation can affect the gestationally immature horses' sympathetic nervous system, leading to over-activation and the anxious or fractious state that appears to be present in these results.

6.5.5 Conclusion

Horses with a history of gestational immaturity are significantly more *Active* and *Aggressive* than their unaffected, related counterparts. Aggression is most strongly expressed in affected Arabians. These horses are also more *Untrusting* and *Intolerant*.

6.5.6 Towards a Phenotype – Behavioural Traits

It is apparent that, despite this study's limitations, an unrelaxed, unsettled, untrusting and sometimes aggressive phenotype is emerging for horses with a

history of gestational immaturity. This finding aligns with anecdotal evidence provided by a number of owner-breeders, including a number who did not participate in this study. However, one owner-breeder of Warmblood x Thoroughbreds, whose horses presented the finer physical phenotype of the Thoroughbred, reported training challenges during consultation, but failed to reflect this in their questionnaire response. This highlights some of the difficulties involved in breeder reporting, particularly when certain aspects of behaviour are perceived as being negative.

Fig. 6.11 This 5 yo Shire mare born prematurely at 296 d differed from siblings with high *Intolerance*, but aligned with siblings with low *Aggression* score. (Image: author's own)

It appears that in addition to breed, genetics, and experiences, temperament may be influenced by the congenital physiological effects of gestational immaturity. This is widely recognized in human research, where the connection between prematurity and emotional and behavioural problems in young people is well documented (Arpi, 2013, Hornman, 2016).

6.6 Expressions of Gestational Immaturity

The results of these pilot studies potentially contribute to development of a phenotype for horses with a history of gestational immaturity.

In paddock horses, basal SCC follows a diurnal pattern and is within the ranges reported by previous studies. The stress startle study was limited in demonstrating a physiological change measurable through SCC; however, it is possible that a biphasic effect is present, in alignment with the earlier findings of the study into the adrenocortical response to exogenous ACTH in Chapter 3.5.

Results of the owner-breeder questionnaire suggested a temperament that is intolerant and untrusting, although in some hot-blood horses this is combined with a level of aggression. It is postulated that intolerance and underlying anxiety may contribute to the responses in the pressure algometry study, with unrelaxed horses being more likely to react swiftly while being handled. Intensive handling in the early days of life may contribute to intolerant behaviours, as is popularly recognised in horses that were bottle-fed as foals. Intravenous administrations during this period may also lead to aversive responses to benign touch, as has been reported in humans.

Behaviours and stress responses may also be affected by the dysregulated HPA axis and, should dysregulation of aldosterone production also be present, by an

electrolyte dysregulation that can cause calcium imbalance and associated anxiety and depression, plus physical discomfort due to joint pain.

Chapter 7

CONCLUSIONS: A SYNDROME OF GESTATIONAL IMMATURITY

"…a significant fraction of them remain at higher risk for neurological, personality and behavioural abnormalities, cardio-pulmonary functional limitations, systemic hypertension and metabolic syndrome compared to their term-born counterparts…" (Raju et al., 2017)

7.1 Introduction

The present research findings indicate that horses with a history of gestational immaturity may be subject to a range of adaptations that lead to subclinical conditions in adult horses. While concurrently present, these adaptations all derive from the foundation of maldevelopment due to intrauterine stress that occurs during the final weeks of gestation. A syndrome of conditions associated with a history of gestational immaturity has started to emerge.

7.2 Adapted Endocrinology

This research has found that the gestationally immature foal can be subject to a complex and multifactorial conditions affecting the endocrine systems, associated with developmental disorders of the glands and organs.

The circadian profile of basal SCC over 24 h of a horse with gestational immaturity was similar to a control and it was found that:

- Amplitude of the SCC elevations was higher in late spring than early spring.
- Peak mean values occurred at 8 am, with lowest mean values at 15:30, 18:30, 23:00 and 00:30 am.
- Basal SCC reflects a circadian pattern over 24 h.

Investigating the adrenocortical response to exogenous ACTH in gestationally immature horses and controls revealed that:

- Gestational stress causes a bilateral adaptation of the adrenocortical response, due to development issues affecting the adrenal cortex, which matures very late in gestation.
- When subjected to a low dose, ACTH challenge, affected horses present a lower or a higher cortisol response than unaffected, related controls.
- All horses in the study that were dysmature or premature were affected.

- Horses with a history of extreme immaturity present extremely high or low values.
- Basal plasma cortisol levels did not differ from controls.

This research established an adaptation of the adrenocortical response in horses with a history of gestational immaturity.

7.3 Adapted Appendicular Skeleton

The association between gestational immaturity, incomplete ossification and Angular Limb Deformities (ALDs) is established in the literature and in practice. The novel use of movement sensors together with analgesics in horses with ALDs and histories of gestational immaturity revealed that lying time in horses is significantly reduced in the presence of ALDs, particularly in extremely cold weather. It was further demonstrated that movement sensors and analgesics can be used to effectively measure paddock behaviour in horses.

7.4 Adapted Growth Patterns

It is often reported that gestationally immature foals fail to achieve anticipated height at maturity. However, the nature of this adaptation has not been investigated in horses. The proportions of adult horses with a history of gestational immaturity and related controls were indexed and analysed to identify growth anomalies and found that:

- The following ratios were significantly lower in case horses than controls:
    - Cannon length to Height at croup;
    - Hock height to Height at croup;
    - Hock height to Body length;
    - Accessory carpal height to Thoracolumbar length.

- The following ratios tended to be lower in case horses than controls:
    - Femur length to Height at croup;
    - Shoulder length (point of shoulder to wither) to Height at withers.

- An effect was also suggested for the following ratios, but the residuals could not be transformed to form a normal distribution:
    - Thoracolumbar length to Wither height (high ratio);
    - Femur length to Height at croup;
    - Hock height to Thoracolumbar length.

It was concluded that horses with a history of gestational immaturity have restricted growth distal to the carpal and tarsal joints, and that this results in a more ‘rectangular’ conformation in adulthood.

7.5 Affected Behaviours

A number of studies and pilots were conducted to assess behavioural and other expressions of gestational immaturity in horses.

