ENDOCRINE TESTING FOR CAUSES OF LAMINITIS IN A GROUP OF AUSTRALIAN HORSES

David Byrne
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*I had to have one
Declaration of originality

I declare that:

a) This thesis is my own account of my research, except where other sources are acknowledged.
b) The extent to which the work of others has been used is clearly stated in each chapter and certified by my supervisors.
c) This thesis is my own account of my research and contains as its main content work that has not previously been submitted for a degree at any tertiary education institution

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Diplomate, ACVIM (Large Animal Internal Medicine)

Statement of contribution of others

The research presented herein was authored primarily by myself, David Byrne, with contributions provided by Profs. Cristy Secombe and Guy Lester, and Drs. Devindri Perera, Rachel Tan, Sally Watts and Jamie Wearn. Their contributions are appreciated.
Acknowledgements

I would like to dedicate this to my Granddad, Michael John ‘Chick’ Gillen, without whom my career in equine medicine would never have even begun.

I would also like to thank my supervisors, Prof. Cristy Secombe and Prof. Guy Lester, for their patience, guidance and good humour in this endeavor.

Finally, I would like to thank my incredibly supportive and loving family. Thank you, Grace, Zoe, and their no doubt ever-suffering baby brother, Oscar, for always giving me perspective and motivation. Most especially of all, I would like to thank my wonderful wife Lisa, who supported me in our move to Perth, my residency and seemingly never-ending Masters. Thank you, darling x
**Foreword**

One of the most cited papers in both Scopus and CrossRef, is by John Ioannidis et al. The paper, titled ‘Why Most Published Research Findings Are False’, evaluated the reproducibility of medical papers published in the previous decade. It argued that individual positive research findings depend massively on the pre-test probability. What matters is the totality of the evidence, rather than any individual results.

Reproducibility refers to the precision of the results of tests carried out under changing conditions of measurement. These changing conditions may refer to different times of testing, locations, populations tested, or laboratory instruments used. This may be relevant in cases where different laboratory assays are available, for instance, or to assess biologic variability over different weeks. It is often confused with repeatability, which is a measurement of closeness of agreement between successive measurements carried out in the same manner, or over a very short period of time. It is thought of as testing the test procedure itself. The reproducibility of research findings in equine medicine needs to be carefully evaluated given the often small sample sizes and limited populations tested.

A second influential paper was published in the Mayo Clinic Proceedings in 2013. This evaluated all original papers published in the New England Journal of Medicine over a 10-year period. In total, there were 146 medical reversals during this time, including C-reactive protein testing for sepsis, and knee arthroscopy for osteoarthritis.

The relevance for laminitis is that many reversals in our understanding of underlying causes of the disease have occurred over the last two decades. Fifteen years ago, hypercortisolaemia was thought responsible for many of the clinical signs of PPID and carbohydrate-mediated gastrointestinal dysfunction was thought the major pathophysiologic contributor to pasture-associated laminitis.

It behooves us to continue to validate prior research findings, and always to question dogma.
Abstract
The objectives of this study were 1) to evaluate the adrenocorticotrophic hormone (ACTH) response to thyrotropin releasing hormone (TRH) administration at different times of the year and in different locations, 2) to determine the effects of insulin dysregulation testing on ACTH concentrations and 3) to determine the reproducibility of two tests of insulin dysregulation.

For the first objective, 13 healthy horses in Perth and 29 in Townsville were identified by clinical parameters and normal monthly endogenous plasma ACTH. TRH-stimulation tests were performed at two time points during the circannual pituitary cycle at both sites. Perth horses were sampled at 10 minutes post-TRH (T10) and Townsville horses were sampled at 30 minutes post-TRH (T30). For the second and third objectives, a group of 15 healthy Thoroughbred and Standardbred horses underwent oral sugar tests (OST) and two-step insulin response tests (IRT) three times, at weekly intervals. For one week, endogenous ACTH was measured at the basal and testing time points in this group and in an additional control group of 15 horses.

For the TRH-stimulation tests, the T10 or T30 time point ACTH concentrations were significantly higher during the dynamic phase of the circannual pituitary cycle at each site, compared to during the dynamic phase (Perth T10 p=0.001; Townsville T30 p<0.0001). The mean T10 ACTH concentration in Perth during the dynamic phase was 248.5 pg/mL (range 80.7 - 511 pg/mL and 95% CI 170.2 - 326.9 pg/mL). The mean T30 ACTH concentration in Townsville during the dynamic phase was 112.3 pg/mL (range 53.6 – 227.0 pg/mL and 95% CI 93.4 - 131.2 pg/mL). For the OST, no substantial increase in insulin concentrations was observed. There was insufficient agreement of the test results between weeks. There was no statistical difference in ACTH concentrations before and at the 60 minutes post-sugar time point (p = 0.27). For the IRT, there was insufficient agreement between glucose concentrations at 25, 30 and 35 minutes post-insulin. There was insufficient agreement of the test results between weeks. There was no statistical difference in ACTH concentrations before and at 30 minutes post-insulin (p = 0.5).
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Abbreviations
2SIRT – 2-step insulin response test
α-MSH – alpha-melanocyte stimulating hormone
ACTH – adrenocorticotropic hormone
AUC – area under the curve
AVP – arginine vasopressin
BCS – body condition score
BM – basement membrane
Bwt - bodyweight
CGIT – combined glucose insulin tolerance test
CHO - carbohydrate
CIRT – complete insulin response test
CLIA – chemiluminescent assay
CLIP – corticotropin-like intermediate lobe peptide
CMax – maximum concentration
CRF – corticotropin releasing factor
CT – computed tomography
CV – coefficient of variation
DST – dexamethasone suppression test
ELISA – enzyme-linked immunosorbent assay
EMS – Equine Metabolic Syndrome
FSIGTT – frequently-sampled insulin-modified glucose tolerance test (with minimal model analysis)
GIP – gastric inhibitory polypeptide
GLP – glucagon-like peptide
GLUT – glucose transporter
HEC – hyperinsulinaemic euglycaemic clamp test
HPA – hypothalamic-pituitary-adrenal
ID – insulin dysregulation
IFGT – in-feed glucose test
IFN-γ – interferon-gamma
IGF-1 – insulin-like growth factor 1
IL-1β – interleukin 1-beta
IL-6 – interleukin 6
IR – insulin resistance
LPS - lipopolysaccharide
MAP – mitogen-activated protein
MIRG – modified insulin-glucose ratio
MMP – matrix metalloproteinase
NGT – nasogastric tube
NO – nitric oxide
NSC – non-structural carbohydrate
OST – oral sugar test
PI3 – phosphoinositide 3
POMC – pro-opiomelanocortin
PPID – pituitary pars intermedia dysfunction
PSSM – Polysaccharide Storage Myopathy
QUICKI - quantitative insulin sensitivity check index
RIA - radioimmunoassay
RISQI – reciprocal index of the square root of insulin
ROC – receiver operator curve
SEL – secondary epidermal lamellae
SGLT – sodium-glucose cotransporter
T0, 60, 90 – Basal, 60- and 90-minute testing time points, respectively
TMax – time to maximum concentration
TNF-α – tumour necrosis factor-alpha
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Chapter 1. Literature review

1.1 Laminitis

Laminitis is a significant cause of morbidity and mortality in horses. It is a painful and debilitating condition of the equine foot that can lead to chronic morbidity, loss of use, and compromised welfare. It occurs in association with pasture access (or other causes of high non-structural carbohydrate (NSC) intake). It can also occur in association with critical illness and contralateral lameness (i.e. support limb laminitis) which are thought to have differing aetiologies and pathophysiologies. As such, only pasture-associated laminitis will be discussed herein.

Clinical signs of laminitis are referable to those of foot pain, namely increased digital pulse amplitude or pulse pressure difference, hoof heat and pain on hoof tester application, along with characteristic lameness and stance alterations. The disease is usually bilateral in the forelimbs but may affect any foot. The clinical signs are due to laminar injury, stretching and subsequent failure, with secondary distal phalangeal rotation and/or sinking. Previous laminitis can be recognized by divergent horizontal ridges on the hoof wall, known as laminar rings, which can represent altered hoof growth subsequent to previous lamellar insult (Fig. 1.1). Clinical findings appear to be among the more accurate prognostic indicators in determining outcome in laminitis cases, with radiologic evidence of distal phalangeal sinking, higher body weight and more severe laminitis (i.e. higher Obel grades) associated with poorer outcomes.

1.1.1 Laminitis epidemiology

A recent systematic review summarized and critiqued the previously published literature with regard to the epidemiology of naturally occurring laminitis. The authors reviewed 69 publications; of these, 10 were considered of sufficient quality to determine prevalence. Within these studies, the prevalence varied between 1.5% and 36.5%. More specifically, of these studies, only 2 involved samples of horses from whole populations (in comparison to subpopulations in other studies). These studies showed quite disparate prevalences, 1.5% at a
hospital in the United States in comparison to 23.5% amongst pony club horses in Australia. These differences may be due to geographic and temporal differences, but they are also likely influenced by signalment and management factors, as well as significant case selection and categorization differences. Other Australian data has also shown a relatively high incidence amongst ponies, in particular with a recent large historical survey in the UK also having a higher prevalence in ponies. In a study of a large UK farm of 1000 horses, 23.5% had at least one episode of laminitis when followed over six years. In that paper, the overall incidence per year varied between 7.9% and 17.1%. There are some papers describing much lower frequencies, with differences attributed to management factors, primarily. In a prospective, longitudinal study

Figure 1.1. Divergent rings visible on the hoof wall are often an indicator of previous laminitis.
of 446 ponies (presumed at increased risk of laminitis in comparison to other breeds) \(^{29}\), the cumulative incidences in years one, two and three were 4%, 6.7%, and 9.9%, respectively.

In some studies, laminitis has occurred most commonly in autumn \(^{30,31}\). Another recent abstract from Australia found an increased incidence in spring \(^{32}\), while a large retrospective study in the UK found a peak in May (northern hemisphere summer) \(^{22}\) and a prospective cohort study in the UK finding a peak in summer \(^{14}\). A large survey in the United States also reported that laminitis was a significant cause of foot pain during spring and summer \(^{9}\). Interestingly, in a large retrospective survey from first opinion practices in the UK, the median duration between recurrence was approximately one year \(^{28}\), suggesting a seasonal effect.

Recurrence rates for laminitis are often considered high, although rates appear to vary significantly between locations, probably reflecting differences in study methodology as much as population differences. An Australian survey revealed that almost 9/10 horses with laminitis had at least one recurrence within one year \(^{26}\). More recent Australian surveys have also revealed high incidences of recurrence \(^{27,32}\). In the study involving the large farm in the UK \(^{22}\), 33.7% of horses had repeated episodes of laminitis, with almost one quarter having recurrences within the same year. A large historical survey of first opinion practices in the UK found a recurrence rate of more than 70% \(^{28}\), while a similar, but smaller survey demonstrated an approximately 40% rate of previous laminitis in cases identified \(^{17}\).

Another key factor in the assessment of the epidemiology of laminitis is the reliance on initial owner recognition. This may be considered poor, with some suggesting an under-recognition of foot pain as laminitis \(^{33}\) and a recent survey supporting this possibility \(^{34}\), while pathologic studies of naturally occurring laminitis have found evidence of prior episodes of subclinical laminitis in these animals \(^{35}\). In horses and ponies diagnosed with *pituitary pars intermedia dysfunction (PPID)*, there is also an under-recognition of laminitis occurrence \(^{36}\).
1.1.1.1 Risk factors

Understandably, given that most pasture-associated laminitis is due to underlying endocrine disease, many of the risk factors for laminitis of this nature reflect either PPID, Equine Metabolic Syndrome (EMS) or both. Indeed, as expected, PPID and EMS are significant risk factors for laminitis.

There is evidence for a sex bias in laminitis with females being more likely to suffer from disease, although not all studies are in agreement. In a multicenter, case-control study of over 250 cases from six teaching hospitals, Alford et al. found that in chronic laminitic cases, Quarter Horses were more at risk than Thoroughbreds, despite recent literature suggestion that they are insulin sensitive, relatively speaking [see section 1.4.6.7 Breed and genetics]. The same survey did not test for insulin dysregulation (ID), however. In a survey of laminitic horses and ponies presented to a veterinary hospital, older age and pony breeds were associated with laminitis. Age has been identified as a risk factor elsewhere, as has pony status. There is conflicting data on whether the risk of laminitis continues to increase with increasing age, with some literature supportive of this, and some suggesting that while middle-age and geriatric horses have an increased odds of laminitis in comparison to young horses, they have similar odds to each other. Several risk factors from Carter et al. are described in Table 1.1.

Obesity has been identified as a risk factor for laminitis, probably because of its association with ID. Similarly, a cresty neck has also been associated with laminitis. One study has shown that diet (low vs high NSC content) was not a risk factor for either current or historical laminitis. This is partially supported by Potter et al., who found that energy-supplying supplementary feed was not associated with laminitis, although access to pasture was. This is in contrast to other literature and expert consensus. Weather may play a role in the incidence of laminitis, likely through its effects on the NSC concentrations of feedstuffs.

Insulin dysregulation is associated with laminitis, given the potential causal link. In a prospective longitudinal study evaluating the incidence of laminitis over time, increased basal
<table>
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<th>Cut-off</th>
<th>Sensitivity, %</th>
<th>Specificity, %</th>
<th>IROC</th>
<th>LR+</th>
<th>LR-</th>
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<tr>
<td>TG (mg/L)</td>
<td>&gt;940</td>
<td>33 (10-70)</td>
<td>99 (92-100)</td>
<td>0.66</td>
<td>22.7</td>
<td>0.7</td>
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<td>(0.54-0.77)</td>
<td>(2.4-215.2)</td>
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<td>Insulin (µU/mL)</td>
<td>&gt;32</td>
<td>100 (61-100)</td>
<td>78 (67-86)</td>
<td>0.89</td>
<td>4.5</td>
<td>0.0</td>
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<td></td>
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<td>(0.80-0.95)</td>
<td>(2.9-7.1)</td>
<td>(0.0-1.5)</td>
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<tr>
<td>RISQI (µU/mL⁻⁰·⁵)</td>
<td>&lt;0.17</td>
<td>100 (61-100)</td>
<td>78 (67-86)</td>
<td>0.89</td>
<td>4.5</td>
<td>0.0</td>
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<td>(0.80-0.95)</td>
<td>(2.9-7.1)</td>
<td>(0.0-1.5)</td>
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<tr>
<td>MIRG</td>
<td>&gt;11.3</td>
<td>67 (30-90)</td>
<td>97 (90-99)</td>
<td>0.82</td>
<td>22.7</td>
<td>0.3</td>
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<td>(µU_{insulin}²/10.I_{glucose})</td>
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<td></td>
<td>(0.72-0.90)</td>
<td>(5.2-99.4)</td>
<td>(0.1-1.1)</td>
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<tr>
<td>Leptin (ng/mL)</td>
<td>&gt;7.3</td>
<td>83 (44-97)</td>
<td>78 (67-86)</td>
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<td>ACTH (pg/mL)</td>
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<td>83 (44-97)</td>
<td>79 (68-87)</td>
<td>0.80</td>
<td>4.0</td>
<td>0.2</td>
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<td></td>
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<td>(0.71-0.90)</td>
<td>(2.2-7.3)</td>
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<td>Body condition score</td>
<td>≥7</td>
<td>100 (61-100)</td>
<td>44 (33-56)</td>
<td>0.72</td>
<td>1.8</td>
<td>0.0</td>
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<td>(0.60-0.81)</td>
<td>(1.4-2.2)</td>
<td>(0.0-2.7)</td>
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<td>Girth:height</td>
<td>&gt;1.3</td>
<td>100 (61-100)</td>
<td>41 (30-53)</td>
<td>0.71</td>
<td>1.7</td>
<td>0.0</td>
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<td>(1.4-2.1)</td>
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<td>Cresty neck score</td>
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<td>83 (44-97)</td>
<td>78 (67-86)</td>
<td>0.81</td>
<td>3.8</td>
<td>0.2</td>
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<td></td>
<td></td>
<td></td>
<td>(0.70-0.89)</td>
<td>(2.1-6.7)</td>
<td>(0.0-1.3)</td>
</tr>
<tr>
<td>Neck circumference:height</td>
<td>≥0.71</td>
<td>100 (61-100)</td>
<td>79 (68-87)</td>
<td>0.90</td>
<td>4.9</td>
<td>0.0</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>(0.80-0.95)</td>
<td>(3.0-7.7)</td>
<td>(0.0-1.5)</td>
</tr>
<tr>
<td>Mean blood pressure (mmHg)</td>
<td>&lt;76</td>
<td>83 (44-97)</td>
<td>69 (57-79)</td>
<td>0.76</td>
<td>2.7</td>
<td>0.2</td>
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<td>(0.64-0.85)</td>
<td>(1.6-4.5)</td>
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</table>

**Table 1.1.** Diagnostic statistics for the prediction of laminitis in ponies using a defined cut-off value for each variable are sensitivity, specificity, area under the local receiver operator characteristic curve (IROC), and log likelihood ratios for the positive (LR+) and negative (LR-) tests. Values in parentheses are 95% confidence intervals (modified from Carter 2009 ⁴²).
insulin, insulin post-dexamethasone and IGF-1 concentrations and decreased adiponectin concentrations were risk factors for laminitis after multivariate logistic regression for all years 29. Understandably, collinearity existed between basal and post-dexamethasone insulin concentrations in that study. Initial basal insulin concentrations were also associated with future laminitis in a prospective cohort study 24, as were the subsequent proxy derivatives. Similarly, leptin concentrations have been used to identify ponies that go on to develop laminitis 42.

Risk factors also appear to vary between first and subsequent laminitis episodes. In a large retrospective study of first opinion practices in the UK, prednisolone was associated with recurrence, but not the initial episode 28. Another study 43 has shown that corticosteroids within the previous 30 days are a risk factor, however. Furthermore, risk factors for critical illness-associated laminitis are quite different to those of pasture-associated or endocrine-mediated laminitis 11.

In summary, increasing age is a consistent risk factor among studies, as has been confirmed by a recent systematic review 46, while other factors appear to vary between populations. Perhaps presciently, several scientists correctly identified insulin resistance as being a risk factor for laminitis more than 25 years ago 47.

1.1.2 Laminitis aetiology

1.1.2.1 Gastrointestinal carbohydrate overload

Previous theories of pasture-related laminitis involved carbohydrate (CHO) overload and caecal dysfunction as a potential aetiology. Oligofructose and enteral starches can reliably induce laminitis in less than 48 hours, as reflected in previous experimental models 48–50. The hypothesis in these cases involved an initial osmotic effect followed by luminal acidosis and thus altered hindgut microbiota 51,52 leading to absorption of endotoxin 50. This endotoxin absorption was suspected of leading to altered laminar perfusion, basement membrane dysadhesion and thus laminitis 48,49 as reviewed by Katz and Bailey 53.
However, this is no longer thought to be a plausible mechanism for several reasons. The histopathology of CHO-induced laminitis is different to naturally occurring or hyperinsulinaemia-induced experimental laminitis [see section 1.2.4 Histopathologic evidence for hyperinsulinaemia]. In addition, most horses administered acute overloads of oligofructose developed acute colitis, with several horses dying, which is not typically seen with pasture-associated laminitis. Although horses and ponies can theoretically ingest enough NSCs from pasture as can induce caecal dysfunction experimentally, in a pasture setting ingestion typically occurs over many hours, rather than as a bolus. Furthermore, when fructans are fed on a meal basis, increased vasoactive amines were detected within the large colon, but not the bloodstream, suggesting that normal meal feeding of fructans may not be comparable to acute nasogastric intubation with high doses. Thus, the acute ingestion of a quantity of NSCs sufficient to cause caecal dysfunction is an unlikely cause of chronic, pasture-associated laminitis. Interestingly, the OF experimental model does not result in changes in IS despite the induction of laminitis.

1.1.2.2 Matrix metalloproteinases

Matrix metalloproteinases (MMPs) have been implicated in the development of laminitis in a variety of experimental settings. In the hyperinsulinaemia model of laminitis, MMP-9 gene expression and concentrations have been shown to be increased. Conversely, in the same study, MMP-2 has been shown not to be increased. Goetzl et al. showed that MMP-9 is primarily derived from leukocytes, and so a pro-inflammatory state might increase the expression of this enzyme. While a pro-inflammatory state has been shown to exist in naturally occurring ID [see section 1.4.4 Systemic inflammation], MMP activation was not thought to be a factor in the hyperinsulinaemia experimental model. The authors hypothesized that insulin may be directly pro-inflammatory in this regard.

1.1.2.3 Reduction/oxidation

The current evidence does not suggest a significant role for reactive oxygen species in the development of experimentally-induced laminitis.
1.1.2.4 Hyperthermia
The current evidence does not suggest that hyperthermia alone plays a significant role in the development of experimentally-induced laminitis\(^6\(^1\). However, it may be a component of insulin-mediated disease\(^4\(^2\),\(^6\(^2\).