A pilot stress stimulus test was conducted to measure the SCC elevations following a startle response in horses with a history of gestational immaturity and controls. This was followed by a field study involving family groups of horses that included a gestationally immature case horse.

- The field study case horses produced higher mean peak and adjusted AUC values than control, but the difference was not significant.
- Mean peak and adjusted AUC values were lower than those produced in the pilot.
- Cases' deltaAUC values appeared to show a moderate biphasic effect.

Algometry was used to measure the sensitivity to pressure of horses with a history of gestational immaturity.

- Horses with a history of immaturity responded to pressure on the lateral pastern of the left forelimb at a significantly lower level of pressure than controls.
- There was no significant difference between the responses of cases and controls to pressure on the lateral pastern of the right forelimb.
- Case horses that responded to significantly lower pressure on the left forelimb had mild fetlock ALDs on that limb. Control horses with mild ALDs did not show greater sensitivity to pressure on that limb.

A questionnaire was completed by the breeder-owners of groups of cases and controls.

- Horses with a history of gestational immaturity showed significantly more *Aggressive* and *Active* traits than controls. A significant interaction with family was present, with *Aggression* being displayed mostly in families of Arabian horses.
- Case horses also tended to be more *Active*, *Intolerant* and *Untrusting*.

7.6 Application of Findings

As the presence of a number of these adaptations is established in individuals, the syndromic effect of adaptations caused by gestational immaturity becomes apparent. A combination of study results and anecdotal evidence obtained by the author suggests how this syndrome might present itself in different horses.

In the present research, a number of horses that were gestationally immature have been included in more than one study, leading to identification of more than one presentation. The following 3 cases present adrenocortical dysregulation, adapted growth patterns, Angular Limb Deformities, and bloodwork that may suggest further endocrine adaptations.

7.6.1 Twin Warmblood Mares

The 8 yo twin Warmblood mares were introduced to the research at an early stage (Fig. 7.1).

- Both demonstrated a blunted adrenocortical response and both presented ALDs (Chapter 3.5).
- The twins' Index 18 (hock height to total body length) ratios were 33.67 and 34 (controls were 40.87, 40.41) (Chapter 5.2).
- Serum biochemistry showed that the more mature twin presented low serum calcium (2.34 mmol/l; normal range 2.6 - 3.3) and low total protein (52 g/L; 55 - 70).
- The more dysmature twin presented high Creatine phosphokinase (444 IU/L; range < 351) and urea (6.8 mmol/L; 3.3 - 6.5), suggesting muscle damage or a chronic kidney or liver condition. Haematology showed high haemoglobin (191 g/DL; range 115 - 175), a low red cell count (5.99 x 10^{12}/ L; 6.0 - 11.0) and low Packed Cell Volume (27.4; 35 - 50), suggesting anaemia, kidney issues and/or infection.
- The mare that was more dysmature at birth was euthanised at 8 yo due to chronic unsoundness. Necropsy revealed advanced osteoarthritis and carpal exostoses, multiple osteochondral lesions, and severe skeletal asymmetry.
- The more mature mare was started under saddle as a sports horse, yet soon after was semi-retired at 10 yo due to unsoundness related to a tarsal valgus.

Fig. 7.1 Twin Australian Warmblood mares born at 321 d (top) In first few days of life and (bottom) at 8 yo, with the most dysmature twin on the left.

7.6.2 Australian Stock Horse Gelding

With a history of immature gestation, this gelding was introduced in the final year of this research (Fig. 7.2).

- Demonstrated an extremely elevated adrenocortical response to low dose ACTH test (Chapter 3.5), registering the second highest deltaAUC SCC value.

- This gelding's Index 18 (hock height to total body length) ratio was 36 (controls: 40.7, 43.92) (Chapter 5.2).
- Serum biochemistry revealed high total proteins (80 g/L; 55 - 70), a low Albumin : Globulin ratio (0.54), low range albumin (28 g/L; 28 - 37), high globulin (52 g/L; 25 - 36) and high Gamma glutamyl transferase (67 umol/L; 1 - 50), with normal bilirubin (37 umol/L; 12 - 62). Suggestive of kidney or liver condition or abnormality.
- At necropsy, hyperplasia of the adrenal and thyroid glands and advanced demineralisation of the skeleton were observed, suggesting the genetic condition Multiple Endocrine Neoplasia Syndrome (MENS) Type-1 (De Cock, 1999). This involves hyperparathyroidism and in humans is associated with gestational insufficiency (Mistry, 2014). Osteochondral lesions and malformed caudal cervical vertebrae were also observed (May-Davis, 2014).

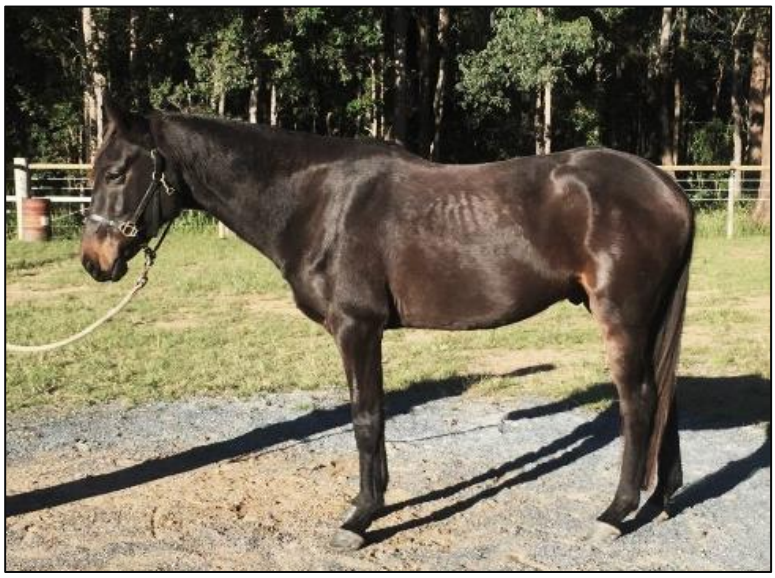

Fig. 7.2 Australian Stock Horse gelding at 5 yo, born dysmature.