1.1.2.5 Endocrine disease
In more recent years, the most commonly identified cause of laminitis in most populations is endocrine disease, specifically PPID\(^3\(^0\),\(^3\(^7\) or EMS\(^3\(^7\). Several studies have shown that the prevalence of PPID in hospitalized laminitis cases can be as high as 70%\(^3\(^0\). One study showed that endocrine causes account for 90% of laminitis admissions\(^3\(^7\). However, that study used relatively insensitive diagnostic methodologies (basal insulin and hypertrichosis) for disease diagnosis, suggesting that the prevalence may actually be higher. Other studies of PPID horses have shown that the prevalence of laminitis within those horses can range from 13-84%\(^6\(^3\)–\(^6\(^6\), with the prevalence being higher than in control horses\(^6\(^6\). In one study that evaluated horses with at least one potential clinical sign of PPID, 61.7% had an endocrinopathy\(^6\(^7\). While there was obvious self-selection of cases, it should be noted even with relatively sensitive inclusion criteria (only one of a multitude of signs), the proportional incidence was only 43% for PPID in this study\(^6\(^7\).
1.2 Insulin Dysregulation

Insulin dysregulation (ID) is defined as any combination of cellular insulin resistance, fasting hyperinsulinaemia, excessive post-prandial hyperinsulinaemia or delayed insulin clearance. Delayed insulin clearance may play a role in the development of ID in horses as demonstrated by increased C-peptide (cleaved from proinsulin to form insulin) concentrations post-dextrose in the insulin-modified, frequently sampled IV glucose tolerance test (FSIGTT). This study was performed in relatively insulin-sensitive, non-obese horses, however, and results should be replicated in ID horses before the role of delayed insulin clearance can be completely defined. In addition, the authors point out that they assume that C-peptide clearance is unaffected by insulin dysregulation. Cellular insulin resistance and hyperinsulinaemia are discussed further below.

1.2.1 Development of hyperinsulinaemia and insulin resistance

Insulin resistance (IR) may lead to hyperinsulinaemia as a compensatory mechanism to maintain normal blood glucose concentrations in the face of decreased peripheral utilization, potentially in a self-perpetuating cycle (so called homologous desensitization). Cellular IR leads to pancreatic beta cell stimulation and thus increased insulin secretion. In horses specifically, IR leads to decreased GLUT-4 expression in equine skeletal muscle; since this is a major route of intracellular glucose transport, a reduction in GLUT-4 expression may lead to glucose-mediated hyperinsulinaemia as compensation.

Hyperinsulinaemia has also been hypothesized as being a precursor to cellular IR. This might occur through receptor downregulation or other mechanisms, although the research on receptor downregulation is conflicting. One study in obese ponies found that an increased number of digital microvascular endothelial cells expressed insulin receptors despite being in a hyperinsulinaemic state. Furthermore, when insulin infusions were administered for 48 hours to induce laminitis in healthy Standardbreds, no reductions in skeletal muscle GLUT-4 expression were identified, making insulin-dependent receptor downregulation less likely. There are data supporting the role of insulin receptor downregulation, however. In a study of mares grazing pastures of various NSC percentages, horses ingesting cultivated grass (assumed to have
higher NSCs) demonstrated down-regulation of insulin receptor gene expression in subcutaneous adipose tissue \(^{84}\), although none of these mares was classed as IR by an FSIGTT. Another study involving hyperinsulinaemia as part of a \textit{hyperinsulinaemic euglycaemic clamp test (HEC)} used to induce laminitis confirmed reduced expression of insulin receptors in lamellar tissue \(^{85}\). It has also been shown that not all ponies that have excessive insulin responses to oral glucose have abnormal \textit{combined glucose-insulin tolerance tests (CGIT); and}

\begin{figure}
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\includegraphics[width=\textwidth]{image.png}
\caption{The cause and effect relationship between insulin resistance and hyperinsulinaemia (modified from Frank and Tadros 2014 \(^{86}\)).}
\end{figure}
Thus cellular IR. Other work has demonstrated that hyperinsulinaemia may lead to IR through alternative mechanisms such as alteration in expression of a mitochondrial uncoupling protein, or through maintenance of the so-called disposition index.

Regardless of which is the initiating factor, there is a close relationship between excessive postprandial hyperinsulinaemia and insulin sensitivity. One study comparing a modified oral sugar test (OST) and the HEC found that the β-cell responsiveness demonstrated by the OST was inversely proportional to the insulin sensitivity in a hyperbolic manner. Thus, the hyperinsulinaemic response to oral sugar depends on the degree of IR present.

1.2.2 Insulin dysregulation and laminitis

There is a reproducible association between hyperinsulinaemia and the presence or subsequent development of laminitis. Ponies with a previous history of laminitis have been shown to be less insulin sensitive than non-laminitic ponies as measured by the FSIGTT. In one experimental study, peak insulin concentrations were associated with the onset of laminitis when ID has been induced with high NSC diets, while another demonstrated clinical cases of laminitis in horses fed high NSC diets that had higher insulin concentrations. There may also be a dose-response effect of insulin on both the risk of laminitis, as well as the degree of histopathologic change, with insulin concentrations of approximately 200 µU/mL being associated with a high risk of impending subclinical or clinical laminitis, depending on the timeframe. A survey performed in the United States has shown that higher Obel grades of laminitis are associated with higher basal insulin concentrations. A preliminary survey in Australia had similar findings, although these data have been presented in abstract form only. Ponies that have had previous episodes of laminitis have also been shown to be more insulin dysregulated than those not thought to be at risk. The numbers in this study were small, and the ponies were all obese. They were well matched with controls, however. Insulin concentrations have also been shown to be associated with mortality in laminitis. In a study of 20 horses with PPID, there was a 90% chance that a horse did not survive when the insulin concentration was ≥188.6 µU/mL, while a concentration of ≤61.4 µU/mL yielded a 90% chance of survival. Encouragingly, when previously
laminitic ponies had strict dietary management to prevent recurrence, their basal insulin concentrations were not significantly different from normal ponies 94.

In a landmark paper in the current understanding of the pathophysiology of endocrine-associated laminitis, induced hyperinsulinaemia (via the HEC) reliably lead to laminitis in ponies without a history of laminitis 95. That group used moderately overweight ponies with normal basal insulin concentrations fed lucerne hay (i.e. relatively low in NSC concentration). Laminitis was reliably induced within 36 hours of hyperinsulinaemia but not in control horses. The hypothesis that prolonged, marked hyperinsulinaemia could cause laminitis was reinforced when the experiment was repeated using healthy Standardbreds 62, a breed thought of as being insulin sensitive 96,97 and thus at low risk of endocrinopathic laminitis. In that study, all treated horses developed histologic evidence of laminitis while none of the control horses did 62. More recently, laminitis (albeit subclinical) has been induced at much lower concentrations of insulin (approx. 200 μU/mL 98 vs >1000 μU/mL in the original papers 62,95). The duration of hyperinsulinaemia seems to be important in the induction of laminitis as well as the peak concentrations 99. The peak insulin doses in a study where insulin was administered to assess the response to glucose in light breed horses 100 was much higher than that used to induce laminitis in either ponies 95 or Standardbred horses 62. Insulin concentrations greater than 800 μU/mL did not lead to significant histopathologic evidence of laminitis after 24 hours 101, whereas the majority of animals had evidence of laminitis when a similar experiment was continued for 48 hours 62. In a study that induced marked hyperinsulinaemia but only for six hours, no laminitis was reported, although this was not a defined endpoint of the study 79. Bamford et al. 102 suggested that once daily high NSC meals may not have led to sufficient downregulation of insulin receptors to IR. Indeed, multiple daily episodes of hyperinsulinaemia may be necessary for IR to develop due to chronic over-stimulation of insulin receptors 79.
1.2.3 How insulin dysregulation might cause laminitis

1.2.3.1 Hyperglycaemia

Laminitis has been induced by prolonged hyperinsulinaemia as part of a euglycaemic clamp procedure. In both studies, euglycaemia was maintained. However, by definition, larger absolute quantities of glucose were delivered to the lamellae. Further research by the same group has shown that infusion of the same supraphysiologic quantities of glucose leads to similar hyperinsulinaemia and subsequently laminitis, albeit subclinically. These two studies suggest that while hyperglycaemia may lead to laminitis, it is probably mediated through hyperinsulinaemia, with direct glucotoxicity less likely. In naturally occurring laminitis, hyperglycaemia is uncommon, further supporting this assertion. While laminitic Andalusians were found to have elevated glucose concentrations relative to healthy controls, the concentrations were not statistically different and the laminitic horses were tested within 24 hours of onset of laminitis. Thus, the elevations may relate more to an acute stress response than underlying insulin dysregulation.

1.2.3.2 Insulin resistance

Cellular IR as a cause of lamellar failure is also not supported by the literature. The lamellae have been found to have very high glucose requirements, even exceeding that of the head at rest. Furthermore, digital lamellae kept in tissue baths without glucose quickly results in laminar separation while the addition of glucose prevents separation for 8 days. Insulin-mediated glucose uptake, through GLUT-4 receptors, is the major mechanism for skeletal muscle uptake of glucose. The digital lamellae, however, have been shown to have minimal or no expression of glucose transporter (GLUT)-4, as demonstrated by mRNA expression by PCR and immunoblotting. Additionally, the digital lamellae do not express insulin receptors, as demonstrated by immunoblotting. The microvascular endothelium, however, do express insulin receptors, as demonstrated by the same study. These receptors have been shown to be increased in association with increased NSC diets. Finally, insulin administration did not increase the glucose uptake of the digital lamellae over basal in hyperinsulinaemic ponies, nor did it alter the expression of GLUT-1 transporters in lamellae in hyperinsulinaemic Standardbreds.
Instead, the lamellae are probably supplied with their glucose requirements through glucose-mediated glucose uptake through GLUT-1 receptors \(^{82,104}\), independently of insulin. Interestingly, one study found that GLUT-8 and -12 were expressed in different quantities between control horses and horses treated with insulin infusions \(^{82}\), although the significance of this is unknown. Thus, the causative role of hyperinsulinaemia in laminitis may be a direct effect of insulin itself.

**1.2.3.3 Hyperinsulinaemia**

Hyperinsulinaemia may lead to laminitis through several potential mechanisms. As previously mentioned, it has been shown that insulin receptors within the foot are found exclusively on the microvascular endothelium; there are no identifiable receptors on the laminar epithelium \(^{81}\). Thus, the role of insulin may relate to its vasoactive effects. In further support of the vasodilator role increased insulin concentrations may play in the normal horse, increased blood flow to the foot has been linked to increased insulin concentrations after meals \(^{107}\). That group suggested that the increased flow may relate to insulin-mediated endothelial-derived nitric oxide (NO) release \(^{107}\). In an experimental model of laminitis, horses that were treated with prolonged insulin infusions had higher hoof wall temperatures in comparison to control horses \(^{62}\). The authors postulated that this increased hoof wall temperature may be associated with insulin-mediated vasodilation \(^{62}\). In humans, the evidence is somewhat contrary to this hypothesis (increased vasodilation in hyperinsulinaemic states). It has been shown that cellular insulin resistance differentially affects the phosphoinositide (PI3) kinase– and mitogen-activated protein (MAP) kinase–mediated signaling systems in the vasculature within human muscle. In obese humans, PI3 kinase (responsible for NO mediated vasodilation) was markedly reduced and absent in people with T2DM \(^{108}\). In contrast, insulin-mediated stimulation of MAP-kinase (which leads to endothelin-mediated vasoconstriction) was found to be normal in these humans \(^{108}\). It has been shown that MAP kinase activation occurs in laminar tissue in the CHO model of laminitis \(^{109}\), although this has not been reported in insulin-mediated laminitis. Horses may also undergo reduced insulin-associated, NO-mediated vasodilation in hyperinsulinaemic laminitis \(^{62}\), although the data is incomplete in this area. Pre-existing hyperinsulinaemia has been shown to alter the response of digital vasculature to insulin, with vasodilatory responses becoming vasoconstrictive.
Alternatively, low adiponectin concentrations may lead to decreased vasorelaxation of the equine digital vasculature. Morgan et al. has also shown a reduction in endothelium-dependent vasodilation in laminar vessels in obese, hyperinsulinaemic laminitic horses, albeit in small numbers. Thus, ID may lead to an imbalance between vasoconstrictive and vasodilatory mediators (Fig. 1.3). These vasoactive mediators are not detectable in peripheral blood after insulin infusions, and may only be active in significant quantities in local tissue beds, such as the lamellae.

**Figure 1.3. Theoretical relationships between insulin sensitivity and vascular tone in horses. Alterations in insulin sensitivity may determine which pathway predominates after activation by circulating insulin (modified from Tadros and Frank 2013).**
An alternative hypothesis involves insulin-like growth factor (IGF)-1 receptor activation. At supraphysiologic concentrations, insulin has been shown to bind and activate IGF-1 receptors in other species. Concentrations of IGF-1 have been shown to be increased in horses fed high NSC diets and non-specific insulin-binding of IGF-1 receptors may also occur in horses. A trend towards increased IGF-1 concentrations has been detected in obese mares. Hyperinsulinaemia leads to insulin receptor and IGF-1 receptor downregulation through specific and non-specific overstimulation, respectively, without any detectable rise in serum IGF-1 concentrations. This IGF-1 receptor stimulation may lead to lamellar cell proliferation and thus lamellar weakening. It has been shown that cell proliferation occurs early in the developmental phase of experimental insulin-induced laminitis. Recent literature has characterized the laminar tissue expression of various insulin and IGF receptors. Those authors suggest that during hyperinsulinaemia, insulin may mediate growth, adhesion and survival, rather than metabolism. One study also suggested that obesity and high NSC diets both influence expression of these receptors, with an inverse relationship between insulin concentrations and receptor expression. Another group has postulated that combined hyperinsulinaemia and hypoadiponectinaemia may lead to altered expression of insulin and IGF-1 receptors in lamellar endothelia and epithelia, respectively, based on human data. Downstream signaling pathway products of IGF-1 receptor stimulation have been identified in the lamellae of obese ponies fed high NSC diets for seven days, with modest correlations with insulin concentrations observed. That study also identified increases in phosphorylated IGF-1 signaling pathway products in Standardbreds that had hyperinsulinaemia-induced laminitis. A recent study has shown that insulin does not bind to IGF-1 receptors at physiologic (or even pathophysiologic) concentrations, however, complicating the current understanding of the role of insulin activation of IGF-1 receptors in the digit.

1.2.4 Histopathologic evidence for hyperinsulinaemia-induced lamellar lesions

In the original paper describing hyperinsulinaemic laminitis, histopathology of the affected feet revealed that the tips of the secondary epidermal lamellae (SEL) were elongated and tapered, the basement membrane (BM) was disintegrated, and basal cell nuclei were rounded. This is
similar to pathologic findings in both experimental hyperinsulinaemic laminitis in Standardbreds \(^{55}\) and naturally occurring, ID-associated laminitis \(^{35}\) and PPID-associated laminitis \(^{119}\). It does not completely agree with histopathologic findings in a CHO-overload model of laminitis, which tends to have more severe BM disruption \(^{49}\). Experimentally, in general, there is SEL lengthening in both models of laminitis \(^{55}\), although there was less evidence of inflammation in the hyperinsulinaemia model of laminitis \(^{54,55}\). Recent research has also shown that lamellar explants weaken in the presence of insulin \(^{120}\). There appears to be a difference with regard to BM disruption between horses \(^{55}\) and ponies \(^{54}\); this has been postulated to be due to differences in bodyweight \(^{55}\). Laminitis occurring secondary to the experimental chronic feeding of high-NSC diets does not lead to leukocyte infiltration or increased inflammatory markers within laminar tissue \(^{121}\), again in contrast to the CHO model of laminitis \(^{122}\). Furthermore, MMP activity in the lamellae in hyperinsulinaemia-induced laminitis is much lower than that in CHO-induced laminitis \(^{58}\).

1.2.5  Insulin dysregulation in EMS

1.2.5.1  Incretins

There are three currently identified incretins – glucagon-like peptides 1 and 2 (GLP-1, 2) and glucose-dependent insulino tropic peptide (GIP) - which are secreted from L and K cells of the small intestine, respectively \(^{123}\). These hormones are released in response to intestinal amino acids, glucose and fats \(^{123}\). In humans, incretins have been found to be significant insulin secretagogues \(^{123}\) and this has recently been confirmed in horses in vitro \(^{124,125}\). Assays for the assessment of GLP-1, GLP-2 and GIP have been validated for use in horses \(^{77}\). The gastrointestinal stimulus for insulin secretion, mediated by these incretin hormones, is referred to as the enteroinsular axis \(^{77}\).

The contribution of incretins to the induction of hyperinsulinaemia in horses has been evaluated, albeit incompletely thus far. There appears to be a breed difference in incretin response to NSCs, with ponies and Andalusians having higher GLP-1 responses post-prandially than Standardbreds \(^{96}\). Earlier research found no difference between horses and Shetland ponies, however \(^{126}\). A relationship between body morphometry and GIP has been identified, with higher
concentrations associated with higher body condition and cresty neck scores \(^\text{124}\). Interestingly, a recent study has shown no difference in GLP-1 responses in insulin sensitive or resistant horses using the OST \(^\text{89}\), although the latter has a much lower sugar dose than the IFGT. In addition, that study demonstrated wide interindividual variability in GLP-1 concentrations. When the incretin response to oral carbohydrates was assessed using the IFGT, the responses were lower than reported in other species \(^\text{77}\), suggesting that glucose is the major determinant of pancreatic release of insulin in horses. When the \textit{in vitro} pancreatic response to GIP and GLP-1 antagonists was investigated, a reduction in insulin release of 30\% \(^\text{124}\) and 27\% \(^\text{125}\) was found, respectively. While the relatively low level of antagonism may be related to various confounding factors, the role of incretins may not be as important in equids in the development of post-prandial hyperinsulinaemia, in comparison to other species, at least with regards to direct effects. A recent paper has demonstrated increased GLP-2 concentrations in ponies with ID \(^\text{127}\). This incretin is thought to be intestinotropic in other species, and may explain why glucose bioavailability is increased in ponies with ID.

\subsection*{1.2.5.2 Genetics}
An alternative, or perhaps complementary, hypothesis relates to a potential genetic predisposition. Initial reports suggest an association with a gene involved with cholesterol metabolism \(^\text{128}\) [see section 1.4.6.7 Breed and genetics].

\subsection*{1.2.6 Insulin dysregulation in PPID}
Insulin dysregulation has long been associated with PPID \(^\text{66,129,130}\). It has recently been reported that between 30 and 60\% of horses with PPID will have concurrent ID \(^\text{36}\). Horses with PPID were more likely to have IR than clinically normal horses in one study \(^\text{129}\). However, age was a potential confounder in that study as ID has been shown to be more common in older horses \(^\text{131,132}\). Similarly, horses with ID were more likely to have PPID than animals with normal insulin statuses \(^\text{67}\). Karikoski et al \(^\text{37}\) showed that the majority of PPID horses are insulin dysregulated in a case-control study of laminitis presentations to their hospital.
Horses with PPID have been shown to have higher basal insulin concentrations in comparison to normal horses or horses with pasture-associated laminitis. They have also been shown to have reduced insulin sensitivity as assessed by the HEC. Furthermore, Frank et al. showed that horses with PPID had elevations in insulin concentrations in autumn in comparison to spring, despite similar pasture ethanol-soluble CHO content. Basal hyperinsulinaemia has previously been found to be a sensitive indicator of PPID in horses selected with specific testing (histopathology and hypertrichosis).

Horses with both PPID and EMS have also been reported to have higher insulin concentrations than those with either condition alone. Furthermore, EMS and PPID horses had similar insulin concentration that were higher than control horses under similar management in a longitudinal, clinical survey. Interestingly, it may be that horses with PPID and ID concurrently are likely to get laminitis, whereas those with PPID but not ID, are not. It has also been found that PPID horses that have laminitis are more likely to have a higher body condition score (BCS), presumably related to metabolic derangements and concurrent ID.

There is also some evidence suggesting that ID might progress with progression of PPID. Keen et al. found that horses with PPID as diagnosed by hypertrichosis (considered an advanced clinical sign) had significantly higher insulin concentrations in comparison to horses with positive dexamethasone suppression test (DST) but no hypertrichosis. This suggests that upregulation of the hypothalamic-pituitary-adrenal (HPA) axis might accentuate insulin dysregulation.

It may be that PPID modifies or accentuates the genetic predisposition to hyperinsulinaemia. However, it has also been found that corticotropin-like intermediate lobe peptide (CLIP) acts as an insulin secretagogue in other species and thus may contribute to hyperinsulinaemia in PPID. Another hypothesis is based on the finding of increased free cortisol in association with hyperinsulinaemia. That group hypothesized that increased 11-beta-hydroxysteroid dehydrogenase activity may lead to increased free cortisol concentrations, thus leading to
hyperinsulinaemia through hepatic gluconeogenesis and failure of GLUT-4 membrane localization.

There is other data that has shown no effect of PPID on OST results ¹⁴⁰. Additionally, in an age-matched study between PPID and control animals using an isoglycaemic hyperinsulinaemic clamp, no differences were identified between groups ¹⁴¹. It may be that certain populations of PPID horses are likely to be insulin dysregulated, independent of other factors. However, a limitation of that study ¹⁴¹ is that the reporting of DST and adrenocorticotropic hormone (ACTH) results in control horses is incomplete, making generalizability difficult.

As previously mentioned, ID has been associated with prognosis in PPID horses. In a longitudinal study of 20 PPID horses, there was a 90% chance that a horse would not survive when insulin concentrations were greater than or equal to 188.6 μU/mL, while those horses with an insulin concentration of less than or equal to 61.4 μU/mL had a 90% chance of survival ⁹³. The presence of more severe ID in PPID-affected animals has also been associated with worse radiographic changes and greater owner recognition of laminitis ³⁶.

There is also a report of pergolide treatment in a horse with minimal model-confirmed type two diabetes mellitus, and concurrent PPID, resulting in lower insulin concentrations after an initial increase, with no other management changes ¹⁴². In certain circumstances, ACTH concentrations have been negatively associated with the presence of laminitis ⁴². It is unclear if animals with PPID were being treated in that study, although the effects on diagnosis of laminitis (i.e. the likelihood ratios) were very small.
1.3 Pituitary Pars Intermedia Dysfunction

Pituitary pars intermedia dysfunction is a neurodegenerative condition of usually older horses that occurs worldwide. The condition is thought to represent the loss of inhibitory signaling to the melanotropes of the pars intermedia of the pituitary gland. This is thought to be due to neurodegeneration of dopaminergic hypothalamic neurons that exert an inhibitory signal on melanotropes. This neurodegeneration is thought most likely due to oxidative damage, although this is not confirmed. The pars intermedia melanotropes undergo hyperplasia, leading to micro- and subsequently macroadenoma development and altered (usually upregulated) pro-opiomelanocortin (POMC) expression. It has been shown that circulating concentrations of POMC itself do not increase in PPID, despite increases in pituitary expression, suggesting post-translational or secretory factors play a role in the pathophysiology of the disease. These factors result in increased circulating concentrations of POMC-derivatives, such as ACTH and alpha-melanocyte-stimulating hormone (α-MSH), however. The altered expression of POMC-derivatives is thought to be responsible for the generation of clinical signs, which include laminitis, hypertrichosis (formerly and erroneously hirsutism), weight loss, polyuria/polydipsia and immunosuppression.

1.3.1 Epidemiology of PPID

In one hospital in the Netherlands in the early 1990s, PPID accounted for up to 0.5% of hospital admissions. This may represent selection bias due to the referral nature of the practice, however. In an international survey of practitioners, an overall estimated prevalence of 1% was reported, that did not appear to differ based on location. In another study, the proportional representation over time increased from 0.25/1000 to 3.72/1000 over a 10 year period. Whether this relates to increased horse longevity, better diagnostic testing or a true increase in the prevalence of disease is unknown. Amongst the general population, one study in Queensland, Australia, demonstrated a prevalence of PPID of 21.2% in horses over 15 years of age, based on ACTH concentrations above seasonal cut-off values while others demonstrated hair coat changes suggestive of PPID in 22% of horses older than 15 years, rising to 39% of horses older than 30 years of age in the UK.
1.3.2 Signalment

As stated above the prevalence of PPID increases with age. In fact, age is a strong independent predictor of PPID status with horses between 15 and 19.9 years of age being 2.2 times more likely of having PPID in comparison to horses <15 years of age. This rises to 4.6 times for horses between 20 and 24.9 years of age and 14 times for horses >25 years of age.

The disease has no apparent sex predilection, although this has recently been challenged based on resting ACTH concentrations. Ponies may be more likely to have PPID than horses, although this is refuted elsewhere. Signalment as described by Ireland and McGowan is described in Table 1.2.

1.3.3 Clinical Signs

Hypertrichosis, previously erroneously called hirsutism, is considered a specific clinical sign of PPID, with a reported positive predictive value of 91%. However, given the association between PPID and laminitis, it seems prudent to diagnose PPID before hypertrichosis develops, given its appearance potentially later in disease than laminitis. Weight loss is also associated with PPID while other clinical signs (such as muscle atrophy lethargy, polyuria and polydipsia, and recurrent or unusual infections) are considered non-specific, but may be overlooked in the early stages of disease. There may also be an infrequent association with suspensory ligament desmitis. Various diagnostics have been evaluated for PPID and most have inadequate sensitivity, specificity or reproducibility. These include basal cortisol, the domperidone stimulation test, loss of diurnal cortisol rhythm, the cortisol response to thyrotropin-releasing hormone (TRH) administration, the cortisol response to ACTH administration, and basal fructosamine concentrations.
<table>
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<th>Heinrichs et al (^{147})</th>
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Table 1.2. Characteristics of 385 horses and ponies with clinical signs or clinicopathologic diagnosis of PPID (modified from Ireland and McGowan, 2018 \(^{153}\)). \(n\), number. NR, not reported.
1.3.4 Diagnosis

1.3.4.1 Histopathology

Histopathology is traditionally thought of as the ‘gold-standard’ diagnostic for PPID \textsuperscript{161,169}. The pituitary changes are graded out of 5, where 1 is within normal limits (Fig. 1.4) and 5 is a macroadenoma (Fig. 1.5) \textsuperscript{170}. However, there are several problems with histopathology being a valid ‘gold-standard’. One study has shown that there are histologic changes in the pituitary gland associated with season \textsuperscript{169}. In this study, grade 4/5 changes (microadenomas) were detected in autumn in horses euthanized for diseases not consistent with PPID. These findings might represent false positives in animals with a low pre-test probability in autumn \textsuperscript{169}. Interestingly, in that study, the pars distalis and pars nervosa histometry findings did not change during autumn. Depending on the time of year, grade 4/5 changes may or may not represent disease. Another study found grade 3/5 (diffuse hyperplasia) and 4/5 changes in the pars intermedia of clinically normal mares at various times of the reproductive cycle \textsuperscript{171}, indicating that this may also affect pituitary gland histopathology. Other factors which may affect pituitary histometry and histology have not been elucidated.

Another potential problem with using histopathology is that agreement between pathologists might vary. One study found that in cases with strong supporting (clinicopathologic) evidence of disease, there was very good to excellent agreement between pathologists \textsuperscript{172}. That study also demonstrated good agreement in cases unlikely to be diseased. The difficult appears to lie in cases where the clinicopathologic diagnosis is unclear \textsuperscript{172}, where a ‘gold standard’ test might be needed to better stratify diseased and healthy animals. While a more recent study found better (but not perfect) agreement \textsuperscript{169}, there are concerns associated with using histopathology as validation of other diagnostic tests. Finally, horses with pituitary microadenomas \textsuperscript{170,171} or adenomas \textsuperscript{154} but no clinical signs have also been reported.
Figure 1.4. Median sagittal section of a haematoxylin and eosin-stained normal equine pituitary gland. The pars intermedia (PI) is a narrow band of endocrine tissue located between the pars distalis (PD) and pars nervosa (PN). The pars tuberalis (PT) surrounds the infundibular stem (IS; modified from McFarlane 2014).  

1.3.4.2 Computed tomography  
There are conflicting reports of computed tomography (CT) being useful in the diagnosis of PPID. In the Pease study, CT was quite well correlated with necropsy findings (size and volume when adjusted for body mass). However, these horses were all quite advanced clinically and were all grade 5/5 on PM. The earlier study used older technology, but did not find the modality as useful, with large variations between CT and PM measurements. With increasing popularity of standing head CT facilities available for horses and improving spatial resolution, CT may become an important research tool in the future. The effects of seasonal changes in pituitary size on the diagnosis of PPID using CT also needs to be investigated. It is unlikely to become a practical clinical diagnostic test except in exceptional cases, however.
Figure 1.5. Median sagittal sections of pituitary glands from horses with PPID. A) Haematoxylin and eosin-stained section of a pituitary gland from a horse with microadenomas of the PI. Minimal compression of the pars nervosa (PN) and pars distalis (PD) is present. Arrows highlight the microadenomas. B) Haematoxylin and eosin-stained section of a pituitary gland from a horse with an adenoma of the pars intermedia. Marked compression of the PN and PD is present and the gland has a rounded appearance (modified from McFarlane 2014).  

1.3.4.3 Biomarkers

Complete blood count and serum biochemical analyses are sometimes abnormal in horses with PPID. However, the abnormalities seen (mild anemia, neutrophilia, lymphopenia, increased γ-glutamyl transferase, creatinine kinase and aspartate aminotransferase activities etc.) are neither specific nor sensitive for the disease. As mentioned, insulin has previously been thought of
as a sensitive test for PPID\textsuperscript{130}, however, this most likely represents those horses that have PPID and ID as comorbidities. It likely misses PPID horses with normal insulin responses and is not specific for PPID\textsuperscript{177}.

\textbf{1.3.4.4 DST}

When first described, the DST was purported to have a sensitivity and specificity of 100\% \textsuperscript{161}. More recent evidence has disputed this, however \textsuperscript{178}, with the original testing likely suffering from selection bias involving advanced cases. Durham et al\textsuperscript{179} has combined the data from three papers \textsuperscript{154,161,180} evaluating the diagnostic accuracy of the DST and found an overall test sensitivity of 89\% and specificity of 88\%. Marked discrepancies have been observed between clinical signs, ACTH concentrations and results of the DST\textsuperscript{137}. Indeed, the post-mortem findings in PPID horses and those with pituitary hyperplasia were not correlated with the results of the DST\textsuperscript{180}. In another study, a significant portion of normal and laminitic ponies had changing test results over time\textsuperscript{181}, which has also been observed elsewhere\textsuperscript{178}. In a study using hypertrichosis as the ‘gold-standard’, the DST was positive in only 50\% of horses, whereas endogenous ACTH concentrations were abnormal in 92\% of horses\textsuperscript{137}. The time of year of testing was not mentioned in this report, however, which may have confounded the results. Part of this poor performance may relate to the fact that the melanotropes of the pars intermedia may not express glucocorticoid receptors\textsuperscript{182}. Additionally, it has been recommended that the DST not be performed as a PPID diagnostic in autumn due to the propensity for false-positives based on current cut-off limits\textsuperscript{183}. Seasonally-adjusted cut-off values may overcome some of this poor specificity, but this data is not available.

While not reported\textsuperscript{181}, some authors have considered the risk of corticosteroid-associated laminitis as a drawback for the DST\textsuperscript{155}. Indeed, the dose of dexamethasone used in the DST has been shown to induce temporary insulin resistance\textsuperscript{184}, which may increase the risk of laminitis in association with concurrent high NSC feed. The test also requires two separate venepunctures on consecutive days, making the test potentially less practical for clients. Thus, there is a need for an accurate, practical ante-mortem diagnostic that removes some of the potential downsides of the DST.
1.3.4.5 **Alpha-melanocyte stimulating hormone**

Alpha-MSH is thought to be an accurate test for PPID diagnosis, and may be released in higher concentrations after TRH-stimulation than ACTH \(^{158}\). The hormone is typically expressed from the melanotropes of the pars intermedia \(^{185,186}\). Like ACTH, its secretion in normal animals is stimulated by **arginine vasopressin (AVP)** and **corticotropin releasing factor (CRF)** and suppressed by cortisol \(^{187}\). Alpha-MSH has also been shown to be more stable than ACTH \(^{188}\). However, it is unclear if its accuracy is superior to ACTH’s, with supporting \(^{185}\) and refuting \(^{189}\) evidence in the literature. A major difficulty with the use of α-MSH as a diagnostic tool is the lack of a commercially available assay.

1.3.4.6 **Adrenocorticotropic hormone**

Adrenocorticotropic hormone was first described as a diagnostic test for PPID in the mid-1990s \(^{64,130}\). It is secreted from the pars distalis in normal horses, with peaks occurring between 2 and 6 times per hour \(^{190–192}\) when pituitary effluent blood is sampled remotely in unperturbed horses. Similarly, when evaluated systemically, adult mares have ultradian rhythms with peaks occurring between approximately 4 and 6 times per hour \(^{192}\). The release of ACTH in normal horses is closely associated with AVP release \(^{190,193,194}\). During physiologically stressful events, CRF appears to modulate this response \(^{195}\), with the two peptides acting synergistically at lower cortisol concentrations \(^{196}\). Interestingly, in horses perceived as having isolation stress (sweating, tachycardia, increased ambulation etc.), those having no significant AVP concentration elevations did not have ACTH concentration elevations \(^{194}\). In normal horses, ACTH is primarily derived from corticotropes within the pars distalis (Fig. 1.6) \(^{197,198}\). In contrast, in PPID, many POMCs are derived from the pars intermedia \(^{143,197–199}\). These POMC products include ACTH, α-MSH, CLIP and beta-endorphin, among others \(^{143,147,198}\). It is thought that the ACTH secreted from the pars intermedia in PPID is less likely to be biologically active, which would explain the lack of hypercortisolaemia in many animals \(^{180,197}\). Furthermore, in horses with PPID, ACTH and cortisol concentrations are only weakly correlated \(^{159}\). Recently, POMC derivatives in PPID have been
shown to be genetically homologous to those produced in normal horses. Thus, the reasons for ACTH inactivity in PPID may relate to post-processing factors.

With regards to the diagnosis of PPID, the sensitivity of plasma ACTH concentration was reported in earlier literature to range from 84 to 100%\(^\text{148}\). The specificity reported varied between 78% using a cut-off of 35 pg/mL and 100% using a cut-off of 50 pg/mL, albeit with different assays\(^\text{64,200}\). More recently, the sensitivity and specificity during the dynamic period [between the

**Figure 1.6.** POMC processing pathway. POMC is processed differently in the corticotropes of the pars distalis than in the melanotropes of the pars intermedia because of the differential expression of the enzymes involved in the posttranslational processing steps. AC-α-MSH: acetyl-alpha-MSH; AC-β-END: acetyl-beta-endorphin (modified from McFarlane 2011\(^\text{201}\)).
summer and winter solstices; see section 1.3.4.6.1.4 Circannual rhythm] are reported as being 100% and 95%, respectively 189.

Discordance has been found between the results of DST, endogenous ACTH concentrations and clinical signs in some horses 137, although generally ACTH concentrations correlate well with histopathologic findings 170.

1.3.4.6.1 Influences on ACTH concentrations
1.3.4.6.1.1 Sex
No association with sex was found when ACTH was measured on several occasions in a group of ponies and horses 183.

1.3.4.6.1.2 Age
Several studies have demonstrated an effect of age on ACTH 180,183,202,203. The cause of this is unknown but in some cases may include horses with subclinical PPID 180. The effect is generally thought to be weak, however 183 and in one study, no horse was classified as having PPID despite increasing ACTH concentrations with age 204. In the Donaldson et al 183 paper, some of the ACTH concentrations of animals in autumn are suggestive of subclinical PPID and may represent improper categorisation. Other studies have not found an association with age 64,205,206. Thus, overall, any effect of age may relate to subclinical PPID or alternatively is unlikely to be diagnostically relevant.

1.3.4.6.1.3 Breed
Some studies have found, when controlled for time of year, that there is no difference between horses and ponies with regard to ACTH concentrations 66,183. Other research has shown that normal ponies may have lower 64 or higher 151 concentrations. The latter study did not discriminate between PPID-affected and normal animals, however. As ponies have been shown to have a higher prevalence of PPID 26, this may confound the breed-effect on ACTH concentrations. Ponies may have a more pronounced ACTH circannual rhythm, however 133.
1.3.4.6.1.4 Circannual rhythm

Possibly the most clinically relevant modifier of ACTH concentrations is that related to time of year. Indeed, a circannual rhythm of ACTH has long been identified\textsuperscript{136,180,183,203,207,208}. More recent research has identified that the increase in ACTH tends to occur around the time of the summer solstice, peaks at the autumn equinox and falls again thereafter\textsuperscript{133,209,210} [hitherto defined as the dynamic phase of the circannual ACTH cycle\textsuperscript{210}]. While the mechanism of this change is unknown, it seems related to the negative change in daylight hours, rather than actual hours, \textit{per se}\textsuperscript{133,209}. During this time of year, basal ACTH concentrations in PPID horses are actually more separated from normal horses than at other times of the year\textsuperscript{211}. As such, the diagnostic accuracy of the test improves, with sensitivity and specificity reported as being 100% and 95%, respectively\textsuperscript{189}. Conversely, a relatively quiescent phase is apparent when endogenous ACTH concentrations are lower and display reduced variability compared to the rest of the year, and occurs between the winter and summer solstices\textsuperscript{179,210}. It should be noted, that there may exist a ‘grey zone’ where the basal ACTH concentrations of early PPID and normal horses may overlap\textsuperscript{179,211,212}.

1.3.4.6.1.5 Location

Several studies have demonstrated effects of latitude on ACTH concentrations, particularly when taking seasonal changes into account\textsuperscript{185,189,211,213}. In general, these studies have shown that ACTH concentrations during the quiescent phase of the circannual ACTH cycle are similar regardless of location. However, during the dynamic phase, the mean ACTH concentrations tend towards higher levels in latitudes closer to the equator\textsuperscript{185,189,204,210,211}, although exceptions have been seen\textsuperscript{204}.

1.3.4.6.1.6 Feeding and diet

The effects of feeding on ACTH concentrations are controversial. Diez de Castro et al\textsuperscript{214} found that fasting increased resting ACTH concentrations. However, in that same study, when replicated in a larger client-owned population, the differences were less obvious. In other
species, feed-associated increases in ACTH concentrations are mediated via alpha-1 adrenergic responses, probably from vasoactive intestinal peptide\textsuperscript{214}. Other studies have found that feeding did not result in significant differences between basal ACTH concentrations when performed as part of a TRH-stimulation test\textsuperscript{215} or as standalone tests\textsuperscript{216}. Type of baseline diet may also affect ACTH concentrations with a starch-rich diet associated with higher resting ACTH concentrations in comparison to an isocaloric fiber-rich diet\textsuperscript{203}. The latter finding has been replicated in a more recent study that showed that starch and sugar diets resulted in higher ACTH concentrations particularly in aged horses\textsuperscript{203}.

1.3.4.6.1.7 Exercise

Intense exercise has been shown to increase ACTH in the short-term\textsuperscript{193,217}. The physiologic drive of this rise in ACTH is unknown. The increase in ACTH concentrations is short-lived\textsuperscript{218}, however, and does not occur with lower intensity exercise\textsuperscript{217}. There is evidence that it may be related to exercise-induced rises in AVP rather than CRF\textsuperscript{193}.

1.3.4.6.1.8 Sickness

Painful and stressful conditions have been shown to increase ACTH concentrations in horses\textsuperscript{219}. This study used a \textbf{radioimmunoassay (RIA)} to determine ACTH concentrations in various grouping of horses including laminitis, acute abdominal syndrome and acute or chronic diseases. All these groups had statistically significant increases in ACTH concentrations. However, the concentrations were within previously published reference intervals using that methodology\textsuperscript{220}. Another study of sick, hospitalized horses demonstrated illness-dependent effects on ACTH concentrations\textsuperscript{221}. In that study, severely ill horses had markedly elevated ACTH concentrations on admission (using an RIA as above) while moderately or mildly ill horses had smaller increases in ACTH concentrations. These concentrations had significantly reduced by day 6 of hospitalization\textsuperscript{221}. It is unknown what influences these increases in ACTH concentrations, although noradrenaline was increased in one study in conjunction with ACTH\textsuperscript{219} while some horses appeared to have critical illness-related corticosteroid insufficiency\textsuperscript{221}.  

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1.3.4.6.1.9 Handling

Stress has been shown to increase the amplitude of ACTH secretion from the pituitary gland, although not the frequency\textsuperscript{195}. Stress associated with short-\textsuperscript{206} and long-distance\textsuperscript{222} transport can also affect ACTH concentrations. In particular, it has been shown that horses that have less experience being transported, and that are less well handled, have higher increases or basal ACTH concentrations, respectively\textsuperscript{206}. It should be noted that while the time of year was not stated, the highest ACTH concentration in that study was 26 pg/mL.

Isolation stress, which has a clinically obvious effect, can cause marked increases in ACTH secretion from the pituitary gland, although this was only observed when marked AVP release was also observed\textsuperscript{194}. Furthermore, this AVP increase has been seen within the first minute of isolation stress\textsuperscript{194}.

Venepuncture has also been evaluated as a cause of elevated ACTH concentrations in the absence of disease\textsuperscript{223}. This study showed that while ACTH concentrations were elevated in many cases after venepuncture, it is unlikely to alter the diagnostic accuracy of the test in most cases\textsuperscript{223}.

1.3.4.6.1.10 Multiple samples

There is controversy about the clinical significance of the ultradian rhythm of ACTH secretion. ACTH is released from the pituitary in a pulsatile manner, with approximately 10 peaks detected per hour when sampled directly from the pituitary circulation\textsuperscript{190}. There is research that has shown that these pulses are not detected at peripheral blood level, however\textsuperscript{191}. Researchers in the USA noted duplicate samples and samples taken at 5 minute intervals could vary by greater than 50 pg/mL in PPID horses, with control horses having generally much lower variability\textsuperscript{180}. Other more recent research found that in most cases, from a clinical point of view, duplicate or paired samples are not required in most cases\textsuperscript{212,224}. Similarly, the clinical relevance of a peripherally detected circadian rhythm is controversial, with studies finding no differences in
ACTH concentrations between times of day \(^{214}\) and others finding differences that are unlikely to be clinically significant \(^{159,224}\).

**1.3.4.6.11 Drugs**

Opioids seem to inhibit ACTH release through a non-CRF factor \(^{225}\). Inhibition of opioids thus leads to increased ACTH concentrations \(^{225}\). Insulin administration, apart from causing hypoglycaemia, leads to increased CRF, ACTH and AVP release, i.e. activation of the HPA axis \(^{226}\). Interestingly, the lower the glucose concentration in that study, the higher the ACTH and cortisol concentrations. Dexamethasone has also been shown to decrease endogenous plasma ACTH concentrations, probably through a negative feedback approach \(^{227}\). That group demonstrated that ACTH concentrations were suppressed for up to three days after a 10 day course of topical dexamethasone.