7.6.3 Arabian gelding

Born prematurely, this 3 yo Arabian gelding was introduced to this research at its start (Fig. 7.3).

- This gelding had a blunted adrenocortical response to exogenous ACTH (Chapter 3.5) and registered the lowest deltaAUC value.
- The Index 18 ratio (hock height to total body length) was 33.45 (controls: 38.82, 39.17) (Chapter 5.2).
- Serum biochemistry: high total proteins (74 g/L; 55 - 70), a low Albumin : Globulin ratio (0.64), low range albumin (29 g/L; 28 - 37); high globulin (45 g/L; 25 - 36) and low bilirubin (10 umol/L; 12 - 62).
- The horse has since developed signs of Equine Metabolic Syndrome (EMS), with fat deposits on the hindquarters and crest of the neck.
- This gelding is highly reactive in the course of regular routines, such as being groomed. Although agreeable, he can be unpredictable during handling.

Fig. 7.3 Premature Arabian gelding born at 305 d gestation (top) aged 2 weeks, and (bottom) as at 3 years.

7.7 A Syndrome of Gestational Immaturity

Research into the Developmental Origins of Health and Disease is bringing focus to the persistent effects of intrauterine and neonatal stress in human adults (Raju et al., 2017). Meanwhile, animal research has shown that adverse conditions in

late gestation have an effect on the metabolic and endocrine functions of small mammals, with increasing research into larger mammals investigating the universality of such adaptations in sheep, pigs and horses (Jellyman, 2015).

The research described in this thesis yields results that suggest additional sequelae to gestational immaturity may be added to those already identified in the literature (Fig. 4). Given developments in both human medical and veterinary science, it is reasonable to postulate that a syndrome of gestational immaturity may persist, both clinically and sub-clinically, in affected adult horses.

With veterinary advances ensuring that more neonatal foals are surviving at gestational ages previously considered unviable, it is important that these persistent effects are both identified and understood so that equine health and welfare can be considered, with appropriate and prognosis-appropriate decisions made in the foal's early life.

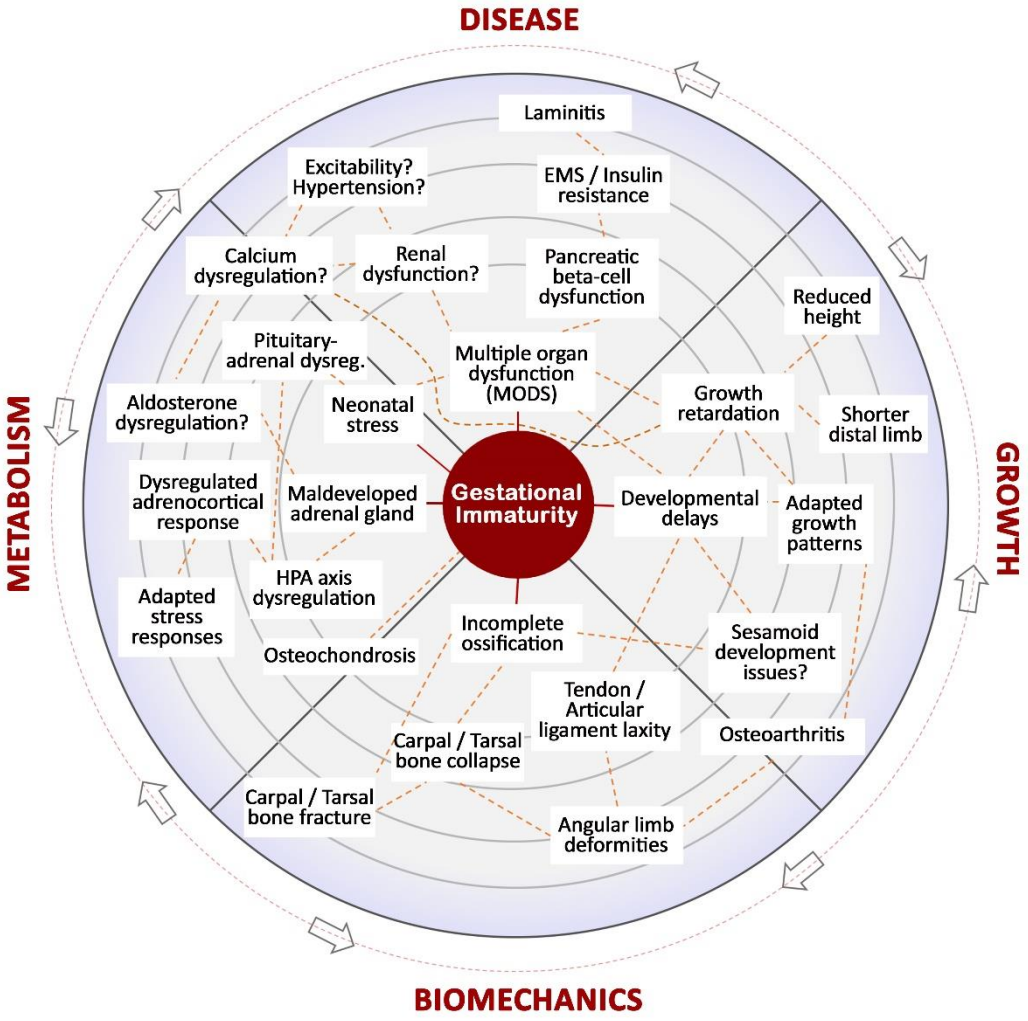

Fig. 7.4 Immature Foal Syndrome: the aetiology of pathologies and disease reported in this research and in the literature.

7.8 Recommendations for Further Work

The purpose of this research was to identify the presence of persistent effects of prematurity and dysmaturity in adult horses. In so doing, the research aimed to create greater understanding of these horses' temperamental and musculoskeletal limitations.

Future research could ameliorate the limitations posed by small numbers, breed variation and limited resources that have affected this research. Research involving Thoroughbred horses in the Australian racing industry would provide the required numbers and remove the variations caused by breed and management practice, while providing extensive retrospective data and longitudinal performance data.