**1.3.4.6.12 Unexplained elevations**

There are reports of ACTH concentrations varying markedly in horses with or without PPID \(^{180}\). One study found that basal ACTH concentration can actually vary in some horses by up to 50 pg/mL \(^{180}\) - this occurred more commonly in PPID horses than in normal horses. Several other papers have also demonstrated occasional, unexpected elevations above previously published reference intervals in otherwise healthy horses \(^{212,228}\) or horses undergoing control experiments \(^{229}\).

**1.3.4.6.13 Stability**

Recent research has shown that ACTH is stable at room temperature for between three and eight hours \(^{64,230,231}\), but degrades even if refrigerated between eight to 24 hours post-collection \(^{230,231}\). From both of these studies, it appears that in most cases the decrease at 24 hours would not lead to erroneous classification. It appears to remain stable when frozen \(^{231}\). Centrifugation (vs gravity separation) and additives do not appear to make a significant short-term difference \(^{230}\), although separation from red blood cells is important after eight hours \(^{231}\). There may be differences between the stability of ACTH from PPID horses in comparison to normal horses \(^{230}\). Several
studies in humans have also identified that human ACTH plasma concentrations remain stable either with or without aprotinin for up to 72 hours when refrigerated 232,233.

1.3.4.6.14 Insulin sensitivity
In contrast to the data on the effect of PPID on ID, there is little data on the effect of ID on ACTH concentrations. One study suggested that there was no effect of ID on ACTH concentrations in 12 mares 229.

1.3.4.6.15 Assay
There are several assays available for the measurement of equine ACTH. There is a CLIA that is commercially available in Australia and validated for horses 200. An alternative assay is an RIA which has also been validated for horses 64. The latter is reported to have different reference intervals to the CLIA 220. Banse et al 234 compared both assays and found that there was poor agreement between the tests. As such, results should not be considered comparable. Those authors also suggested that the ACTH assay may measure more than the functional ACTH1-39 fragment, leading to false positives 234. Knowles et al 235 reported a large positive bias using the CLIA in autumn in non-laminitic ponies in comparison to an immunoassay. Significant differences were not observed in spring. The authors suggested that cross-reactivity with circannually variable POMC-derivatives, such as CLIP, might be responsible for this difference. Whether this non-specific cross-reactivity explains some of the spurious elevations observed is unknown.

1.3.4.7 TRH stimulation test
The TRH-stimulation test (measuring ACTH) is a development of the now superseded TRH-stimulation test of cortisol 236. The latter suffered from variability associated with cortisol measurement 4 and so is no longer recommended. TRH receptor mRNA can be detected in both the pars intermedia and the pars distalis of the equine pituitary gland 199. The end-products of this stimulation in healthy horses, however, are α-MSH and ACTH, respectively 199. When TRH was injected in post-mortem confirmed normal horses, ACTH concentrations at 10 minutes post injection were all <81 pg/mL 180. In clinically normal horses with pituitary hyperplasia, however,
values can exceed this\textsuperscript{158,180}. The clinical significance of the latter finding is unconfirmed but may relate to earlier stages of PPID, given the correlation between basal ACTH and histopathologic findings\textsuperscript{170}. Given the circannual variability in pars intermedia size\textsuperscript{169}, it may be that the increased variability of post-TRH ACTH concentrations relates to increased variability of TRH receptor expression. Others have also suggested individual differences in the secretion of various POMC derivatives\textsuperscript{237} which may explain some of the variability.

The current cut-off of 100 pg/mL at 10 minutes was derived from Beech et al.\textsuperscript{180} although the methodology for generation of this cut-off from the data is unclear. That paper originally described the 30 minute time-point as not being statistically different from baseline, and thus used a cut-off of 35 pg/mL\textsuperscript{180}. It should be noted that that lab uses a different methodology and reference interval\textsuperscript{236} to the work presented herein. More recently, 60 or 65 pg/mL at 30 minutes were suggested\textsuperscript{238}. Again, however, the source of this cut-off is not clear from the available data.

While the concentrations of ACTH obtained 30 minutes after TRH injection in the earlier papers fall under 65 pg/mL\textsuperscript{180}, it is unclear what statistical analyses were performed to obtain this cut-off. ACTH concentrations increase in response to TRH in all horses, although the responses are markedly greater in PPID horses, both with regards to duration and magnitude\textsuperscript{180}. Overall, it is assumed that due to the maximal responses of ACTH to TRH administration within the first few minutes post-injection, an arbitrary time point of 10 minutes was chosen to be practical and standardized.

Previous literature has used 36 pg/mL at 30 minutes as an ACTH concentration cut-off at 30 minutes\textsuperscript{158,180}. This results in a sensitive test (88%), although in that study, 16/60 TRH-stimulation tests in clinically normal horses were considered positive (although 3 of these tests occurred during autumn), including 2/23 in histologically confirmed normal horses\textsuperscript{158}. This would likely lead to false positive diagnoses which has implications regarding potentially lifelong treatment for positive horses. When these histologically-normal horses had α-MSH measured at the same time (30 minutes post-TRH administration), the concentrations were considered normal.
1.3.4.7.1 Influences on the TRH-stimulation test

1.3.4.7.1.1 Effect of dose of TRH

When using a blanket dose of TRH (1mg) there was no evidence to suggest an influence of body mass, condition score or breed (horses vs. ponies) \(^{180}\). This dose has also been considered as saturating or near-saturating for the pituitary gland in vivo \(^{229,239}\). Furthermore, 1mg is accepted as the standard dose regardless of weight \(^{180}\) and, more recently, 0.5mg has been shown to give similar results to 1mg \(^{240,241}\).

1.3.4.7.1.2 Effect of feeding

There is literature showing that fasting prior to TRH-stimulation testing may result in recategorization of horses (i.e. PPID or normal), although statistically, overall, there was no significant difference \(^{215}\). However, one horse had a discrepancy of 82 pg/mL \(^{215}\) – other causes of this bias cannot be excluded.

1.3.4.7.1.3 Effect of circannual rhythm

There is scant information available on the effect of time of year on TRH-stimulated ACTH concentrations \(^{180,214,242,243}\). Previous data in one study was log-transformed, from different horses and different disease categories, making interpretation difficult \(^{180}\). In another study \(^{242}\), the variability associated with the points of central tendency were not presented. In addition, the post-TRH stimulation testing ACTH concentrations during the dynamic testing time point was markedly lower than previously reported in other studies \(^{242}\). Haffner et al \(^{243}\) more robustly attempted to stratify horse results into two populations based on TRH-stimulation test results in autumn. Again, the variability is unclear from the information provided, as is the likelihood of the population with higher post-TRH ACTH concentrations having PPID \(^{243}\). Some authors recommend evaluating the test based on changes in daylight hours, rather than \textit{a priori} decided seasonal groupings \(^{180}\).
1.3.4.7.1.4 Lack of rises

There are reports of horses having atypical or lower than expected ACTH concentration rises to TRH administration. The significance of this is unknown but may relate to insulin sensitivity as in horses that were insulin resistant there was no substantial ACTH concentration elevation at all.

1.3.4.7.1.5 Concurrent testing

Concurrent testing with the OST is not recommended at this time as the latter appears to affect the stimulated ACTH results, with a significant negative bias.
1.4 Equine Metabolic Syndrome

Traditionally, EMS was defined as obesity or regional adiposity (Figs. 1.7, 1.8), laminitis or a history of laminitis, and insulin resistance/dysregulation. More recently, the roles of dyslipidaemia, systemic inflammation, hypertension and infertility have been better defined in this disease. Many animals with EMS are classified as ‘easy keepers’, with these animals able to maintain or even gain weight eating diets on which other breeds of comparable size may lose weight. In addition, many clinicians will diagnose this condition a priori - that is, before laminitis is ever diagnosed – in acknowledgement of the desire to identify at risk animals prior to laminitis development. Additionally, many clinical signs associated with insulin dysregulation such as body condition score, are not useful in non-obese but dysregulated animals.

Figure 1.7. Regional adiposity demonstrated by a cresty neck.
1.4.1 Epidemiology of EMS

It has been reported that ID can occur in up to 10% of horses\textsuperscript{253}, although another study in Australia has shown that the prevalence of resting hyperinsulinaemia may be as high as 28%\textsuperscript{110}.

1.4.1.1 Prevalence of obesity

The prevalence of obesity has been reported from several locations. In a survey of randomly chosen clients of first opinion practices in the UK, overall obesity was reported as 31%, with ponies being more frequently obese than horses\textsuperscript{254}. In Scotland, Wyse et al.\textsuperscript{255} found that 45% of horses were obese out of 319 pleasure horses. The authors identified that owners in this study tended to underestimate their horse’s body condition. In Virginia, USA, Thatcher et al.\textsuperscript{256} found that almost one third of 300 light breed, mature horses were overweight in summer, with almost one fifth being obese. Geor\textsuperscript{257} reported a prevalence of obesity of 19% an unpublished survey of
300 randomly chosen horses. Jaqueth et al.\textsuperscript{258} found that approximately 40% of horses and ponies in Maryland, USA are obese. In a recent longitudinal evaluation of laminitis incidence, the prevalence of obesity or greater than ideal bodyweight was 72%, with only 27% of animals being considered at ideal bodyweight.\textsuperscript{29} The prevalence in Australia has been reported as 24.5%, although ponies were overrepresented in that study.\textsuperscript{259} In keeping with owner under-recognition of laminitis, owners have also been found to underestimate their horses’ body condition.\textsuperscript{259} The prevalence of laminitis amongst EMS horses is unknown although Morgan et al.\textsuperscript{110} found an prevalence of 12% amongst ponies with hyperinsulinaemia.

1.4.2 Signalment

The signalment of EMS typically reflects that of ID [see section 1.4.6 Influences on insulin dysregulation].

1.4.3 Dyslipidaemia

Leptin is an adipokine that is excreted by adipocytes in a constitutive manner.\textsuperscript{260} It functions to indicate satiety (and hence energy balance) and is important in the maintenance of bodyweight.\textsuperscript{260} Leptin concentrations have been shown to increase with obesity.\textsuperscript{102,114,245} In addition, hyperleptinaemia is associated with cellular insulin resistance as evaluated by the complete insulin response test (CIRT).\textsuperscript{100} In that study, obesity was not associated with hyperleptinaemia.\textsuperscript{100} Similarly, in horses with high body conditions, leptin concentrations were associated with insulin concentrations and the insulin response to glucose, but not all obese horses in that study were hyperleptinaemic.\textsuperscript{246} Hyperleptinaemia has also been associated with obesity and ID as evaluated by the CGIT.\textsuperscript{261} and insulin concentrations post-TRH, vehicle or sulpiride administration.\textsuperscript{229} However, from a patient-oriented endpoint, leptin has not been associated with laminitis in a clinical cohort study.\textsuperscript{29}

Adiponectin is a cytokine secreted from adipose tissue that is anti-inflammatory and protective against IR.\textsuperscript{117} Adiponectin is negatively correlated with body mass.\textsuperscript{262,263} IR \textsuperscript{245,263} and laminitis
and this is true independently of leptin concentrations. There is some clinical evidence showing that adiponectin might not always be associated with body morphometry.

**Triglycerides (TG)** are involved in lipid storage and energy utilisation. One study has demonstrated excellent specificity of elevated triglyceride concentrations at discriminating between clinically laminitic ponies and healthy ponies. Triglycerides were also used as part of a scoring system that had a positive predictive value of 78% in differentiating previously laminitic from normal ponies. A curvilinear relationship between BCS and TG concentration was also found by a group of researchers in Australia, while increased TG concentrations have been identified in previously laminitic ponies in a clinical longitudinal study. The authors in the latter study suggest that due to overlap between groups, that TG concentration may not be useful as a diagnostic test. Hypertriglyceridaemia has also been reported from clinical cases of ID, albeit with no evidence of associated clinical disease.

### 1.4.4 Systemic inflammation

Elzinga et al. found that EMS horses have increased concentrations of inflammatory markers including interferon gamma (IFN-γ), interleukin 6 (IL-6), interleukin 1 beta (IL-1β) and tumour necrosis factor alpha (TNF-α) 60 minutes after oral CHO challenges. Burns et al. evaluated mRNA expression from various adipose tissues in both insulin resistant and insulin sensitive horses. They found that there was increased mRNA expression for IL-6 and IL-1β in nuchal ligament adipose tissue; however, there was no difference between insulin sensitive or resistant mares. Thus, while the nuchal ligament adipose tissue may be more likely to have a pro-inflammatory profile, the significance as it pertains to ID is unknown. Higher concentrations of serum amyloid A, a positive acute phase reactant, have been associated with both obesity and insulin concentrations in mixed light breed horses. The same study failed to identify an association between obesity and other inflammatory markers, however. Increased TNF-α concentrations have been associated with IR, although there was no difference in other inflammatory markers in two studies of previously laminitic and normal ponies. In the Wray et al study, the authors postulate that one reason for not identifying significant differences
between groups may relate to the strict management of the previously laminitic ponies aimed at reducing its recurrence.

1.4.5 Cardiovascular changes

Equine metabolic syndrome has also been associated with effects on the cardiovascular system. In one group of age- and body condition-matched ponies, ID was associated with hypertension during summer \(^{249}\). Conversely, clinically laminitic ponies have been shown to be relatively hypotensive, in comparison to non- and previously-laminitic ponies \(^{42}\). The cause of this phenomenon is unknown, although there was considerable overlap in blood pressure values between groups and this was a relatively closed population. However, laminitic ponies were also shown to have lower blood pressure than normal ponies after insulin administration \(^{269}\), which may support this phenomenon. While these ponies did not have ‘gold-standard’ assessment of their ID status, they did have versions of insulin response tests, with all laminitic ponies having reduced glucose responses to insulin injection \(^{269}\). More recently, myocardial hypertrophy has been identified in ponies with EMS and with relative cardiac wall thickness correlated with serum insulin concentrations \(^{270}\).

1.4.6 Influences on insulin regulation

1.4.6.1 Season

There is conflicting literature on the effects of season on insulin sensitivity and regulation. Banse et al \(^{39}\), Bailey et al \(^{249}\) and Place et al \(^{207}\) found no significant difference in basal insulin concentrations between seasons whereas Beech et al \(^{133}\) found higher insulin concentrations in summer and Hart et al \(^{139}\) and Wray et al \(^{94}\) found higher concentrations in spring. Borer et al \(^{99}\) found that insulin peaks were higher and more variable during autumn, and that ponies were more insulin resistant during winter \(^{271}\). Beythien et al \(^{272}\) recently found seasonal changes in insulin concentrations in response to a modified in-feed glucose test (IFGT) during summer while Funk et al \(^{273}\) found no significant seasonal differences in combined glucose-insulin tolerance test (CGIT) results in normal horses. Using the HEC, Fitzgerald et al \(^{274}\) found that horses became more insulin resistant in winter. Turner et al \(^{275}\) evaluated fasting insulin concentrations at various
times during the year and found higher concentrations in summer, however the horses in that paper were exercised in the non-summer months, and thus its effects may be responsible for the apparent circannual differences observed in that paper. DeBoer et al \(^{276}\) found higher insulin and glucose responses to cool-season grass ingestion in autumn and spring. Schreiber et al \(^{204}\) found a poor overall seasonal response, although individual animals had more variability in autumn and early winter, with one horse have markedly elevated concentrations during this time, and low concentrations for the rest of the year.

When NSC concentrations have been evaluated concurrently with insulin concentrations, the seasonal patterns tend to correlate well in horses kept at pasture \(^{136}\). Thus, seasonal changes in insulin concentrations may be due to circannual changes in NSC concentration. This may account for variability between studies.

Regardless of any seasonal effects, it has been shown that there is variation in basal insulin concentrations from year to year amongst both normal and previously laminitic ponies \(^{181}\), as well as within a year in clinically normal geldings \(^{204}\).

Overall, while certain populations may have circannual fluctuations in insulin sensitivity, some of this change may relate to other factors such as pregnancy, pasture and feed NSC concentration, and PPID comorbidity.

### 1.4.6.2 Sex

A difference between sexes in insulin concentrations \(^{139}\) or insulin responses to a modified IFGT \(^{272}\) has not been identified.

### 1.4.6.3 Age

Age plays a role in ID. Insulin dynamics may follow a J-curve distribution in horses over their lives whereby a nadir occurs after the zero point, with a sharp rise in the curve thereafter. Standardbred foals have been shown to be relatively insulin resistant for the first few months of
life. As mature ponies age, insulin sensitivity, as tested by a version of the IFGT, tends to decrease, while the prevalence of hyperinsulinaemia increases. Similarly, insulin responses to sweet feed have been shown to be higher in older horses and insulin sensitivity as measured by the HEC is reduced in older mares. Nielsen et al found that older horses had higher post-prandial insulin concentrations, although the mature animals ate the meals more quickly, possibly confounding the results. Some have suggested that the rate of decline of insulin sensitivity may be affected by environment, diet, genetic or other factors, although much of this was supposition based on human data. Given that horses with PPID can be more insulin resistant than normal horses, and the strong association between age and PPID, it may be that PPID is a potential confounder in some cases. However, when PPID is controlled for, the relationship remains true. Furthermore, in Standardbred and Thoroughbred horses screened for PPID using the DST, age-related effects were found when ID was assessed using the FSIGTT, a modified OST and a dietary challenge model. In clinical cases, it thus may be important to separate PPID effects from true age-related effects. There are also data suggesting a lack of age effect on insulin concentrations.

1.4.6.4 Pain and disease
Several researchers have suggested that insulin is not significantly affected by stress and pain. It has been postulated that differences in IR testing (specifically a version of an insulin response test) were not due to pain or stress associated with laminitis, but were more likely to be associated with intrinsic metabolic differences. Similarly, hyperinsulinaemia in previously laminitic EMS ponies did not correlate with laminitic episodes. Additionally, in the oligofructose experimental model, Obel grade 3 laminitis was generally only associated with small, or no significant, increases in insulin concentrations. Similarly, in the hyperinsulinaemic model, Obel grade 2 laminitis was experimentally induced, without significant cortisol increases. In a study evaluating the diagnostic accuracy of basal insulin concentrations, no effect of current laminitis on insulin concentrations was observed. Thus, acute laminitis may not preclude testing of ID. Other stressful events such as nasogastric intubation have not been found to cause elevated insulin concentrations. Some authors dispute these findings, advising that
stressful events may affect ID testing using basal testing and dynamic testing such as the OST and two-step insulin response test (2SIRT). Others have also found effects of hospitalization on ID as measured by the FSIGTT, although diet may be a confounding factor in this study. Testing should be withheld in the case of severe systemic illness, as the infusion of endotoxin has been shown to dramatically lower insulin sensitivity, although that study was not formally published or peer-reviewed.

Transport and hospitalization are two potential stressors that may affect the assessment of ID as it pertains to testing. Bröjer et al evaluated the effects of novel stabling and transportation as a simulation of hospitalization and found no effect on either basal insulin concentrations or insulin curves as assessed by the CGIT.

1.4.6.5 Time of day
There is conflicting evidence for a circadian effect on insulin concentrations with one paper supporting an effect and another refuting it.

1.4.6.6 Pregnancy status
Pregnant mares have been shown to be more insulin resistant than non-pregnant mares up to 270 days of gestation, although after this date glucose disposal into the mammary gland is improved. Another study has also shown that insulin sensitivity (as measured by FSIGTT) is lower in mid-gestation in comparison to non-pregnant mares, and that this difference is accentuated with the feeding of a high NSC diet. Interestingly, mares did not become more insulin resistant in later pregnancy without a high NSC diet.

1.4.6.7 Breed and genetics
There is thought to be a genetic component to ID, as a presumed dominant trait with epigenetic influences. Recently, a genome-wide associated study found that a gene associated with cholesterol metabolism is associated with various phenotypic and clinicopathologic features of
EMS \textsuperscript{128}. It should be noted that genome-wide association studies, because of their inherent risk of a type-I error, are most at risk of irreproducibility \textsuperscript{1}.

With regards to breed differences, Standardbreds are widely thought of as being insulin sensitive \textsuperscript{96,97}. In addition, Quarter Horses are thought to be relatively insulin sensitive in comparison to other breeds \textsuperscript{39,40}. This appears to be exaggerated in those Quarter Horses who have polysaccharide storage myopathy (PSSM), a disease associated with insulin sensitivity \textsuperscript{290}. There are reports, however, of EMS-affected Quarter Horses, and their crosses \textsuperscript{139}. Ponies \textsuperscript{96,133,291,292} and Andalusians \textsuperscript{96} are considered relatively insulin resistant or dysregulated and this IR state was found in animals not previously adapted to a high NSC diet. Icelandic horses have been shown to be less insulin sensitive than Standardbreds \textsuperscript{251,286} while warmbloods are more insulin sensitive than ponies \textsuperscript{291}. Tinworth et al \textsuperscript{292} found that ponies were more insulin dysregulated than horses, although body condition was an important potential confounder in that study. Thoroughbreds are generally thought of as being relatively insulin sensitive \textsuperscript{278}, although they can certainly be susceptible to ID with (in)appropriate management \textsuperscript{72,74,293}. It should be noted that even Standardbreds have been shown to have cellular IR when tested by the FSIGTT \textsuperscript{294}. The breed difference does not appear to be related to differences in feed digestibility \textsuperscript{251}.

\subsection*{1.4.6.8 Obesity}

Obesity is commonly associated with ID (Fig. 1.9) \textsuperscript{24,47,72,73,139,251,261,268}. More than half of overconditioned ponies were insulin dysregulated as assessed by a modified IFGT by one research group \textsuperscript{47,295}. Using the HEC, obesity markedly reduces insulin sensitivity in comparison to lean animals \textsuperscript{296}, while similar findings have been found using the FSIGTT \textsuperscript{72}. In a survey of mixed, light-breed horses, obesity was positively correlated with body condition \textsuperscript{247}. In a survey of an inbred group of native UK ponies \textsuperscript{9}, those animals predisposed to laminitis were more likely to be obese and insulin dysregulated as assessed by proxy analysis. However, the association has been found to vary between populations. Obesity may lead to cellular IR through one or both of two mechanisms \textsuperscript{112}: adipokine-mediated downregulation of signalling pathways, or direct lipotoxicity.
due to their accumulation in insulin-sensitive tissues. There is some data suggesting that obesity has a greater influence on insulin sensitivity in younger mares in comparison to older mares\textsuperscript{268}.

1.4.6.8.1 Insulin dysregulation without obesity

There are several studies demonstrating that ID can occur in animals of normal body condition, depending on various factors\textsuperscript{72,74,249,297–299}. Bailey et al\textsuperscript{249} compared non-obese, age-matched ponies with or without previous laminitis, and demonstrated differences in insulin sensitivity based on the reciprocal of the square root of insulin (RISQI) and the modified insulin-glucose ratio (MIRG) despite their body conditions. Similarly, differences in insulin sensitivity between ponies having previously been diagnosed with laminitis and control ponies were identified when fed fructans or administered dexamethasone\textsuperscript{297}. In one study, previously laminitic ponies tended to be of leaner body condition than normal ponies, with neither group being obese\textsuperscript{181}. Interestingly, one group found IR, as measured by the HEC, at both ends of the body condition spectrum, including in underweight animals\textsuperscript{274}. Morgan et al\textsuperscript{110} found that, in a survey of 188 ponies, body condition was not associated with insulin status.

Postulated reasons for the development of ID in the absence of obesity include animals that have previously been obese and whose weight is being managed through diet and exercise\textsuperscript{298}, animals who have regional adiposity without obesity\textsuperscript{127,298}, and the effects of NSC on the enteroinsular axis in the absence of excess calorific intake\textsuperscript{72,74,297}.

1.4.6.8.2 Obesity without insulin dysregulation

There is also evidence that insulin dysregulation need not be present in all obese animals. Bamford et al\textsuperscript{102} induced obesity through high fat diets without inducing ID. It should be noted that the obesity in this study should was of a short-term nature, which may not fully allow for the development of IR over time. Quinn et al\textsuperscript{293} found that mature Thoroughbred geldings fed high NSC or high fat diets gained weight but only the high NSC diet induced changes in insulin sensitivity; this study was performed over a longer period than the Bamford et al study. Another paper found that there was no relationship between indices of obesity and ID\textsuperscript{39}. When 20 light
Figure 1.9. Putative relationships among hyperinsulinaemia, obesity, insulin resistance and the exacerbating factors of pregnancy, inflammation and PPID (modified from Frank and Tadros 2014). 

Breed horses were evaluated for IR based on the FSIGTT, obese but insulin sensitive horses were identified. When a high fat diet was used to induce weight gain in Standardbreds, no change in insulin sensitivity measured by the HEC was observed, although these effects should be repeated in laminitis-susceptibility animals. In a study comparing insulin concentrations to CGIT results, no effect of obesity was observed. A cross-sectional survey also found no significant
association between obesity and basal insulin concentrations in light breed horses. Perhaps most clinically relevant, in a prospective, longitudinal study of 446 ponies, body morphometry was not associated with the risk of developing laminitis over three years.

1.4.6.9 Exogenous and endogenous glucocorticoids

Exogenous steroid administration has been shown to result in ID. In one paper examining the effects of topical dexamethasone therapy on various hormone concentrations, a relatively conservative dexamethasone dose (17mg total per day) lead to markedly elevated insulin concentrations (>300 μU/mL) that persisted for several days post-administration. These Thoroughbreds had surprisingly high basal insulin concentrations, however, and so there may have been an element of underlying ID. In addition, they were fed concentrates (composition unknown) three times daily which may have induced the condition. Regardless, steroid administration has been shown to induce ID in breeds considered insulin sensitive. Another paper evaluating IR in non-obese ponies demonstrated ‘unmasking’ of an insulin resistant phenotype after dexamethasone administration as part of a DST. Brennan and Urschel demonstrated that a 21 day course of oral dexamethasone resulted in elevated insulin concentrations for three days after dexamethasone cessation and abnormal proxy indices for between four and 15 days while Tiley et al described increased insulin concentrations during a course of the same in healthy Standardbreds on a low NSC diet. Unfortunately, that paper did not evaluate the duration of the effect after cessation of the medication. Even a single dose of dexamethasone leads to ID as assessed but the CGIT, although the effect typically lasts less than three days.

The relationship of endogenous glucocorticoids, outwith that investigated with regards to perceived external stress, has not been fully elucidated. There is evidence showing that while total cortisol concentrations between healthy and hyperinsulinaemic (both PPID and EMS) horses are similar, free cortisol fractions are higher in the latter group. The authors hypothesized that this may be directly related to obesity (as either a cause or effect) or may be due to reduced cortisol-binding globulin concentrations.
associated with mild inflammation. However, that study found a stronger association with body condition than ID, and so its relevance to the development of ID in PPID or EMS is unclear. Others have confirmed the lack of a relationship between total cortisol concentrations and EMS status 207.

The mechanism of ID development secondary to glucocorticoids is incompletely defined. Glucocorticoids counter insulin’s effects by altering GLUT-4 membrane expression peripherally (leading to cellular IR), as well as stimulating hepatic gluconeogenesis, leading to hyperglycaemia and subsequent hyperinsulinaemia 139. Altered 11-β-HSD1 activity in PPID or EMS may also lead to altered total or free cortisol concentrations, further leading to ID 139.

1.4.6.10 Sepsis
Infusion of lipopolysaccharide (LPS) is a reliable method for inducing ID, with a 75% reduction in insulin sensitivity as measured by the FSIGTT demonstrated 24 hours after its infusion into lean, healthy horses 306. A second study identified similar changes 20 hours post-LPS infusion 284. Another study has replicated this effect, as measured by the HEC 307, with a potentially biphasic response reported. When LPS infusion has been performed in both healthy and EMS horses, greater reductions in insulin sensitivity were identified in EMS horses 308. As in interesting aside, in the Tóth et al. study 284, pretreatment with thyroxine, a commonly prescribed medication for EMS in certain parts of the world, mitigated the effects of LPS on insulin sensitivity. The clinical significance of this is unknown however, as few septic patients would be concurrently pretreated with thyroxine, and an immediate effect of treatment may not occur.

1.4.6.11 Diet
While obesity has been associated with insulin dysregulation [see section 1.4.6.8 Obesity], it may be that diet is a more important factor in the development of ID in genetically predisposed animals.
The feeding of NSCs can cause both obesity\textsuperscript{73,245,293} as well as ID\textsuperscript{72–74,78,281,297,300}. When horses or ponies are fed high calorie diets consisting primarily of fat and fibre (and thus low in NSCs), these animals become obese, but do not develop ID\textsuperscript{245,293}. If they are exercised and thus remain the same body mass, the results are similar\textsuperscript{309}. Similarly, when ponies are randomized to isoenergetic fat and fibre- or oat-supplemented rations to which they are acclimatised, higher glucose and insulin responses are seen in the oat-supplemented ponies\textsuperscript{310}. Arabian horses fed isocaloric diets rich in either fat and fibre or sugar and starch gained weight on the sugar and starch diet, potentially related to the metabolic effect of high glycaemic feed\textsuperscript{311}. When previously laminitic ponies were changed from pasture to hay diets, basal insulin concentrations reduced significantly\textsuperscript{181}. Similarly, in an experiment evaluating the effects of obesity and diet on insulin sensitivity, when obesity was induced in mature Arabian horses through the feeding of high NSC diets, the horses became insulin resistant\textsuperscript{73}. These geldings then had their obesity maintained through the feeding of low NSC hay but their ID status was not different to baseline (i.e. it improved with the withdrawal of high concentrations of NSCs in the diet)\textsuperscript{73}. The feeding of fructans leads to a marked increase in IR in previously laminitic non-obese ponies, but not significantly so in ponies with no history of laminitis\textsuperscript{297}. When hay with a high NSC concentration was fed to both normal and PSSM-affected horses, higher insulin concentrations were observed than when a low NSC hay was fed\textsuperscript{290}. One confounding factor in the latter study was that the high NSC hay was eaten more quickly than the low NSC hay. Others have also confirmed that hays of different types induce different insulin and glucose responses\textsuperscript{276}. Another study evaluating the effects of high NSC diets on insulin receptor expression revealed no difference between basal insulin concentrations in both lean and obese ponies, but did reveal an increase in both groups within seven days of beginning the high NSC diet\textsuperscript{81}. Importantly, the control ponies (both lean and obese, and kept on a high fat and fibre diet) had no significant increase in basal insulin concentrations over the same period\textsuperscript{81}. It should be noted that four ponies in this study developed clinical laminitis after ingestion of the high NSC diet, but the oligofructose concentration ingested was lower\textsuperscript{81} than that previously used to induce laminitis through caecal dysfunction\textsuperscript{48}. The effect seems to be somewhat variable between studies, however, with one study demonstrating only trends towards IR as determined by the FSIGTT\textsuperscript{84}. This reduction of
insulin sensitivity can occur after the feeding of high NSC diets for as little as four to six weeks.

The opposite has also been seen – that a reduction in dietary NSCs can lead to improved insulin sensitivity or insulin responses. In an experiment evaluating the effects of exercise on insulin sensitivity and bodyweight in ponies, dietary modification alone resulted in comparable weight loss and improvements in insulin responses to a modified IFGT. Similarly when previously laminitic ponies are fed hay instead of high NSC grass, their insulin concentrations lower to being clinically similar to ponies with no previous history of laminitis. When Standardbreds were moved to pasture after having gained weight with a high fat diet, their insulin concentrations decreased. However, the change in housing (and thus potentially exercise) may have confounded these results. Alternatively, as no further weight gain occurred whilst at pasture, the diets may not have been isocaloric. It is estimated that a 50% reduction in dietary NSCs would result in a 25% reduction in insulin responses.

Dietary sugar has been found to reduce insulin sensitivity as assessed by FSIGTT or HEC even in relatively insulin-sensitive breeds, such as Thoroughbreds and Standardbreds, respectively. This effect appears to be repeatable when using basal glucose and insulin concentrations in Thoroughbred mares fed high-starch diets long-term. Interestingly, dietary influences on insulin sensitivity appear to be more influential than that of breed.

Glucose bioavailability has also been shown to be higher in insulin dysregulated ponies in comparison to normal ponies. Given the relative importance of glucose-mediated insulin release in horses, this may explain some of the breed susceptibility differences observed. In addition, it has been shown that GLP-2 is increased in ponies with ID, and this incretin may have intestinotropic effects which might explain the increased glucose bioavailability.
Fasting can lead to decreased insulin sensitivity in as few as 3 to 8 hours, when evaluated as part of basal or dynamic ID testing, although one study found no effect of prior fasting on insulin concentrations.

Dietary fat may have some modulating effects on insulin dysregulation, aside from its ability to provide energy in a low-CHO diet. Fish oil added to the diet of exercising Thoroughbreds and Standardbreds lead to trends towards lower insulin and glucose concentrations, although the results were not statistically significant. Additionally, in another study of exercising horses, dietary fat reduced insulin concentrations when combined with exercise, although not at rest. Psyllium has been reported to have some modulating effects on insulin concentrations, despite having almost 25% NSC. The study may be misleading, however, as the control diet was grain-based with approximately 43% NSC.

1.4.6.12 Biologic variability
Borer et al noted considerable variation of basal glucose and insulin concentrations when assessed during different seasons with different diets, with AUC of insulin over 60 hours varying by more than six-fold in normal ponies.

1.4.6.13 Exercise
Several postulated mechanisms for improved insulin sensitivity associated with exercise have been proposed. These include a) a reduction in muscle and liver glycogen; b) increased insulin receptor binding of insulin; and c) increased GLUT expression in skeletal muscle leading to increased glucose uptake peripherally.

However, in vivo, the effects of exercise on measures of ID are controversial. Exercise has been shown to lower insulin concentrations, although the decrease persisted only for the day of exercise in one study, and for less than that in another. Conversely, when evaluating insulin responses to an IFGT, reduced responses were seen when ponies were exercised five days per week for six weeks, with some of these improvements lasting for four weeks after cessation of
exercise. Turner et al. confirmed that as little as 15 days of exercise can reduce fasting insulin concentrations, although paddock turnout alone is unlikely to be effective. Similarly, when evaluated using the HEC, obese and non-obese mares had improved insulin sensitivities that persisted for up to nine days after cessation of exercise. Menzies-Gow et al demonstrated that post-exercise spikes in plasma insulin concentration were less marked after 14 days of low-intensity exercise in non-laminitic and previously laminitic ponies, as well as finding an acute reduction during exercise. Insulin sensitivity has been shown to improve during moderate intensity exercise in endurance horses, over and above the effects of diet. The same group has also found similar results in Thoroughbreds despite weight gain, and mixed, unknown breeds of horses at both low and moderate intensities of exercise. Exercise may also be able to mitigate some of the IR induced by a high NSC diet with weight gain. A recent paper described the use of a novel feeding device designed to increased daily exercise and showed that weight loss was improved in those ponies that used this device. However, in keeping with the literature on diet and obesity, while exercise may reduce bodyweight over time, it is unlikely to lead to additional improvements in insulin regulation status over a low NSC diet alone. There is also literature demonstrating no effect of exercise on insulin dynamics.

1.4.7 Testing of insulin dysregulation

1.4.7.1 Basal insulin

Basal hyperinsulinaemia, particularly after fasting, is generally considered specific for ID at commonly used cut-offs. While in many populations it is considered insensitive, there are data demonstrating that resting hyperinsulinaemia (>32 μU/mL) has been 100% sensitive for predicting future laminitis occurrences when exposed to high NSC pastures. This study used pony breeds considered at risk of ID, however, and may not be representative across all populations. A more recent Australian study demonstrated that basal insulin concentrations (after 12 hours of fasting) greater than or equal to 8.5 μU/mL were associated with the development of laminitis in 75% of ponies subsequently exposed to high NSC. Limitations of using basal insulin concentrations to detect ID mostly reflect its relative insensitivity as well as intrahorse variability. When compared to the CGIT, insulin concentrations have
a sensitivity of 16.5% \(^{44}\). An ROC in that study identified an insulin concentration of 3.03 μU/mL as being the most diagnostically accurate. In this author’s opinion, that cut-off is clinically impractical. It should be noted that this paper used a CLIA that has been shown to have a negative bias in comparison to the more commonly reported RIA \(^{323–325}\) [see section 1.4.7.5]. It has been also shown that single, random, non-fasting serum insulin concentrations are not necessarily an accurate reflection of an individual’s insulin sensitivity \(^{93,280}\).

There are various influences on endogenous insulin concentrations that may confound resting insulin concentrations, as has mostly been described in the previous section (see section 1.4.6 Influences on insulin dysregulation). Briefly, several factors that have been shown to affect basal insulin concentrations include the following: A high fat diet has been shown to reduce insulin concentrations in exercising, but not resting, Thoroughbreds \(^{316}\). Metformin has been shown to reduce fasting insulin responses \(^{305,326}\), although this effect is not necessarily repeatable when assessed using the FSIGTT \(^{327}\). In Thoroughbreds fed high NSC or fat and fibre diets for eight weeks, basal insulin concentrations did not differ between groups, despite differences in insulin sensitivity as measured by the FSIGTT \(^{72}\). Insulin concentrations are often associated with body condition score and tend to be higher in obese animals \(^{268}\). Insulin has also been correlated with neck circumference, as a proxy for regional adiposity \(^{261}\). Insulin sensitivity as assessed by the FSIGTT has been found to be lower in obese Thoroughbreds \(^{72}\). Others have found no effect of obesity on either insulin concentrations or results of the CGIT \(^{44}\).

1.4.7.2 Insulin proxies

Several proxies for insulin sensitivity have been developed based on basal glucose and insulin concentrations. These include the MIRG, RISQI and the quantitative insulin sensitivity check index (QUICKI) \(^{70}\). The former is used to estimate the beta-cell insulin response to glucose while RISQI is used to estimate insulin sensitivity \(^{70}\). Unfortunately, these proxies cannot accurately differentiate between previously laminitic and normal ponies with only small differences between, and large variability within, groups \(^{271}\). Other researchers have also found unacceptable variability \(^{322}\). When these proxies were compared with the HEC as a ‘gold standard’ \(^{328}\), fair
correlation, at best, was identified (with an $R^2$ of 0.71 in the best comparison). This is similar to the comparison with the FSIGTT in the original paper of their description, where $r = 0.77$ in the best comparison of RISQI to insulin sensitivity \(^70\). In addition, the proxies were not able to accurately predict insulin sensitivity changes when a subgroup of Standardbreds underwent weight gain \(^328\). Other researchers have described the use of ratios: the insulin: glucose estimates the quantity of insulin per glucose concentration while the glucose: insulin estimates the effect of insulin on glucose concentrations \(^280\). In summary, proxies are no better than the non-proxied variables from which they are derived \(^42\).

1.4.7.3 Tests of cellular insulin resistance

1.4.7.3.1 Hyperinsulinaemic euglycaemic clamp test

The HEC is referred to as one of the ‘gold standards’ for assessing insulin resistance \(^135,329\). A modified version has also been used to induce laminitis \(^62,95\). The HEC involves the administration of supraphysiologic concentrations of insulin (typically 45 µU/kg followed by 6 µU/kg/min \(^135\)). Glucose is measured frequently, and a constant rate infusion of glucose administered to maintain euglycaemia. Once a steady state is achieved, insulin concentrations are measured on several occasions. The quantity of glucose administered is used to calculate the glucose metabolism rate, on the assumption that the high insulin concentrations suppress endogenous insulin release \(^330\), and accounting for urinary glucose loss \(^135,291\). The glucose metabolism rate to insulin concentration ratio is also calculated. The HEC is not considered a clinically practical test and is used primarily for research. It is considered more repeatable than the FSIGTT in horses \(^330\). However, if glucose concentrations are not effectively ‘clamped’ in steady state, results may not accurately represent the true glucose disposal rate \(^329\). Furthermore, higher insulin concentrations lead to lower measured insulin sensitivity \(^111\) and so studies using the HEC should be critically evaluated prior to their comparison.

1.4.7.3.2 Insulin-modified, frequently sampled IV glucose tolerance test

The FSIGTT is sometimes considered the alternative ‘gold standard’ method of determining insulin sensitivity \(^321\). When used with minimal model analysis \(^72,311,331\), the FSIGTT has theoretical
advantages over the HEC: it estimates insulin-dependent and -independent glucose utilization as well as pancreatic beta-cell function. It has been argued that it is technically more straightforward than the HEC as glucose infusion rates do not have to be continually adjusted. The FSIGTT involves collecting basal samples and then administering a known quantity of glucose by IV infusion (typically 0.3 g/kg). Samples are collected every 1-3 minutes until 20 minutes, at which time insulin is administered IV, typically at 30 µU/kg, with continued frequent sampling until 180 minutes. Measured variables include glucose and insulin concentrations. Calculated variables include insulin sensitivity, glucose effectiveness, the acute insulin response to glucose and the disposition index. This test is also considered a research tool rather than a clinical tool. It should be noted that the FSIGTT is an indirect measure of insulin sensitivity.

The HEC and FSIGTT have been shown to give results that correlate, but do not necessarily agree. It should be noted that some have argued that neither the HEC or FSIGTT are necessarily appropriate for determining ID status. Some authors have argued that the tests are not true representations of physiologic processes as the glucose kinetics are in response to typically supraphysiologic concentrations of insulin. While the tests might effectively identify cellular IR, cases of horses with normal CGIT or HEC results, but with excessive postprandial hyperinsulinaemia have been identified. Specifically, they may not adequately assess the role of the enteroinsular axis in ID in a particular individual. Additionally, it has been argued that the FSIGTT suffers at extremes of insulin sensitivity, returning potentially nonsensical values, and so is best reserved for mild cases of IR. It should also be noted that both of these dynamic tests of insulin sensitivity can have significant inter-day variability, with a large reported coefficient of variation (CV) for the FSIGTT.

1.4.7.3.3 Combined glucose-insulin tolerance test

The CGIT involves the rapid, sequential infusion of glucose and insulin and the frequent measurement of blood glucose thereafter. The test is characterized by an initial, rapid hyperglycaemic phase followed by a slower hypoglycaemic phase, and was initially thought to be
an accurate but straightforward test of IR. However, the CGIT has been shown to have considerable variability in diagnostic accuracy depending on what cut-offs are used. It also suffers from inadequate reproducibility. The CGIT is typically performed with intermittent IV sampling, although a subcutaneous glucose monitor has been used successfully to perform a modified version of the test, with a 15-minute lag time suggested to compare with IV glucose samples.

1.4.7.3.4 Complete and 2-step insulin response test

The CIRT (also known as the insulin tolerance test) was developed as an assessment of insulin sensitivity that was more easily performed in a clinical setting. The test involves measuring blood glucose before, and frequently after, insulin administration. In that paper, a variety of doses of insulin were administered to both normal and hyperleptinaemic horses and their glucose concentrations (as a direct response) determined. Horses that were hyperleptinaemic showed clear separation from those that had normal leptin concentrations. The authors also evaluated the reproducibility of the test four months later, using the 40- and 50-minute time points post-insulin. Interestingly, they found that all horses were less insulin sensitive, although variations in pasture NSC content may have affected results. The authors deemed the test results appropriately reproducible; although the test results over time were correlated, there was bias present that might make comparisons between time points difficult in individual horses.