- The findings from the study into adrenocortical dysregulation should be validated with larger numbers, with concurrent study of different points along the HPA axis and sympathoadrenal nervous system.

- The effects of gestational immaturity on calcium dysregulation via the renin-angiotensin-aldosterone system should be investigated.

- The effects of gestational immaturity on growth patterns of Thoroughbred foals, weanlings and yearlings should be studied, with inclusion of performance data.

- A behavioural study should investigate the horses' response to prolonged stress, e.g. transport or separation.

- A biomechanical study into the effects of adapted growth patterns would provide further knowledge on how gestational immaturity impacts upon future athletic performance.

Such a research programme could potentially inform management of gestationally immature foals in the early months of life, rending it possible for interventions to be developed that would minimise the extent of syndromic sequelae in the maturing horse.

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Appendix A. Horses included in the research and studies. Horse ID shows group and individual. GL Status shows P (Premature), D (Dysmature) and C (Control).

Horse ID	GL Status	Sex	Breed	Adrenal (Ch.3.5)	Sensors (Ch.4.5)	Skull (Ch.5.3)	Skeletal (Ch.5.2)	React. (Ch.6.3)	Algom. (Ch.6.4)	Quest. (Ch.6.5)	Haem. (Ch.7.6)
GFT-ER	P	M	Arabian				x		x		
GFT-ET	C	M	Arabian				x		x		
GFT-EX	C	M	Arabian				x		x		
GFT-P	C	G	Arabian				x		x		
TA-SH	D	M	Arabian	x			x	x	x	x	
TA-OP	C	M	Arabian				x		x	x	
TA-KA	C	G	Arabian				x	x	x		
TA-MS	C	G	Arabian x					x		x	
TA-RL	C	S	Arabian				x				
OLD-RO	D	G	WB				x			x	
OLD-M	C	M	WB							x	
OLD-RU	C	S	WB				x			x	
BU-PO	P	M	Shire	x			x	x		x	
BU-RA	C	G	Shire	x							
BU-ED	C	G	Shire	x							
BU-SA	C	M	Shire				x	x		x	
BU-MO	C	G	Shire					x		x	
FV-VR	C	M	Arabian				x	x	x		
FV-VB	C	M	Arabian				x	x	x		
FV-VV	P	M	Arabian				x	x	x		
CH-BU	P	G	Arabian	x	x		x	x			
CH-LA	C	M	Arabian				x				
CH-FA	C	S	Arabian				x				
Ch-CL	C	M	Arabian				x				

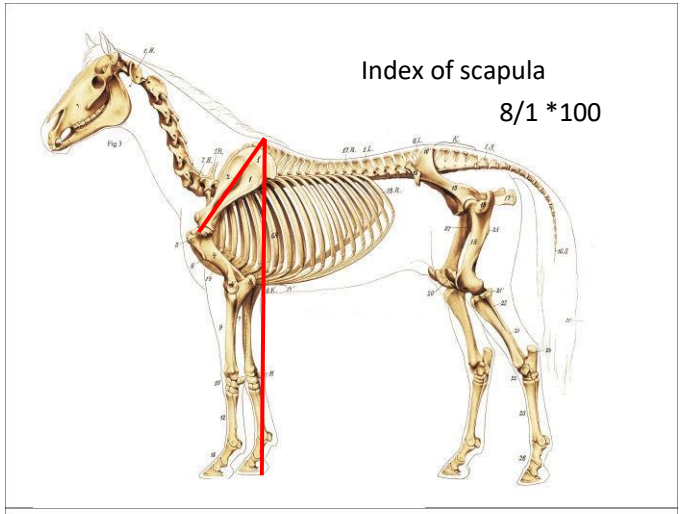
CH-CO	C	G	Brumby	x	x			x			
CH-RA	C	M	Brumby	x				x			
CH-TA	C	M	Arab x	x							
CH-AS	C	M	STB	x	x			x			
HV-F	C	M	Waler				x	x	x	x	
HV-S	P	G	Waler				x	x	x	x	
GV-AN	P	M	Arabian				x	x	x		
GV-AP	P	M	Arabian				x	x	x		
GV-SO	C	G	Arabian				x	x	x		
TWC-O	D	G	ASH x Clyd				x	x			
TWC-R	C	M	ASH x Clyd				x	x			
WA-MA	C	M	WB			x	x	x		x	x
WA-TH	C	G	WB				x			x	x
WA-SW	C	M	WB	x						x	
WA-RI	D	M	WB	x			x	x		x	x
WA.CH-MG	D	M	WB	x	x		x	x		x	x
AN-KI	D	M	Andalucian				x			x	
AN-KS	C	M	Andalucian				x			x	
AN-EG	C	G	Andalucian							x	
AN-KL	C	S	Andalucian				x			x	
KW-IL	D	M	WB (D)				x			x	
KW-SL	C	M	WB (D)							x	
KW-UT	C	S	WB (D)				x				
KW-BR	C	G	WB (D)				x				
KW-GT	C	M	WB (D)				x				
KW-AL	C	G	WB (D)							x	
QH-JB	P	M	QH				x			x	
QH-JD	C	M	QH							x	
QH-LB	D	M	QH							x	