The 2SIRT was first described as an abbreviated version of the CIRT. The test involves the measurement of effect of insulin on glucose concentrations, as a measure of tissue insulin sensitivity, at one time point (30 minutes after insulin injection) instead of at multiple times. The authors described complete separation between insulin resistant and sensitive horses based on the 2SIRT. However, this was an anticipated response given that the horses were initially categorized using the CIRT. Of note, the median time to less than 50% of basal glucose concentrations was less than 30 minutes in insulin sensitive horses; this may have implications for the repeatability of the 30-minute testing time point. The true ID status of the horses in a follow-up paper by the original authors was unknown as the 2SIRT was not compared to a
‘gold standard’. Interestingly, in the same paper, the glucose responses to insulin were not reproducible between fasting periods with many horses having different categorical results\(^{283}\). Fasting has been shown to affect the 2SIRT, with reduced glucose responses to insulin injections observed after 12-13 hours of fasting\(^{283,314}\). This was also observed in an older study\(^{339}\) where plasma glucose concentrations were reduced by 72% in fed ponies after insulin administration, in comparison to only 30% when the ponies were fasted for three days. The cause of this may relate to a relatively increased GI absorption of volatile fatty acids and a relatively reduced GI absorption of NSCs, reduced hepatic glycogen stores or altered endogenous insulin concentrations\(^{283}\). As expected, sympathetic responses (in this case mimicked by exogenous adrenaline administration) increase insulin insensitivity, although this effect was mitigated by exercise due to increased muscular GLUT-4 expression\(^{314}\). Sympathetic responses should perhaps be avoided prior to testing for cellular IR.

### 1.4.7.4 Tests of the enteroinsular axis

#### 1.4.7.4.1 Oral sugar test

The OST was first described as a practical method of assessing insulin dysregulation in a field setting\(^{340}\). It is primarily a test of pancreatic beta-cell responsiveness\(^{88}\). The original study\(^{340}\) compared EMS horses and controls and differentiated them based on their insulin responses to a brand of corn syrup\(^b\). The paper described the administration of 0.15 mL/kg corn syrup to fasted horses, and the subsequent measurement of serum insulin concentrations at 60 and 90 minutes post-syrup. However, selection bias may confound the conclusions drawn in this paper. The control group consisted of Quarter Horses, which have been recognized as being relatively insulin sensitive\(^{39,40}\). In contrast, the EMS group consisted of several breeds overrepresented in epidemiological studies of laminitis [see section 1.4.6.7 Breed and genetics]. Thus, the differences between groups may represent intrinsic breed differences rather than disease states. In another paper examining the effects of fasting on the OST\(^{283}\), insulin concentrations after corn syrup administration were much lower in a group containing Thoroughbreds than in the original paper. Similarly, in a paper examining the incretin effect of glucose administration\(^{89}\), normal horses had a median fasting insulin of 3 μU/mL using the same RIA as the original paper.
The OST appears to be specific, but insensitive when evaluating horses for ID. The original paper compared the OST results to those of an intravenous glucose tolerance test in the same animals. There was considerable overlap in the area under the curve (AUC) of insulin in the OST results, despite separation in the glucose tolerance test results, as well as large intrahorse variability in the OST results in normal horses. When the OST has been compared to the HEC, no significant relationship was found between the tests, although other authors have argued good correlation exists between results of a modified OST and insulin-dependent glucose disposal rates. When compared to the FSIGTT, poor correlation was also found, although some variability exists between studies and calculated variabilities. Specifically, all horses in the former study were classes as having normal insulin responses to the corn syrup despite several being classed as insulin resistant based on the FSIGTT; sensitivities of 0% and 14% were found in the latter study using insulin cut-offs of 60 μU/mL and 45 μU/mL, respectively. Interestingly, more recent research by the group that first described the OST has defined ID horses using means that were below the original cutoff. The same research group later evaluated the repeatability of the OST in two different populations in both control and EMS horses. The investigators found that there was agreement between insulin concentrations in between 83 and 91% of test results. However, the 95% CI of the mean biases were markedly wide, making the true repeatability of the test poorer than was reported. Indeed, the authors state that the OST may not be useful for horses with results near the cut-off, and that individual insulin results vary too much for monitoring the response to any management or therapy. Another study by the group also suggested that variable insulin concentrations as part of the OST are due to within-horse variability. The potentially poor reproducibility of the test has been further shown in ponies subjected to four tests over three weeks using slightly different testing conditions. In that study, inadequate agreement between samples taken at the same time points in the same animals were found, along with wide limits of agreement and large CV. Minimal increases in insulin concentrations have also been seen in ponies in a modified version of the OST. This same modified OST found satisfactory diagnostic accuracy in comparison to the HEC but suffers from similar selection bias, whereby severely insulin resistant horses and
ponies were used as the EMS group. This OST also had less than satisfactory repeatability, with relatively high mean CVs for both TMax and CMax of insulin. When compared to the FSIGTT, this modified OST has been shown to be poorer at evaluating changes in insulin sensitivity at or near the normal sensitivity range \(^{88}\), suggesting that it is not accurate at detecting mild disease.

Varied dosing \(^{343}\) (up to 0.45 mL/kg) and insulin cut-offs \(^{40}\) (normal < 30.2 µU/mL when administered at 0.25 mL/kg) have been recently described for the OST which may be more reproducible in other populations, although the follow-up work by Frank and Walsh \(^{140}\) found poor repeatability despite using a lower cut-off. This less-than-satisfactory repeatability has also been demonstrated elsewhere when using higher doses of corn syrup \(^{343}\). The authors of that paper suggested that the diagnostic accuracy of the test could be improved by calculating the AUC of insulin over at least three measurements, although this may be impractical in a clinical setting. Poor repeatability has been found elsewhere \(^{313}\).

In keeping with the effects of fasting on the 2SIRT, fasting for greater than 3 \(^{283}\) to 8 \(^{313}\) hours has been shown to increase stimulated insulin concentrations, with potential increases in insulin concentrations of 40 µU/mL estimated in some cases after fasting \(^{313}\). Interestingly, significant increases in insulin concentrations post-syrup administration were not observed in non-fasted horses, although most of these horses would not be considered to be predisposed to ID \(^{283}\). Again, however, the true ID status of these horses was not assessed by a current ‘gold standard’. Interestingly, the effect of fasting on OST results were disputed by another study recently, with only small effects on glucose concentrations observed in some horses \(^{215}\). The same study did not identify differences in insulin concentrations when the OST was performed concurrently with the TRH-stimulation test \(^{215}\).

There is no apparent effect of season on results of the OST \(^{39}\), although results were not available for the entire year and only five horses were assessed. In agreement with the original paper describing the OST \(^{340}\), when Quarter Horses were assessed to evaluate the effects of pasture housing on the OST, insulin concentrations were well below the limits used \(^{140,340}\) to categorise
horses as insulin dysregulated\textsuperscript{39}. Results were similar when horses were tested at pasture and while stabled\textsuperscript{39}.

There may be breed differences with regards to the time to maximum post-syrup insulin concentrations in the OST with longer (approx. 68 minutes) and shorter (approx. 58 minutes) TMax seen in horses and ponies, respectively\textsuperscript{344}. Knowles et al\textsuperscript{313} found that the most common time to the CMAx of insulin was 30 minutes, in contrast with the original study in horses\textsuperscript{340}.

1.4.7.4.2 In-feed glucose test
The IFGT has been described using 0.75 g/kg\textsuperscript{292,345}, 1 g/kg\textsuperscript{99} and 1.5 g/kg\textsuperscript{96} bodyweight (bwt) glucose. It was originally used to evaluate small intestinal absorption of glucose\textsuperscript{346} but has more recently been used to characterize insulin responses to known quantities of glucose\textsuperscript{96,99,292,345}. At 1.5 g/kg, the IFGT was moderately well correlated with results from the FSIGTT\textsuperscript{96}. There is some evidence that the IFGT can predict\textsuperscript{32,91} or categorise\textsuperscript{99} horses and ponies at risk of laminitis (a more patient-oriented outcome than simply ID). Of interest, the insulin cut-offs that separated previously-laminitic ponies and normal ponies in a recent study\textsuperscript{99} were much higher than the cut-off used to classify a horse as insulin dysregulated\textsuperscript{68}. From personal observations, some horses do not eat the entire glucose-containing meal either at all, or sufficiently quickly. While cut-offs are also available for a lower dose of glucose (0.5 g/kg bwt)\textsuperscript{68}, the recent description of a lower dose of glucose leading to similar insulin concentrations\textsuperscript{345} is a potentially helpful modification. The authors of that study actually reported lower insulin concentrations in ponies that ate the larger dose of glucose (1 g/kg bwt) and attributed this to slower meal consumption\textsuperscript{345}. The IFGT has also been shown to be a repeatable test\textsuperscript{345}, arguably more so than the OST\textsuperscript{140}.

Younger horses have been shown to have a longer time to peak insulin after ingestion of various high NSC meals\textsuperscript{279} (as a proxy IFGT), while different CHO sources also lead to various TMax in the same study. A variant of the IFGT (with the glucose administered by NGT), insulin concentrations at two hours were well correlated with those of the FSIGTT and insulin sensitivity as measured by the HEC\textsuperscript{282}. When insulin was measured four hours after a high NSC feed, insulin
concentrations were able to characterise not only the risk of laminitis, but also the speed of onset of disease. While not an orthodox IFGT, this nevertheless demonstrates the use of post-NSC insulin measurement.

The OST and IFGT have been directly compared, albeit in ponies of previously unknown ID status. This study found that results were not completely comparable, with the IFGT categorising more animals as insulin dysregulated. The IFGT did have more variability, but importantly had larger insulin concentrations and AUC in comparison to the OST.

While enteroinsular axis testing might better evaluate the natural pathophysiology of disease, concerns exist about confounding factors on glucose absorption, and hence insulin release. These include rates of feed consumption, gastric emptying and intestinal absorption.

1.4.7.5 Insulin assays and sample handling

Insulin is a relatively stable analyte. When stored at room temperature for up to 3 days, either centrifuged and separated or in whole blood, there was no significant difference in comparison to results obtained, in the same clinical cases, after immediate centrifugation and refrigeration. In that study, the largest bias observed was 2 μU/mL, which would not be clinically significant.

From a research point of view, the Siemens Coat-a-Count RIA is one of the most frequently cited methodology for insulin concentration determination in horses. This RIA is not available in Australia. In clinical practice, a CLIA is available at several commercial laboratories. The precision of this assay, as determined by its intraassay CV, is considered excellent. The CLIA has been used in clinical research involving hyperinsulinaemia secondary to oral glucose intake and in generating reference intervals for ACTH in ponies. When comparing the CLIA with the RIA, Banse et al. found that the CLIA had a positive bias (i.e. gave higher concentrations) of approximately 13 μU/mL. It should be noted that when 5 discordant results were removed, the bias was considered clinically insignificant. Both tests in that paper had good linearity. However, another study has found that the Coat-a-Count RIA has poor
linearity. That paper did describe a technique of improving the performance of the assay by using charcoal-stripped plasma, which subsequently was superior to the Mercodia ELISA as well as other ELISAs. Interestingly, that paper demonstrated only moderate concordance between the RIA and the ‘gold standard’ of mass spectrometry/gas chromatography. In contrast, a more recent paper comparing the same CLIA and RIA identified a negative bias at lower concentrations, but confirmed the positive bias at higher concentrations. This negative bias has been corroborated in another study comparing the CLIA to two different RIAs and in another study comparing a different CLIA to an RIA and ELISA.
1.5 Manufacturers’ Details

a Immulite 1000/2000, Siemens Healthcare Pty Ltd, Bayswater, VIC, Australia

b Karo light corn syrup; ACH Food Companies, Inc, Memphis, TN, USA

c Siemens Coat-A-Count Insulin Radioimmunoassay, Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA

d Bio-One 4 mL plastic whole blood tube with spray-coated K2EDTA, Greiner, Germany

e TRH, Sigma Pharmaceuticals Ltd, North Liberty, IA, USA

f Bio-One 4 mL plastic whole blood tube with spray-coated K2EDTA, Greiner, Germany

g Medcalc Statistical Software version 16.4.3, MedCalc Software, Ostend, Belgium

h RStudio, R Studio Inc, Boston, MA, USA

i Sealed Envelope Ltd, www.sealedenvelope.com

j Accutrend Plus, Roche Diagnostics, Risch-Rotkreuz, Switzerland

k Humulin R 100, Eli Lilly, West Ryde, NSW, Australia
1.6 Justification

Endogenous ACTH is a readily available method to diagnose PPID in Australia. However, diagnostic uncertainty can arise when endogenous ACTH concentrations fail to clearly characterise a horse as either PPID-positive, or PPID-negative. As PPID exists as a continuum of disease, early recognition of disease might be difficult. A so-called ‘grey zone’ can be described when ACTH concentrations fall near the upper cut-off limit, particularly given the numerous non-PPID influences on ACTH concentrations, as previously discussed. The interpretation of results within a ‘grey zone’ depends on the pre-test probability of disease in an individual and the likelihood ratios of a test. Based on existing published literature, positive likelihood ratios during the quiescent phase and dynamic phases are 3.36 and 20, respectively. Negative likelihood ratios during the quiescent and dynamic phases are 0.21 and 0, respectively. Assuming a pre-test probability of 20% (based on a prevalence of approximately the same), and using the two-step Fagan’s nomogram, this would translate into the following approximate post-test probabilities: positive test during the quiescent phase – 48%; positive test during the dynamic phase – 83%; negative test during the quiescent phase – 6%; and, negative test during the dynamic phase – 0%. Thus, while negative tests perform adequately, positive tests, particularly during the quiescent phase, may not be appropriate in subclinical cases.

The TRH-stimulation test holds promise as an alternative test to endogenous ACTH. Although there is some data from the northern hemisphere proposing diagnostic cut-offs for the dynamic phase for ACTH concentrations post-TRH administration, a consensus has yet to be reached. Diagnostic cut-offs and seasonality of the TRH-stimulation test have not been reported in the southern hemisphere. Recent data at this institution and by others suggest geographic location affects basal endogenous ACTH upper reference limits. While this may be due entirely to photoperiod differences, geographic or other climactic influences may also be involved. Previous research has identified differences in basal pars intermedia secretions in different locations and climates, although their relative effects could not be separated.
Previous work on the effects of feeding on basal ACTH concentrations has demonstrated mixed results \(^{214,215}\). It is unknown if the CHO administered in the OST affect ACTH concentrations. Furthermore, it has been shown that intravenous insulin can increase ACTH concentrations \(^{226}\), but this effect as part of the 2SIRT is unknown. This may have implications for field-based testing with regard to the timing of blood collection for ACTH determination, such as where basal insulin is omitted during an OST, or if a TRH-stimulation test is to be combined with the 2SIRT.

Finally, performing diagnostic test experiments in different locales using different testing methods is important to robustly evaluate the reproducibility of these tests.
1.6.1 Aims

The aims of this study are to better characterize the results of various tests of endocrine causes of laminitis in an Australian setting, and to evaluate their reproducibility. The study is designed to reflect clinical practice in Australia, using resources that are widely available in this country.

The aims of the study are as follows:

- to characterise the endogenous plasma ACTH response to TRH-stimulation during both phases of the circannual pituitary cycle in a group of clinically normal horses in two disparate geographic and climatic locations in Australia;
- to assess the reproducibility of the OST and 2SIRT in a group of horses of unknown ID status;
- to determine the repeatability of the 2SIRT when glucose samples are obtained at 25, 30 and 35 min post-insulin (to allow for imperfect field testing conditions); and
- to determine the effects of OST and 2SIRT testing on ACTH concentrations thus determining the suitability of post-test sampling in diagnosing concurrent PPID.

1.6.2 Hypotheses

The hypotheses of the study are as follows:

- that there will be a significant difference between the plasma ACTH concentrations at 10 min and 30 min post-TRH administration between the dynamic phase and the quiescent phase of the circannual pituitary cycle;
- that the 2SIRT will be poorly reproducible, that there will be differences between results of the 2SIRT when testing at different post-insulin time points, and that there will be significant effects of the test protocol on ACTH concentrations; and
- that the OST will be poorly reproducible, and that there will be significant effects of the test protocol on ACTH concentrations.
Chapter 2. Circannual variability in adrenocorticotropic hormone responses to administration of thyrotropin-releasing hormone in clinically normal horses in Australia

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2.1 Abstract
Thyrotropin releasing hormone-stimulation (TRH) testing for pituitary pars intermedia
dysfunction (PPID) is recommended at times when pituitary gland activity is quiescent. Current
diagnostic cut-offs reflect testing in the northern hemisphere during this time. The objectives of
this study were to evaluate TRH-stimulation testing during two different phases of the circannual
pituitary cycle, and to determine whether diagnostic cut-offs developed in the northern
hemisphere are appropriate in Australia. Thirteen clinically normal horses at Perth and 23 at
Townsville had TRH-stimulation tests performed during the quiescent and dynamic phases of the
circannual pituitary cycle. At both locations, post-TRH adrenocorticotropic hormone (ACTH)
concentrations were significantly different between testing time points (Perth \( P=0.001 \);
Townsville \( P<0.0001 \)). In Perth, the mean ACTH concentrations 10 min post-TRH during the
quiescent and dynamic phase were 51.4 pg/mL (95% CI 46.4-56.4) and 248.5 pg/mL (95% CI
170.2-326.9) respectively. The median percentage change in ACTH concentrations between T0
and T10 in the dynamic phase was 361.9%. In Townsville, the mean ACTH concentrations 30 min
post-TRH during the quiescent and dynamic phase were 35.3 pg/mL (95% CI 29.6 to 40.9) and
112.3 pg/mL (95% CI 93.4 to 131.2), respectively. The median percentage change in ACTH
concentrations in the dynamic phase was 144.7%. The ACTH cut-off after TRH-stimulation in
normal horses during the quiescent phase in Perth and Townsville appears similar to the values
established in the northern hemisphere. However, TRH-stimulation testing during the dynamic
phase was extremely variable at both locations, complicating its interpretation at this time of
year.

*Keywords:* ACTH; Diagnosis; PPID; Reference value; TRH
2.2 Introduction

The circannual pituitary pars intermedia cycle affects basal adrenocorticotropic hormone (ACTH) concentrations in both normal horses and horses with pituitary pars intermedia dysfunction (PPID) \textsuperscript{133,183,185,189,207,211,238}. More recently, this circannual cycle has been linked to changes in daylight length \textsuperscript{209,210}, although other factors may also influence this rhythm. Broadly, between the summer and the winter solstice, endogenous ACTH concentrations increase, reach a peak (acrophase) and then decline – this is defined by the authors as the dynamic phase \textsuperscript{210}. In contrast, a relatively quiescent phase is apparent when endogenous ACTH concentrations are lower and display reduced variability compared to the rest of the year, and occurs between the winter and summer solstices \textsuperscript{179,210,351}. There are increased ACTH responses to thyrotropin releasing hormone (TRH) administration during the dynamic period of the circannual pituitary cycle in clinically normal horses compared to the quiescent period of the cycle \textsuperscript{180,214,242}.

The ACTH response to TRH administration is a more sensitive test for PPID than basal endogenous ACTH at certain times of the circannual pituitary pars intermedia cycle (Beech et al., 2007, 2011a, 2011b). Several studies have shown that ACTH concentrations peak in less than 15 min post-TRH administration \textsuperscript{158,180,236}. Current recommendations in the northern hemisphere support a diagnostic cut-off of 110 pg/mL ACTH at 10 min and 65 pg/mL ACTH at 30 min post-TRH administration \textsuperscript{238}. The test may be used to differentiate normal horses from PPID-affected horses whose basal endogenous ACTH concentrations fall within a ‘grey-zone’ \textsuperscript{179}. In contrast to basal endogenous ACTH testing, TRH-stimulation testing is currently recommended at times when ACTH concentrations are quiescent \textsuperscript{238}.

Although there is some data from the northern hemisphere proposing diagnostic cut-offs for the dynamic phase \textsuperscript{243}, a consensus has yet to be reached. Diagnostic cut-offs and seasonality of the TRH-stimulation test have not been reported in the southern hemisphere. Recent data by the authors and others suggest geographic location affects basal endogenous ACTH upper reference limits \textsuperscript{189,210}. While this may be due entirely to photoperiod differences, geographic or other climactic influences may also be involved. Previous research has identified differences in basal
pars intermedia secretions in different locations and climates (McFarlane et al., 2011), although their relative effects could not be separated.

The aim of the study was to characterise the endogenous plasma ACTH response to TRH-stimulation during both phases of the circannual pituitary pars intermedia cycle in a group of clinically normal horses in two disparate geographic and climactic locations in Australia. The hypothesis was that there would be a significant difference between the plasma ACTH concentrations at 10 min (T10) and 30 min (T30) post-TRH administration between the dynamic phase and the quiescent phase.