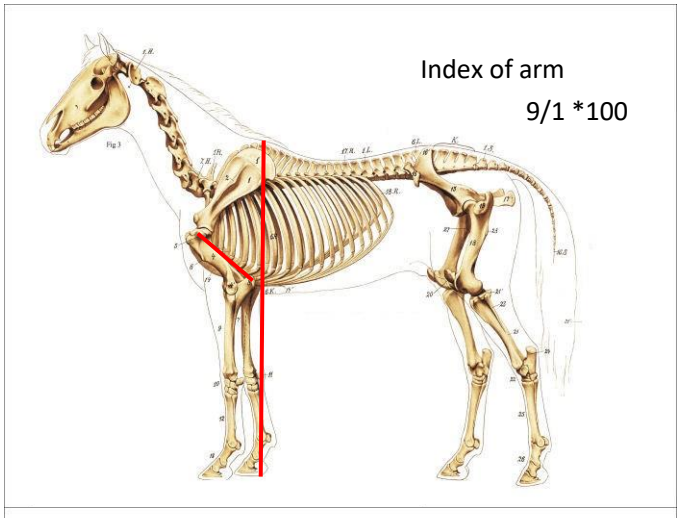
IS-ZM	D	M	Irish SH				x			x	
IS-ZD	C	M	Irish SH				x			x	
CFH-EZ	P	G	WB				x				
CFH-WK	C	S	WB				x				
MS-F	D	G	Apaloosa				x				x
CHQ-K	P	G	QH x WB								x
CP-RO	P	G	WB x TB	x			x			x	
CP-DI	P	G	WB	x			x			x	
CP-MI	D	M	ARP x ASH	x			x			x	
CP-DD	C	M	WB x	x			x			x	
CP-CL	C	M	WB x	x			x			x	
NZ-N	D	G	Arabian				x			x	
NZ-T	C	M	Arabian				x			x	
ASH-D	D	G	ASH	x	x		x				x
ASH-S	C	S	ASH				x				
ASH-Q	C	S	ASH				x				
MK-A	P	M	Arabian				x				
MK-S	C	S	Arabian				x				
MK-F	C	M	Arabian				x				
CAM-Y	P	M	AU Pony				x				
CAM-D	C	M	AU Pony				x				
CAM-S	C	S	AU Pony				x				
URAL-G	C	G	WB			x					

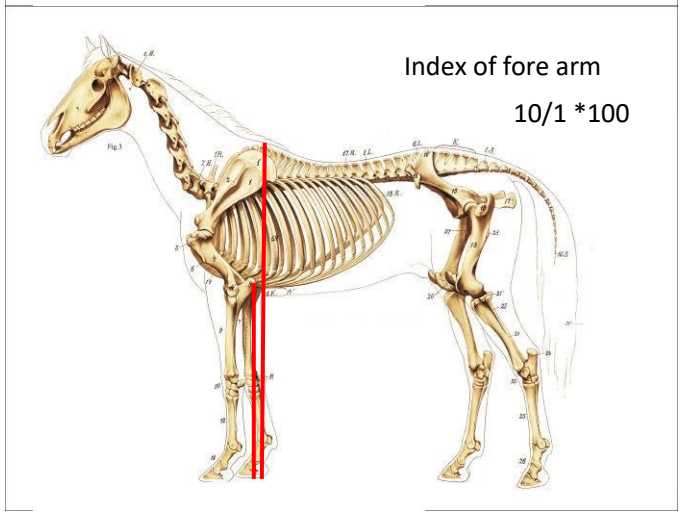
Appendix B. List of measurements adapted from Komosa et al (2009) for the study into growth retardation (Chapter 5.2), with illustrations of measurements utilized in ratios.

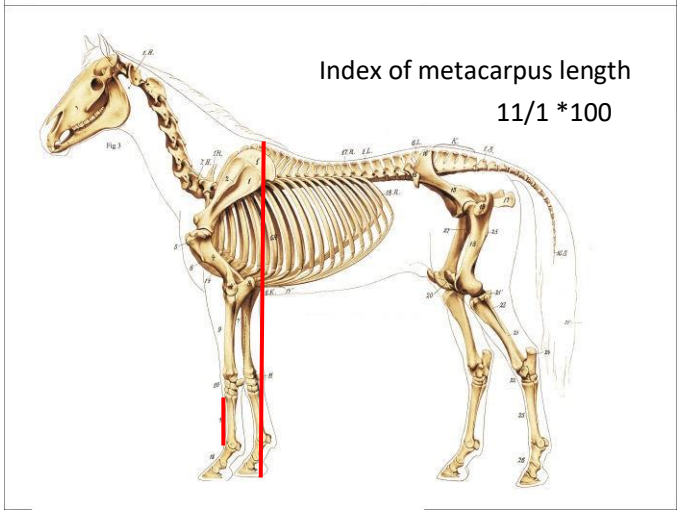
Measurement Number	Name	Index Number	Index name
1	Height of wither	1	Index of scapula
2	Depth of girth	2	Index of arm
3	*Removed from study*	3	Index of forearm
4	Height of croup	4	Index of hand
5	Point of Shoulder to Ischial Tuberosity	5	Index of MC3 length
6	Point of Shoulder to Point of Hip	6	Index of Forepastern
7	*Removed from study*	7	Index of trunk (great)
8	Point of Shoulder via Scapula to Withers	8	Index of trunk (short)
9	Point of Shoulder to Olecranon	9	Index of femur
10	Olecranon to Accessory Carpal	10	Index of anterior hock
11	Length of Third Metacarpal, dorsal aspect	11	Index of MT3 length
12	Length of Paster, dorsal aspect	12	Index of hind pastern
13	*Removed from study*	13	Index of foot
14	Accessory carpal to ground	14	Index of pelvis/patella
15	Point of hip to Ischial tuberosity	15	Index of forearm/hand
16	*Removed from study*	16	Index of arm/hand
17	Point of hip to external patella	17	Index of hand/trunk
18	Croup to Ischial tuberosity	18	Index of foot/trunk GR
19	Greater trochanter of femur to Patella	19	Index of foot/trunk SM
20	Patella to angerior aspect of Talus	20	Index of croup
21	Length of Third Metatarsal	21	Index of head
22	Hind pastern length	22	Index of neck (neck to trunk)
23	*Removed from study*		
24	Point of hock to ground		
25	*Removed from study*		

Appendix B. Indexes adapted from Komosa et al (2009)

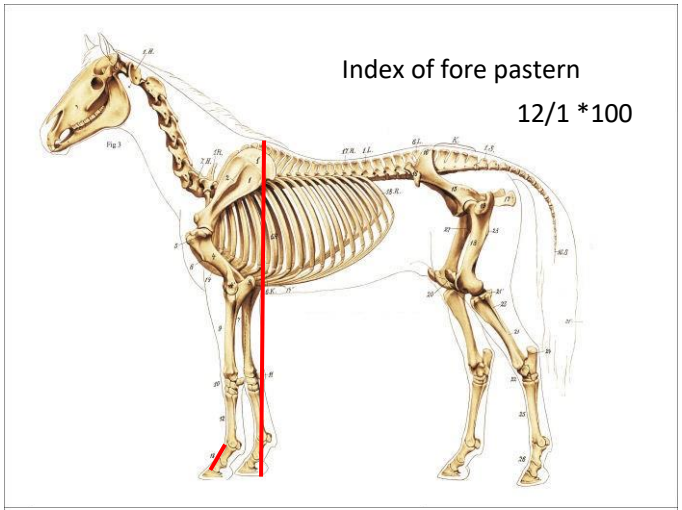


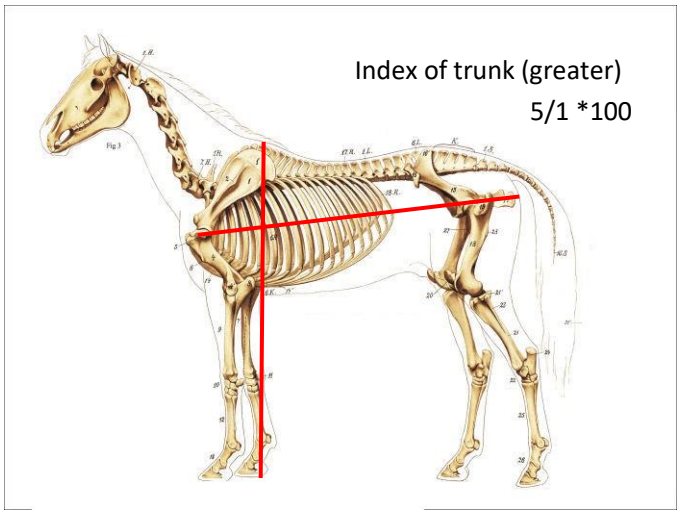


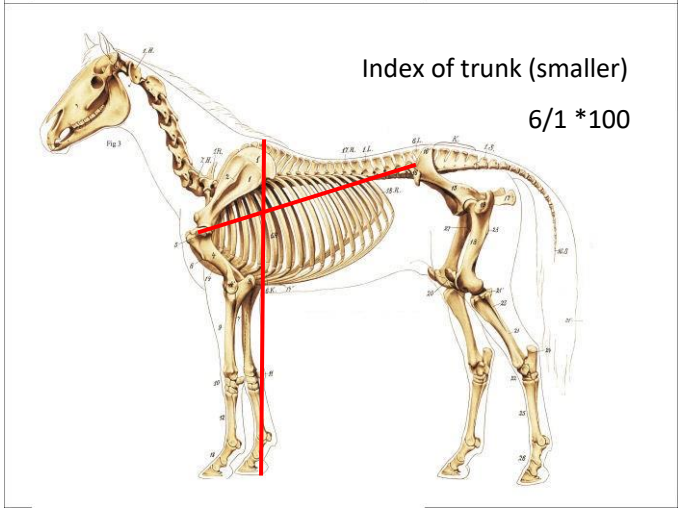


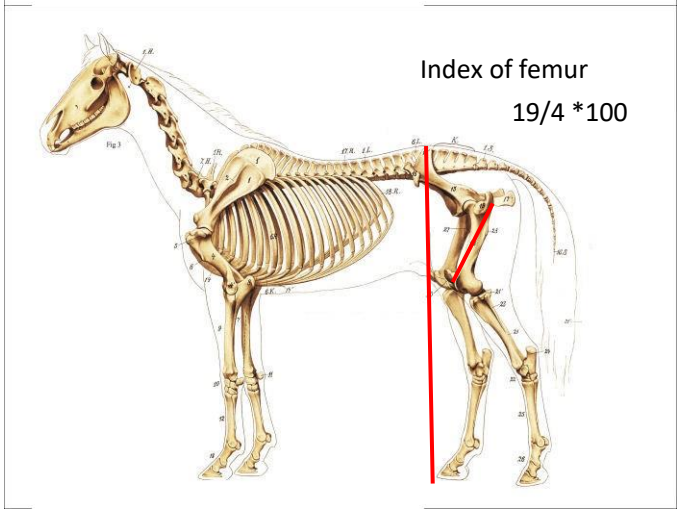


Appendix B. Indexes adapted from Komosa et al (2009)