### 2.3 Materials and methods

#### 2.3.1 Horses

The study protocol was approved by the animal ethics committees of Murdoch University (R2702/14 8 December 2014) and James Cook University (A2127 7 November 2014). Horses were selected at each of two geographical locations within Australia; Perth in southern Australia (31°57’S, 115°52’E; considered a hot-summer Mediterranean climate) and Townsville in northern Australia (19°26’S, 146°81E; considered a tropical savanna climate). In Perth, 13 horses were tested once in September 2015 and once in March 2016. Horses were chosen on the basis of age (< 15 years), lack of clinical signs of PPID and normal basal quiescent & dynamic phase endogenous ACTH concentrations. There were five Thoroughbreds (four geldings and one mare) and eight Standardbreds (four geldings and four mares). The mean age (range) was 10.4 (4-14) years. All horses were clinically well for at least six months prior to each time point. Horses were housed in irrigated Kikuyu pasture in larger herds all year round with supplemental oaten hay. All horses were in moderate body condition at both testing times (body condition score median 5/9 (range 4-6) when tested by two independent observers.

In Townsville, 23 horses were tested once in September 2015 and once in April 2016. Horses were similarly chosen on the basis of age, lack of clinical signs of PPID and normal basal quiescent &
dynamic phase ACTH concentrations. Breeds represented were Thoroughbreds \( n = 14; 13 \) mares, one stallion), Standardbreds \( n = 5; \) mares), Quarter Horses \( n = 3; \) mares) and an Australian Stock Horses \( n = 1; \) mare). The mean (range) age of these horses was 11.4 (3-17) years. All horses were clinically well for at least six months prior to each time point. Horses were dry lot paddock housed and received supplemental feed of roughage only or a balanced pelleted ration and roughage depending on their level of physical activity. Horses were body condition scored during the April testing period by two independent observers with the median body condition score being 6/9 (range 4-8).

2.3.2 Experimental Methodology

Horses were normally housed together in paddocks and were moved to smaller paddocks together on the morning of each test day at least 2 h prior to testing being performed. Evidence of excessive exercise or physiologic stress was not observed in any of the horses. No supplemental feed (other than pasture) was supplied for the duration of the test. The horses were regularly housed as such as part of their teaching duties and were considered acclimatized to this procedure. The TRH-stimulation test was performed as previously described. Blood was collected into sterile plastic vacutainer tubes containing ethylenediaminetetraacetic acid (EDTA) for measurement of ACTH concentrations prior to intravenous injection of 1 mg of TRH. The TRH was reconstituted under a laminar flow hood with sterile water for injection to a final concentration of 1 mg of TRH/mL. The solution was filtered with a 0.22-\( \mu \)m syringe filter and stored in 1 mL aliquots at \(-80^\circ\)C until use (2-9 months). Blood was again collected into sterile plastic vacutainer tubes containing EDTA at 10 min (Perth) or 30 min (Townsville) post-TRH injection and immediately placed on ice. The horses were monitored for 1-2 h and returned to their respective herds thereafter. The samples were centrifuged in a refrigerated centrifuge for 5 min at 4000 g within 2 h of collection. The plasma was then separated and frozen at \(-80^\circ\)C until analysis. Plasma samples were transported on dry ice and ACTH concentrations analysed using a chemiluminescent assay previously validated for use with equine plasma. Quality control materials provided by the manufacturer were used daily. The interassay coefficients of variation for lower and upper level quality control materials were 4.16% and 5.07% respectively.
2.3.3 Statistical analysis
The data were assessed for normality using the Shapiro-Wilk test; all raw data were normally distributed either before or after logarithmic transformation. Normally distributed data are presented as means (± standard deviations; SD) or means (range). The data for percentage change in ACTH concentration, evaluated between T0 and T10 or T30 at each time point, were not normally distributed and are presented as median (range). A paired, two-tailed t-test was performed to determine if a difference existed between the T10 or T30 ACTH concentrations in the dynamic and quiescent time points at each location. An unpaired two-tailed t-test was performed to compare basal ACTH concentrations between Perth and Townsville at both the quiescent and dynamic time points. A Wilcoxon signed-rank test was performed to determine if there were differences in the percentage increase in ACTH concentration after TRH stimulation between testing time points at each location. Statistical significance was set at \( P<0.05 \). All statistical analyses were performed on commercially available software.

2.4 Results
Four horses had one to two coughs post-TRH administration. No other adverse effects were noted. In Perth, all basal ACTH concentrations were within previously published reference intervals during both the quiescent and dynamic phases of the circannual pituitary pars intermedia cycle at this location \(^{210}\). Results are presented in Table 1 and Fig. 1. The T10 ACTH concentrations in September were all below the upper reference limit (110 pg/mL) previously described in clinically normal horses \(^{238}\). The T10 ACTH concentrations in March were significantly higher than the T10 ACTH concentrations in September \((P=0.0001)\). The median percentage changes (Table 2) between time points were significantly different \((P<0.0001)\).

For Townsville, results are presented in Table 3 and Fig. 2. All basal ACTH concentrations were within previously published reference intervals during both the quiescent and dynamic phases of circannual pituitary pars intermedia cycle at this location \(^{210}\). All but one T30 ACTH concentration was <65 pg/mL in September, as previously described for normal horses \(^{238}\). One horse had a 30
min post-TRH ACTH concentration of 68 pg/mL. This horse was characterised as normal based on monthly ACTH concentrations and the proximity to the previously described decision limit and subsequently included in further analysis. The T30 ACTH concentrations in April were significantly higher than the T30 ACTH concentrations in September ($P<0.0001$). The median percentage change (Table 2) between time points were significantly different ($P<0.001$).

Basal T0 ACTH concentrations at each time point were significantly different between sites during the dynamic phase (March/April $P=0.036$) but not during the quiescent phase (September $P=0.063$).

### 2.5 Discussion

When TRH-stimulation tests were performed in a group of clinically normal horses with a low likelihood of PPID in two geographic locations in Australia during the quiescent phase (September) of the circannual pituitary pars intermedia cycle, the ACTH concentrations were consistent with those previously reported for normal horses as confirmed by pituitary histology $^{180,236}$. This is of note as the reference intervals for basal ACTH concentrations at these locations in Australia differ from other locations (in the northern hemisphere) where the TRH-stimulated ACTH concentration cut-offs have been created $^{210,238}$. However, despite the low variability, too few horses were tested at 10 min post-TRH administration to determine a more robust reference interval. Specifically, Friedrichs et al. (2012) recommend that a minimum of 20 animals, and ideally 40, be used to create reference intervals.

The results of the TRH-stimulation tests performed at March in Perth and April in Townsville suggest these time points are within the dynamic phase of the circannual pituitary cycle as marked variation in post-TRH ACTH concentrations occurs. Although a recent abstract $^{243}$ suggested post-TRH cut-offs during the dynamic phase of the circannual pituitary cycle, the study numbers were small. Another study evaluated circannual ACTH concentration responses to TRH administration and found marked dynamic phase variability in ACTH concentrations, regardless of age $^{281}$. Given the marked variability in endogenous ACTH concentrations after TRH stimulation
in this study, further work is required to determine the results of TRH-stimulation testing in PPID horses during the dynamic phase of the circannual pituitary cycle.

We also examined the percentage change in ACTH pre- and post-TRH administration. In both locations, the percentage change was significantly different between the quiescent and dynamic time points. Our results support the premise that percentage change in ACTH post-TRH administration is affected by circannual cycle and is in agreement with Diez de Castro and colleagues. However, these results are in contrast to Funk and colleagues where the percentage change in ACTH after stimulation with TRH did not appear to be affected by the circannual rhythm. The reason for this is unknown, although the latter study had lower ACTH responses to TRH than the current study and used a different ACTH assay. From these data, it may not be appropriate to use percentage change in ACTH concentrations as a method of diagnosing PPID in horses having undergone TRH stimulation tests.

The marked variability in endogenous ACTH post-TRH stimulation during the dynamic phase of the circannual pituitary cycle may make testing and interpreting results of a TRH-stimulation test in this phase difficult. Several studies have reported the results of TRH-stimulation tests performed in clinically normal horses during the dynamic phase and also demonstrated variability. Apart from time of year, variability in resting ACTH concentrations may be caused by breed, time of day, stress, illness, long-distance transport, feeding, laminitis & high intensity exercise, as well as PPID. Causes of variability in TRH-stimulated ACTH concentrations other than time of year and PPID are unknown. All of the horses studied were free of clinical signs of disease, fed only pasture, did not exercise within 2 h of sample collection and were housed in a manner to which they were acclimatized. Furthermore, the basal concentrations obtained in this study demonstrated little variability. While it is thought that the circannual pituitary cycle may be related to changes in photoperiod, geographic or other environmental factors may contribute to the observed variations. When the circannual pituitary cycle has been evaluated in other parts of the world, less obvious circannual differences have been identified in ACTH concentrations at locations closer to the equator (Alabama, USA —
Schreiber et al., 2012; Florida, USA – McFarlane et al., 2011; Townsville, Australia - Secombe et al., 2017), which might reflect changes in photoperiod.

Several horses had normal results but with minimal ACTH elevations post-TRH administration. This has been reported elsewhere. The cause is unknown but may relate to altered TRH receptor expression in the pituitary gland or temporary exhaustion of ACTH stores at the time of TRH administration. The TRH receptor mRNA has been identified in tissue explants of both the pars intermedia and pars distalis. In normal horses, TRH-stimulated ACTH derives from the pars distalis, while in PPID horses it derives from the pars intermedia (McFarlane et al., 2006). Given the circannual variability in pars intermedia size (Cordero et al., 2012), it may be that the increased variability of post-TRH ACTH concentrations relates to increased variability of TRH receptor expression. Others have also suggested individual differences in the secretion of various pro-opiomelanocortin derivatives (Sojka-Kritchevsky and Johnson, 2014) which may explain some of the variability.

The horses used in our study were not confirmed to be free of PPID based on histology. However, they all had 12 months of ACTH measurement, all of which were deemed to be within normal limits of previously determined regional and seasonal reference intervals. Additionally, the results of the TRH-stimulation tests during the quiescent phase are similar to those obtained in histologically normal horses.

There are several limitations associated with this study that may reduce its external validity. Unfortunately, standardisation of testing times was not achieved. Logistical limitations did not enable T10 testing in Townsville. However, T30 testing results may still be clinically useful in situations where T10 testing is missed. The literature to date supports an early ACTH peak post-TRH injection of less than 15 min. Furthermore, during the quiescent phase, the difference between normal and PPID horses is greatest at 10 min post-TRH injection. While our previous work has shown there to be differences in resting ACTH concentrations...
circannually between these locations\textsuperscript{210}, the effect of location on post-TRH ACTH concentrations is still unknown. Testing at both time points in both locations would have allowed analysis of this.

Only two time points during the year were investigated for logistical reasons. It has recently been shown\textsuperscript{209,210} that the circannual rise and fall in ACTH concentrations occurs over a longer phase, with a more gradual rise and steeper fall, than what is considered autumn. The purpose of this paper was not to determine monthly ACTH responses to TRH stimulation. However, given that the length of each phase may vary with geographic location, more complete characterisation of the ACTH response to TRH throughout the year may be important with regards to testing at transition times between the quiescent and dynamic phases.

2.6 Conclusions

In this population of clinically normal horses in Australia, TRH-stimulation testing during the dynamic phase of the circannual pituitary pars intermedia cycle demonstrated marked variability. In contrast, and similar to other locations, TRH-stimulation testing during the quiescent phase demonstrated lower variability and existing cut-off values as recommended by the Equine Endocrinology Group appear applicable. Significant differences were identified between post-TRH ACTH concentrations at both locations during the dynamic phase. Variability in the dynamic phase results makes interpreting results and proposing a point estimate cut-off at which a horse would be considered to have a normal test result difficult. Until TRH-stimulation tests are performed on large numbers of PPID-confirmed horses to establish appropriate diagnostic cut-offs, basal ACTH concentrations measured during the dynamic phase and TRH-stimulation tests performed during the quiescent phase may be more appropriate for the diagnosis of PPID, particularly in those horses with early disease.
### 2.7 Table 1
Means, 95% CI of the means and ranges of ACTH concentrations for both the dynamic phase and quiescent phase TRH-stimulation tests in Perth.

<table>
<thead>
<tr>
<th></th>
<th>T0 Quiescent Perth</th>
<th>T10 Quiescent Perth</th>
<th>T0 Dynamic Perth</th>
<th>T10 Dynamic Perth</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>13</td>
<td>13</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>Mean</td>
<td>26.7</td>
<td>51.4</td>
<td>49.6</td>
<td>248.5</td>
</tr>
<tr>
<td>95% CI</td>
<td>24.0 to 29.3</td>
<td>46.4 to 56.4</td>
<td>43.5 to 55.7</td>
<td>170.2 to 326.9</td>
</tr>
<tr>
<td>Minimum</td>
<td>18.9</td>
<td>36.0</td>
<td>35.6</td>
<td>80.7</td>
</tr>
<tr>
<td>Maximum</td>
<td>33.7</td>
<td>69.7</td>
<td>69.6</td>
<td>511.0</td>
</tr>
</tbody>
</table>
2.8 Table 2
Median (range) percentage increases in ACTH concentrations after TRH administration during the
dynamic and quiescent phases at both locations. Note that Perth post-TRH samples were
collected at 10 min and Townsville post-TRH samples were collected at 30 min. The median
percentage change was significantly different between testing time points at each location.

<table>
<thead>
<tr>
<th>n</th>
<th>Quiescent Perth</th>
<th>Dynamic Perth</th>
<th>Quiescent Townsville</th>
<th>Dynamic Townsville</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>13</td>
<td>13</td>
<td>23</td>
<td>23</td>
</tr>
<tr>
<td>Median</td>
<td>85.8</td>
<td>361.9</td>
<td>40.0</td>
<td>144.7</td>
</tr>
<tr>
<td>Minimum</td>
<td>33.5</td>
<td>68.8</td>
<td>-35.1</td>
<td>-7.2</td>
</tr>
<tr>
<td>Maximum</td>
<td>164.0</td>
<td>977.1</td>
<td>135.7</td>
<td>521.9</td>
</tr>
</tbody>
</table>
### 2.9 Table 3
Means, 95% CI of the means and ranges of ACTH concentrations for both the dynamic phase and quiescent phase TRH-stimulation tests in Townsville. *Backtransformed after logarithmic transformation.

<table>
<thead>
<tr>
<th></th>
<th>T0 Quiescent Townsville</th>
<th>T30 Quiescent Townsville</th>
<th>T0 Dynamic Townsville</th>
<th>T30 Dynamic Townsville</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>23</td>
<td>23</td>
<td>23</td>
<td>23</td>
</tr>
<tr>
<td>Mean</td>
<td>24.0*</td>
<td>35.3*</td>
<td>49.3</td>
<td>112.3</td>
</tr>
<tr>
<td>95% CI</td>
<td>20.4 to 27.6*</td>
<td>29.6 to 40.9*</td>
<td>41.7 to 56.9</td>
<td>93.4 to 131.2</td>
</tr>
<tr>
<td>Minimum</td>
<td>14.7</td>
<td>18.1</td>
<td>25.6</td>
<td>53.6</td>
</tr>
<tr>
<td>Maximum</td>
<td>52.5</td>
<td>68.0</td>
<td>86.0</td>
<td>227.0</td>
</tr>
</tbody>
</table>
Box and whisker plot of quiescent (September) and dynamic (March) phase TRH-stimulation testing in Perth. Individual results are denoted by shapes. The box denotes the first and third quartiles and the bars denote the inner fences. The central line in the box is the median. The horizontal line denotes the suggested cut-off at 10 minutes post-TRH administration in the quiescent period in the northern hemisphere.
Figure 2
Box and whisker plot of quiescent (September-November) and dynamic (April) phase TRH-stimulation testing in Townsville. Individual results are denoted by shapes. The box denotes the first and third quartiles and the bars denote the inner fences. The central line in the box is the median. The horizontal line denotes the suggested cut-off at 30 minutes post-TRH administration during the quiescent phase in the northern hemisphere.
Chapter 3. Reproducibility of the two-step insulin response test in a group of Western Australian Thoroughbreds and Standardbreds, and its effects on endogenous ACTH concentration

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3.1 Abstract

Insulin dysregulation can occur concurrently with PPID. Field-testing methods of detecting insulin dysregulation should be reproducible in a variety of populations. The objectives of this study were to determine the effects of insulin dysregulation testing using a two-step insulin response test (IRT) on ACTH concentrations and to determine the reproducibility of this test. A group of 15 Thoroughbred and Standardbred horses underwent an 2SIRT three times, at weekly intervals. For one week, ACTH was measured at the basal and testing time points in this group and in an additional control group of 15 horses. Agreement was deemed satisfactory when a difference beyond twice that of the standard deviation of the mean would not result in clinically significant differences. There was unsatisfactory agreement between the glucose concentrations at 25, 30 and 35 min post-insulin. There was unsatisfactory agreement of the test results between weeks. There was no clinically significant difference in ACTH concentrations before and at 30 min post-insulin (P = 0.05). A limitation of the study is that selection bias limits external validity in the target population. Our results indicate that the 2SIRT is not adequately reproducible. The test did not appear to significantly affect ACTH concentrations.
3.2 Introduction

The recognition of insulin dysregulation is essential in the management of laminitis. Current methods to assess insulin dysregulation involve stimulation of the enteroinsular axis (in addition to blood glucose-mediated insulin release) or evaluation of the cellular response to insulin with some authors suggesting testing both aspects of insulin dysregulation in horses suspected of having endocrinopathic laminitis.

Reproducibility refers to the precision of the results of tests carried out under changing conditions of measurement. These changing conditions may refer to different times of testing, locations, populations tested, or laboratory instruments used. This may be relevant in cases where different laboratory assays are available, for instance, or to assess biologic variability over different weeks. Testing of the cellular response to insulin, namely the ability of endogenous or exogenous insulin to maintain normoglycaemia, can be achieved via several methods. The two-step insulin response test (IRT) was developed from the complete insulin response test as a practical means of assessing whole body glucose responses to exogenous insulin. It involves measuring basal glucose, administering regular insulin, measuring glucose 30 min after insulin administration, and expressing the results as a ratio where a result > 0.5 suggests insulin resistance. While the 2SIRT has not been validated against the frequently-sampled insulin-modified glucose tolerance test (FSIGTT) or the hyperinsulinaemic euglycaemic clamp test (HEC; both considered the ‘gold-standards’ for assessing insulin sensitivity), the parent complete insulin response test has been shown to correctly identify horses as being either insulin sensitive or resistant. The test offers a rapid, inexpensive assessment of insulin dysregulation with near-instant results which is attractive from a practical point of view. The complete insulin response test described testing glucose at 15, 30 & 45 min post-insulin administration. The 2SIRT further refined this to testing glucose levels prior to, and 30 min post-insulin administration. However, it is unknown how imprecision of the 30 min post-insulin administration sampling time point would affect the interpretation of the test results (i.e. if the sample was taken at 25 or 35 min post-insulin instead). This might be relevant in a field testing environment.
Insulin dysregulation is also a component of pituitary pars intermedia dysfunction (PPID)\textsuperscript{135}. Testing for PPID most commonly relies on basal endogenous adrenocorticotropic hormone (ACTH) concentrations\textsuperscript{189,211}. There are various elucidated influences on ACTH concentrations\textsuperscript{194,203,214,219,221} but the effects of insulin dysregulation testing on ACTH concentrations require evaluation. Specifically, previous work has shown that intravenous insulin can increase ACTH concentrations\textsuperscript{226}, but this effect as part of the 2SIRT is unknown. This may have implications for field-based testing with regard to the timing of blood collection for ACTH determination.

The study was designed to reflect clinical practice in Australia, using resources that are widely available in this country. The first aim of this experiment was to assess the reproducibility of the 2SIRT in a group of horses of unknown insulin dysregulation status. A second aim was to determine the repeatability of the 2SIRT when glucose samples were obtained at 25, 30 and 35 min post-insulin (to allow for imperfect field testing conditions). The final aim was to determine the effects of testing on ACTH concentrations thus determining the suitability of post-test sampling in diagnosing concurrent PPID. Our hypotheses were that the 2SIRT would be poorly reproducible, that there would be differences between results of the 2SIRT when testing at different post-insulin time points, and that there would be significant effects of the test protocol on ACTH concentrations.

### 3.3 Materials & methods

The use of animals in this study was approved by the animal ethics committee of Murdoch University (R2702/14).

#### 3.3.1 Horses

Thirty horses of unknown insulin dysregulation status underwent testing in August (southern hemisphere winter). Horses were randomly assigned\textsuperscript{4} with blocking by age into equally-sized treatment (test) or control (sham test) groups. Thirty horses were chosen on the basis of \textit{a priori} power calculations using existing ACTH concentration variability\textsuperscript{211,214,236}, to reject the null hypothesis that there would be no difference between treatment and control groups with
regards to ACTH concentrations post-2SIRT with a power of 0.8 and alpha set to $p < 0.05$. Three replicates of the 2SIRT over three weeks were chosen to allow evaluation of fixed and random effects. Horses with historical or current evidence of laminitis (based on clinical examination and foot radiography where necessary) were excluded due to its potential confounding effects on ACTH concentrations. Horses were also excluded if they demonstrated clinical signs of PPID; a subset of the horses had monthly ACTH testing and TRH-stimulation testing with results supportive of disease-free status. There were 12 Thoroughbreds (six geldings & six mares) & 18 Standardbreds (seven geldings & 11 mares). The mean age ($\pm$ SD) was 14 ($\pm$ 5) years. All horses were clinically well for at least six months prior to the study. Horses were housed in irrigated Kikuyu pasture in larger herds all year round with supplemental ryegrass hay. All horses were in moderate body condition (body condition scores 4-6/9). The mean ($\pm$ SD) body weight was 564 ($\pm$ 33) kg.