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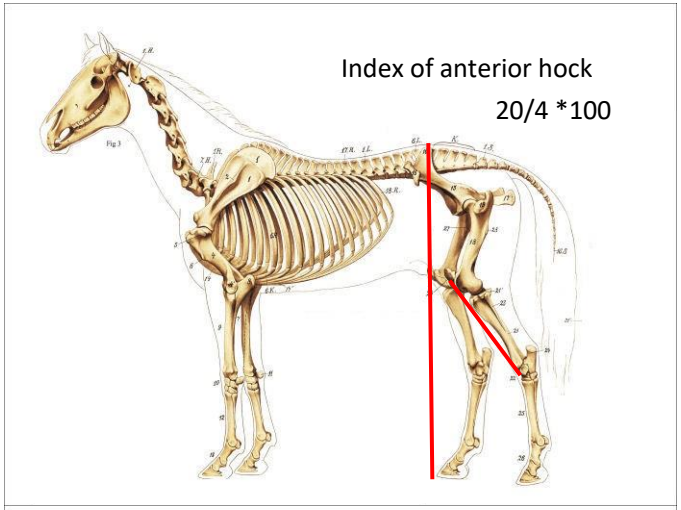
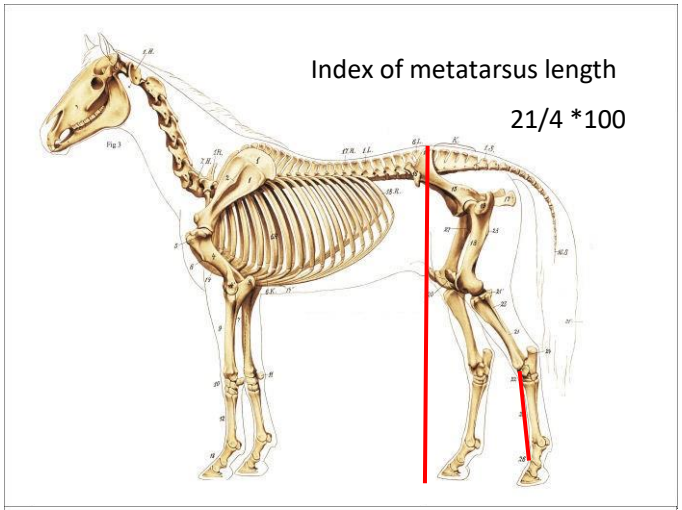
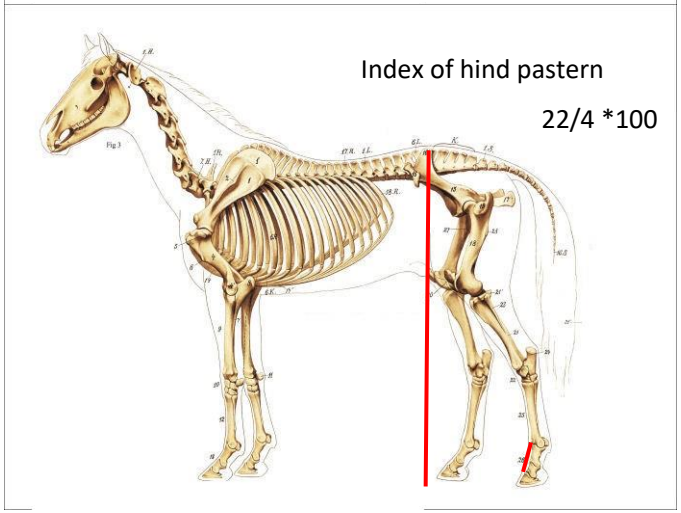
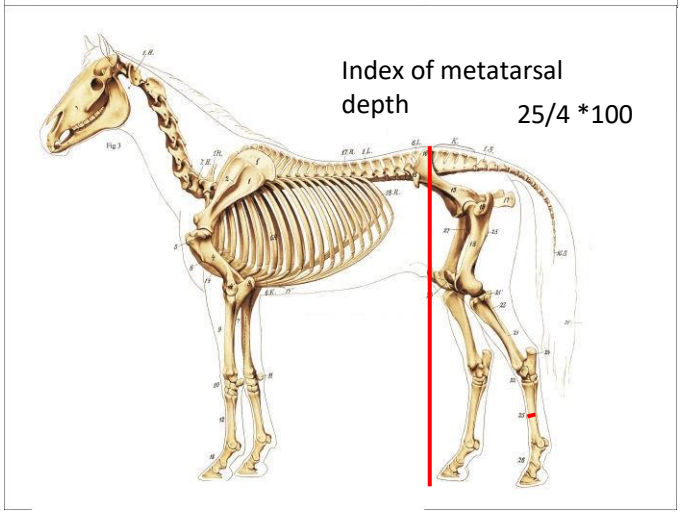
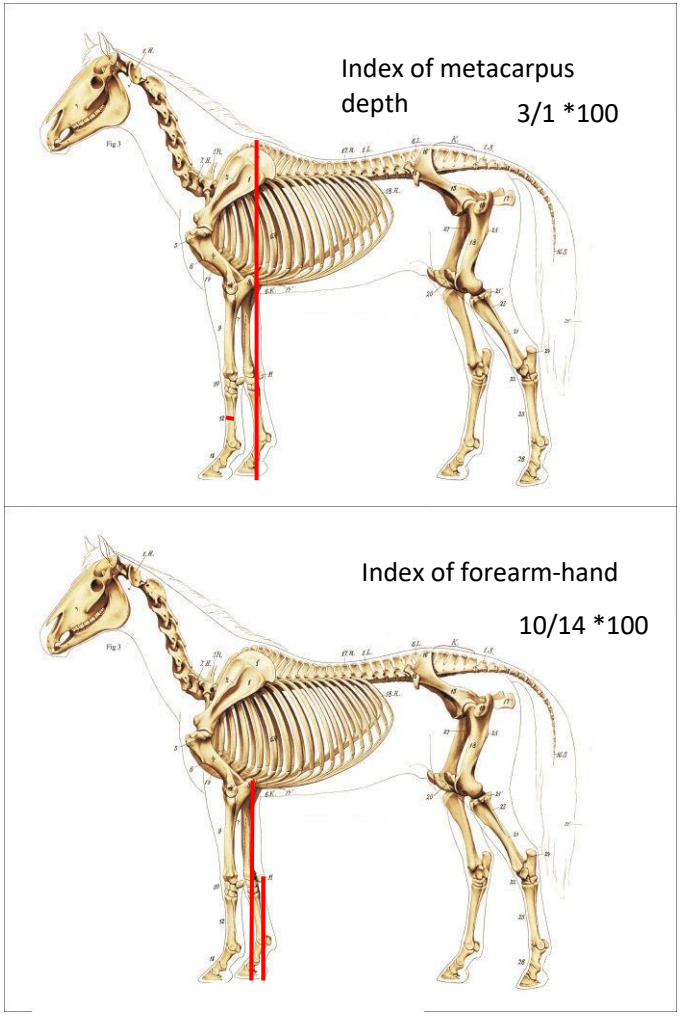








Appendix B. Indexes adapted from Komosa et al (2009)



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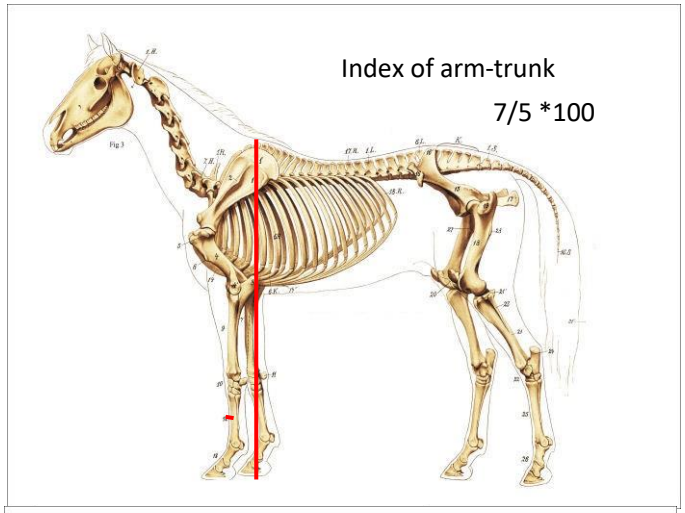

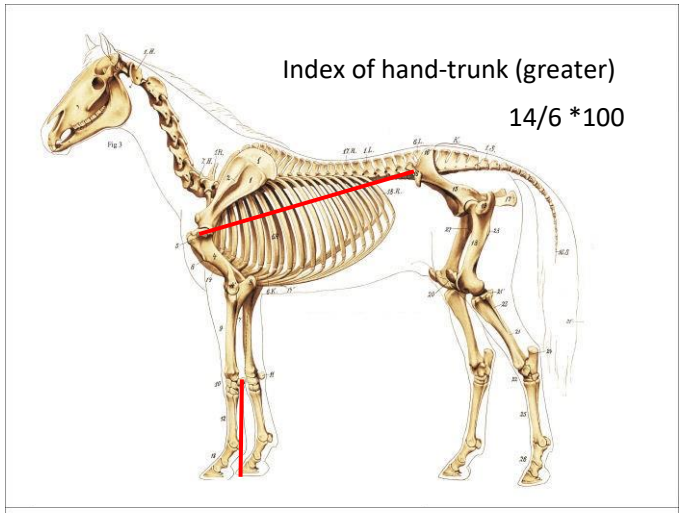

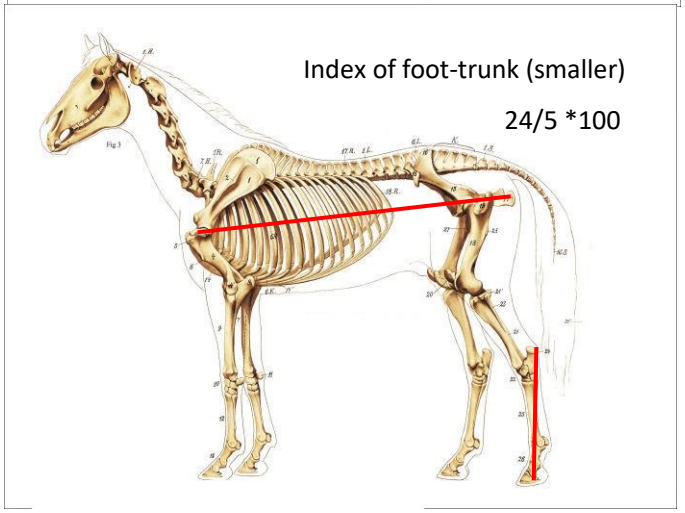

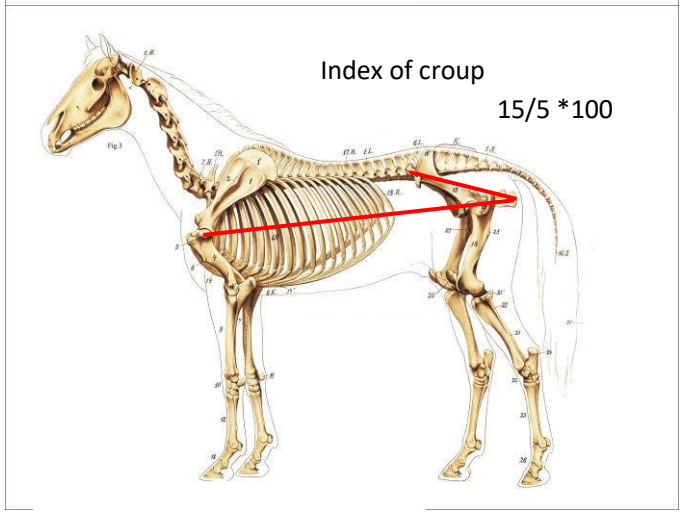

Appendix C. Questionnaire completed by owner/breeders for groups of horses, including cases with history of gestational immaturity, for Study into Behavioural Traits (Chapter 6.5).

Horse name:

Owner name:

Personality/behaviour assessment. These questionnaires are designed as part of the study into behavioural traits in horses born prematurely or as dysmature foals. Please tick one box in each row to reflect your horse's usual behaviour.

	Very	Fairly	Slightly	50/50	Slightly	Fairly	Very		*Don't Know*
Aggressive Quick to threaten harm or cause harm to others. Low tolerance.								**Agreeable** Reacts to others in an even, calm way. Is not easily aggravated.	
Anxious Becomes worried easily. Fears or avoids any kind of risk or disturbance.								**Curious** Readily explores new situations. Appears to be stimulated by novelty.	
Active Moves around a lot, lively, does not like being still for long.								**Inactive** Moves in relaxed manner, moves slowly and deliberately, unhurried.	
Submissive Gives in quickly to others. Submits easily and does not fight to defend self.								**Dominant** Gets own way quickly. Can control others quickly and easily.	
Cooperative Responds in appropriate manner to behaviour of other horses & humans.								**Uncooperative** Responds in obstructive manner to behaviour of other horses & humans.	
Untrusting Does not trust others easily. Trusts few individuals.								**Trusting** Trusts others readily and after a short time.	
Intolerant Reacts negatively with only slight provocation.								**Tolerant** Is not easily provoked.	
Excitable Over reacts to any change. Is easily excited, highly strung. 'Spooky'.								**Stoic** Steady, little physical reaction to change or stimuli. 'Safe' horse to be around.	
Fast learner Learns new things easily and quickly. Benefits from mental stimulation.								**Slow learner** Needs extra time and repetition to learn something new.	
Insecure Hesitates to act alone. Seeks reassurance from others.								**Confident** Goes forward alone. Acts boldly.	