### 3.3.2 Experimental design

Horses were housed in paddocks together on the morning of each test day. No supplemental feed (other than pasture) was supplied for the duration of the test. The horses were regularly housed as such as part of their teaching duties and were considered acclimatized to this procedure. To minimise the effects of exercise from moving paddocks on ACTH concentrations, testing began at least two hours after movement to the test paddock. All horses had either a real or sham 2SIRT as per randomisation. The 2SIRT was performed as previously described. Venous blood was obtained by direct venepuncture using a 21-gauge needle and blood glucose determined by use of a handheld glucometer previously validated for use in horses. Direct venepuncture was chosen to best reflect standard clinical practice. Fifty units of regular insulin were administered intravenously using the same venepuncture. Control horses were administered 0.5 mL of saline intravenously. To assess the repeatability of the 30-min glucose result of the 2SIRT (T30) in the treatment group, glucose samples were measured from whole blood at 25 (T25), 30 and 35 (T35) min post-insulin administration. Basal and T30 EDTA-anticoagulated blood samples for ACTH concentration determination were also collected.
and immediately placed on ice. The samples were centrifuged in a refrigerated centrifuge for five minutes at 4° C at 4600g within two hours of collection. The plasma was then separated and frozen at -80° C until analysis. Blood glucose concentrations were monitored for at least 30 min after insulin administration or until normal. Intravenous 50% glucose was available for rapid administration in case of clinical signs of hypoglycaemia. A high energy grain feed was provided to all horses once the 35-min glucose sample had been collected. To assess the reproducibility of the IRT, the treatment horses underwent testing under the same conditions at one-week intervals, for a total of three tests.

3.3.3 Laboratory assays
Concentrations of ACTH were measured using a chemiluminescent assay as previously described\(^a\) as previously described\(^{200,211}\). The coefficients of variation (CV) for lower and upper level quality control materials were 4.16% and 5.07% respectively. The analytical sensitivity was 9 pg/L.

3.3.4 Statistical analysis
Data were assessed for normality using the Shapiro-Wilk test. If normality was not met, then a reciprocal transformation was used to achieve normality of data. Random glucose samples were assessed in duplicate or triplicate to ascertain the CV of the glucometer. The CV of the glucometer was 6%.

To determine if ACTH concentrations before and after each test were significantly different, a mixed model ANOVA was used, with time (pre- and post-test) as the within-subject measure and the test group (treatment vs control) as the between-subject measure. Box’s Test of Equality of Covariance Matrices were performed to confirm that the ANOVA assumptions were met for each test.

A linear mixed effects model was used to examine whether there was an effect of time (between weeks 1, 2 and 3) on the results of the IRT. Time (weekly replicates) and breed of horse were treated as fixed effects and horse as the random effect. Results are presented as ratios of T30 to
basal glucose levels over three weekly replications (week 1, week 2 and week 3). Agreement analysis based on the method suggested by Bland and Altman \textsuperscript{356} was performed to examine whether week 1, week 2 and week 3 measurements were in agreement with each other. We performed three agreement tests: week 1 and week 2, week 1 and week 3 and finally week 2 and week 3. Mean values of the differences for each combination of weeks, lower and upper agreement levels for each combination of weeks and 95\% confidence intervals for the mean differences, upper agreement levels and lower agreement levels were obtained. Agreement was deemed satisfactory when a difference beyond twice that of the standard deviation of the mean would not result in clinically significant differences \textsuperscript{356}.

For all tests, statistical significance was set at $P < 0.05$. All statistics were performed on commercially available software packages\textsuperscript{6, h}.

### 3.4 Results

All data were available for 12 horses in the treatment group that underwent repeated testing, with one week’s data missing for one horse and two weeks’ data missing for two horses. Horses with incomplete data were excluded from the analysis. Data were available for all but five horse-week T30 glucose replications. Four horses developed blood glucose concentrations less than 1.1 mmol/L during testing. However, no clinical signs of hypoglycaemia were observed. There was insufficient agreement between T25, T30 and T35 during each week of testing (Tables 1-3). Nine out of 15 horses were classed as insulin resistant in at least one week \textsuperscript{338}. Only seven horses were categorized the same (either insulin resistant or insulin sensitive) each week. The mean glucose ratio for week 1 was significantly different to week 2 ($P = 0.04$) but not week 3 ($P > 0.05$). No effect of breed was identified ($P = 0.18$). Agreement was not satisfactory between weeks (Table 4).

The mixed model assumptions evaluating the effects of the 2SIRT on ACTH concentrations were met ($P = 0.975$). There was a significant difference in the mean ACTH concentration between the T0 and T30 ($P = 0.004$) with T30 being higher (by 6.61 pg/mL for the controls and by 7.56 pg/mL
for the treatment group; fig. 1). There was no significant difference in mean ACTH concentrations between the two test (treatment and control) groups of horses ($P = 0.5$). Finally, there was no significant interaction effect between time and test group for mean ACTH concentrations ($P = 0.87$).

### 3.5 Discussion

In this population of horses, the 2SIRT was not adequately reproducible. The 2SIRT was assessed continuously (using the individual ratios), whereas the original paper describes a categorical cutoff with results >0.5 indicating insulin resistance $^{338}$. Recent data have shown that, particularly using categorical cut-offs, some horses can have inconsistent classifications $^{283}$. It was decided to use continuous data in case there were horses that had similar results centred around the cutoff, thus increasing the chances of finding the test reproducible. Indeed, some horses crossed categories each week or had ratios equal to 0.5. Even allowing for this, there was insufficient agreement between weeks. Overall, there was insufficient agreement between glucose concentrations obtained at T25, T30 and T35. This suggests that being consistent regarding the post-insulin glucose measurement timing is important when comparing tests for individual animals. This study did demonstrate the safety of the 2SIRT when being performed in presumed insulin sensitive horses.

Given that most of these horses have had several negative OSTs [see Chapter 4], it is difficult to determine the clinical significance of one or several positive 2SIRTs in this population of horses. Based on the findings here, more research is required to determine the accuracy and appropriateness of the 2SIRT in evaluating clinical laminitic cases for underlying ID. Causes of poor reproducibility might relate to endogenous sympathetic drive (e.g. from venepuncture or herd dynamics), insulin bioavailability differences or variable fasting $^{283,314}$.

Field-based tests such as these are important to develop and validate as the current ‘gold-standard’ tests are logistically challenging and not relevant for clinical practice. Some have argued that the HEC test and the FSIGTT are not physiologic assessments of insulin resistance in horses
Furthermore, excessive post-prandial hyperinsulinaemia may occur prior to the development of tissue insulin resistance \(^{33}\), suggesting that some horses may have normal HEC or FSIGTT but have a higher risk of laminitis nonetheless.

We found no significant effects of the 2SIRT on ACTH concentrations. Hypoglycaemia secondary to intense exercise is associated with increased ACTH concentrations in humans \(^{357}\), and glucose and ACTH concentrations are inversely proportional in foetal sheep \(^{358}\). It may be that the mechanism of hypoglycaemia is more important rather than the absolute concentration, or that there are species differences explaining the results. The reason for the increased ACTH concentrations observed over time is unknown. These horses were observed continuously during the test and there was no perceived illness or physiologic stressor that would increase ACTH concentrations \(^{193,354}\). While the treatment horses had additional venepunctures to assess the reliability of the test, venepuncture alone is unlikely to increase ACTH concentrations significantly in most horses \(^{223}\), these horses were accustomed to multiple venepunctures and the control horses had only one venepuncture. Regardless, the effects are unlikely to lead to recategorisation of horses as false positives in the majority of horses given the bias, using regionally appropriate reference intervals \(^{210}\).

There are several limitations of this research limiting its external validity, chiefly selection bias. Standardbreds have been shown to have increased insulin sensitivity in comparison to breeds typically affected by endocrinopathic laminitis \(^{96,97}\). It could be argued that testing should be limited to those horse breeds more likely to have genetically-predisposed insulin resistance, respectively (i.e. the target population \(^{359}\)). Unfortunately, the study was limited by breed availability. The sample in this study, albeit small, could not identify a difference between Standardbreds and Thoroughbreds regarding reproducibility of the IRT. Thoroughbreds are not typical of the breeds associated with insulin dysregulation \(^{360}\) but may be susceptible to it under certain circumstances \(^{72,74,111}\). Furthermore, PPID horses were excluded from analysis; the response of endogenous ACTH concentrations to exogenous insulin may be different in that population. Given that all of these horses had three negative OSTs [see Chapter 4], however, it is
still relevant that many horses had some or all IRTs give results consistent with evidence of IR. Whether this reflects IR in the absence of enteroinsular axis abnormalities, or measurement error, or both, is unknown.

3.6 Conclusions
In this population of horses in Western Australia, the 2SIRT was not adequately reproducible. There does not appear to be a clinically significant effect of performing the 2SIRT on endogenous ACTH concentrations.
## 3.7 Table 1

Agreement analysis for the 2SIRT for week 1 across T25, T30 and T35. Results are expressed as glucose concentrations in mmol/L.

<table>
<thead>
<tr>
<th>Differences</th>
<th>Mean (95% CI)</th>
<th>Standard Deviation</th>
<th>Lower agreement limit (95% CI)</th>
<th>Upper agreement limit (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T25 and T30</td>
<td>0.22 (-0.08, 0.47)</td>
<td>0.46</td>
<td>-0.71 (-1.16, -0.27)</td>
<td>1.15 (0.70, 1.60)</td>
</tr>
<tr>
<td>T25 and T35</td>
<td>0.01 (-0.47, 0.44)</td>
<td>0.76</td>
<td>-1.5 (-2.26, -0.78)</td>
<td>1.6 (0.81, 2.29)</td>
</tr>
<tr>
<td>T30 and T35</td>
<td>-0.225 (-0.58, 0.08)</td>
<td>0.55</td>
<td>-1.33 (-1.86, -0.80)</td>
<td>0.88 (0.35, 1.42)</td>
</tr>
</tbody>
</table>
### 3.8 Table 2
Agreement analysis for the 2SIRT for week 2 across T25, T30 and T35. Results are expressed as glucose concentrations in mmol/L.

<table>
<thead>
<tr>
<th>Differences</th>
<th>Mean (95% CI)</th>
<th>Standard Deviation</th>
<th>Lower agreement limit (95% CI)</th>
<th>Upper agreement limit (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T25 and T30</td>
<td>0.06 (-0.21, 0.30)</td>
<td>0.43</td>
<td>-0.80 (-1.22, -0.39)</td>
<td>0.93 (0.51, 1.34)</td>
</tr>
<tr>
<td>T25 and T35</td>
<td>0.14 (-0.19, 0.43)</td>
<td>0.52</td>
<td>-0.89 (-1.3, -0.4)</td>
<td>1.18 (0.68, 1.67)</td>
</tr>
<tr>
<td>T30 and T35</td>
<td>0.08 (-0.13, 0.26)</td>
<td>0.33</td>
<td>-0.60 (-0.92, -0.27)</td>
<td>0.75 (0.43, 1.08)</td>
</tr>
</tbody>
</table>
### 3.9 Table 3

Agreement analysis for the 2SIRT for week 3 across T25, T30 and T35. Results are expressed as glucose concentrations in mmol/L.

<table>
<thead>
<tr>
<th>Differences</th>
<th>Mean (95% CI)</th>
<th>Standard Deviation</th>
<th>Lower agreement limit (95% CI)</th>
<th>Upper agreement limit (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T25 and T30</td>
<td>0.14 (-0.39, 0.61)</td>
<td>0.84</td>
<td>-1.54 (-2.35, -0.73)</td>
<td>1.18 (1.02, 2.63)</td>
</tr>
<tr>
<td>T25 and T35</td>
<td>0.30 (-0.26, 0.78)</td>
<td>0.87</td>
<td>-1.45 (-2.29, -0.61)</td>
<td>2.05 (1.21, 2.90)</td>
</tr>
<tr>
<td>T30 and T35</td>
<td>0.16 (-0.58, 0.08)</td>
<td>0.29</td>
<td>-0.43 (-0.71, -0.15)</td>
<td>0.74 (0.46, 1.02)</td>
</tr>
</tbody>
</table>
### 3.10 Table 4

Agreement analysis for the 2SIRT. Results are expressed as the ratio of T30 glucose concentrations to T0 glucose concentrations.

<table>
<thead>
<tr>
<th>Differences</th>
<th>Mean (95% CI)</th>
<th>Standard Deviation</th>
<th>Lower agreement limit (95% CI)</th>
<th>Upper agreement limit (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 1 and Week 2</td>
<td>0.10 (0.016, 0.179)</td>
<td>0.13</td>
<td>-0.16 (-0.29, -0.017)</td>
<td>0.36 (0.21, 0.49)</td>
</tr>
<tr>
<td>Week 2 and Week 3</td>
<td>0.03 (-0.14, 0.21)</td>
<td>0.27</td>
<td>-0.51 (-0.80, -0.21)</td>
<td>0.57 (0.28, 0.87)</td>
</tr>
<tr>
<td>Week 2 and Week 3</td>
<td>-0.06 (-0.18, 0.05)</td>
<td>0.18</td>
<td>-0.43 (-0.63, -0.23)</td>
<td>0.31 (0.10, 0.51)</td>
</tr>
</tbody>
</table>
3.11 Figure 1
Comparison between basal and T30 time points in test and control groups demonstrating no significant difference between groups at either test time point ($P = 0.54$). There was a clinically insignificant effect of time on ACTH concentrations, however ($P = 0.004$). Columns and bars represent the mean and standard deviation, respectively.
Chapter 4. Reproducibility of the oral sugar test in a group of Western Australian Thoroughbreds and Standardbreds, and its effects on endogenous ACTH concentration

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b School of Engineering and IT, Murdoch University, Perth, Western Australia, Australia
4.1 Abstract

Insulin dysregulation can occur concurrently with PPID. Field-testing methods of detecting insulin dysregulation should be reproducible in a variety of populations. The objectives of this study were to determine the effects of insulin dysregulation testing using an OST on ACTH concentrations and to determine the reproducibility of this test. A group of 15 Thoroughbred and Standardbred horses underwent an OST three times, at weekly intervals. For one week, ACTH was measured at the basal and testing time points in this group and in an additional control group of 15 horses. For the OST, no substantial increase in insulin concentrations was observed in our horses. Despite this, there was unsatisfactory agreement of the test results between weeks. There was no statistical difference in ACTH concentrations before and at the 60 min post-sugar time point. A limitation of the study is there were minimal increases in insulin concentrations in response to sugar identified in all horses. In addition, selection bias limits external validity in the target population. The results suggest that the OST was not a test likely to detect early ID in this population or with this assay, nor was it reproducible. It did not significantly affect ACTH concentrations; however, this should be assessed in animals with higher insulin responses to the OST. Reproducibility of the OST should be assessed in breeds that demonstrate higher insulin responses.
4.2 Introduction

The enteroinsular axis is tested by oral administration of simple carbohydrates.\(^{40,96,99,340,343,345}\). The oral sugar test (OST) was originally described\(^ {340}\) in North America using a radioimmunoassay that is no longer available in Australia. The test involves administering a specific brand of corn syrup\(^ b\) and measuring endogenous insulin concentrations 60 or 90 minutes later\(^ {340}\).

Previous work on the effects of feeding on basal ACTH concentrations has demonstrated mixed results\(^ {214,215}\). It is unknown if the carbohydrates administered in the OST affect ACTH concentrations. This may be relevant as omission of a basal insulin concentration time point may allow owner administration of the corn syrup and thus only one venepuncture by the veterinarian 60 or 90 minutes later.

The study was designed to reflect clinical practice in Australia, using assays that are widely available in this country. The first aim of this experiment was to assess the reproducibility of the OST in a group of horses of unknown insulin dysregulation status. A second aim was to determine the effects of testing on ACTH concentrations thus determining suitability of post-test sampling in diagnosing concurrent PPID.

The hypotheses were that the OST would be poorly reproducible, and that there would be significant effects of the test protocol on ACTH concentrations.

4.3 Materials & methods

4.3.1 Horses

Thirty horses of unknown insulin dysregulation status underwent oral sugar testing in October or December (southern hemisphere spring to summer). Horses were randomly assigned\(^ i\) with blocking by age into equally-sized treatment (test) or control (sham test) groups. Groupings were the same as in the 2SIRT experiment. Control horses were administered 75 mL water orally. Thirty horses were chosen on the basis of \textit{a priori} power calculations using existing ACTH concentration variability\(^ {211,214,236}\), to reject the null hypothesis that there would be no difference between
treatment and control groups with regards to ACTH concentrations post-OST with a power of 0.8 and alpha set to \( p < 0.05 \). Three replicates of the OST over three weeks were chosen to allow evaluation of fixed and random effects. Horses with historical or current evidence of laminitis (based on clinical examination and foot radiography where necessary) were excluded due to its potential confounding effects on ACTH concentrations. Horses were also excluded if they demonstrated clinical signs of PPID; a subset of the horses had monthly ACTH testing and TRH-stimulation testing with results supportive of disease-free status. There were 12 Thoroughbreds (6 geldings & 6 mares) & 18 Standardbreds (7 geldings & 11 mares). The mean age (± SD) was 14 (± 5) years. All horses were clinically well for at least 6 months prior to the study. Horses were housed in irrigated Kikuyu pasture in larger herds all year round with supplemental ryegrass hay. All horses were in moderate body condition at both testing times (body condition scores 4-6/9). The mean (± SD) body weight was 564 (± 33) kg.

### 4.3.2 Experimental design

Horses were housed in paddocks together on the morning of each test day. No supplemental feed (other than pasture) was supplied for the duration of the test. The horses were regularly housed as such as part of their teaching duties and were considered acclimatized to this procedure. To minimise the effects of exercise from moving paddocks on ACTH concentrations, testing began at least two hours after movement to the test paddock.

All horses had either a real or sham OST in October or December 2015, as per randomisation. The OST was performed as previously described, with 0.15 mL/kg bodyweight corn syrup administered by dosing syringe. Basal (T0) EDTA and serum samples were collected by plastic vacutainer system for measurement of ACTH and insulin concentrations, respectively, prior to oral administration of either corn syrup or water, and at 60 min post-administration (T60). Serum samples for insulin determination were also obtained at 90 min post-administration (T90). EDTA samples were immediately placed on ice. The samples were centrifuged in a refrigerated centrifuge for five min at 4°C at 4000g within two hours of collection. The plasma was then separated and frozen at -80°C until analysis. Plain blood samples were allowed to clot at room
temperature, were centrifuged and the serum separated and frozen at -80° C until analysis. All samples were analysed within 9 months. To assess the reproducibility of the OST, the treatment horses underwent testing under the same conditions at one-week intervals, for a total of three tests.

### 4.3.3 Laboratory assays

Concentrations of ACTH were measured using a chemiluminescent assay\(^a\) as previously described \(^{200,211}\). The coefficients of variation (CV) for lower and upper level quality control materials were 4.16% and 5.07% respectively. The analytical sensitivity was 9 pg/mL. Insulin concentrations were measured using a chemiluminescent assay\(^a\) as previously described \(^{323}\). The intra-assay and interassay coefficients of variation were both <8%. The analytical sensitivity was 2 μU/mL.

### 4.3.4 Statistical analysis

Data were assessed for normality using the Shapiro-Wilk test. If normality was not met then a reciprocal transformation was used to achieve normality of data.

To determine if ACTH concentrations before and after each test were significantly different, a mixed model ANOVA was used, with time (pre- and post-test) as the within-subject measure and the test group (treatment vs control) as the between-subject measure. Box’s Test of Equality of Covariance Matrices were performed to confirm that the ANOVA assumptions were met for each test.

A linear mixed effects model was used to examine whether there was an effect of time (between weeks 1, 2 and 3) on the results of the OST. Time (weekly replicates) and breed of horse were treated as fixed effects, horse as the random effect and testing time points (T0, T60 & T90) within each week as nested effects.

Results are presented as T60 or T90 insulin concentrations. Agreement analysis based on the method suggested by Bland and Altman \(^{356}\) was performed to examine whether week 1, week 2...
and week 3 measurements were in agreement with each other. Three agreement tests were performed: week 1 and week 2, week 1 and week 3 and finally week 2 and week 3. Mean values of the differences for each combination of weeks, lower and upper agreement levels for each combination of weeks and 95% confidence intervals for the mean differences, upper agreement levels and lower agreement levels were obtained. Agreement was deemed satisfactory when a value beyond twice that of the standard deviation of the mean would not result in clinically significant differences.

For all tests, statistical significance was set at $p<0.05$. All statistics were performed on commercially available software packages.

### 4.4 Results

Data were available for all horses at all time points each week. No adverse effects were noted in any horse. Basal insulin concentrations at each week were $< 2 \mu U/mL$ in all horses. In five horses, no insulin response was detected at any time point in any week. The maximum serum insulin in any horse at any time point in any week was 26.2 $\mu U/mL$. As such, no horse was classed as insulin resistant. The mean week 2 and week 3 post-syrup insulin concentrations were significantly different from week 1 ($P = 0.004$ and 0.027, respectively). No effect of breed was identified ($P = 0.06$). Agreement was not satisfactory between weeks (Tables 1 and 2).

The mixed model assumptions evaluating the effects of the OST on ACTH concentrations were met after the removal of one outlier ($P = 0.296$). There was no significant difference in the mean ACTH concentration between T0 and T60 ($P = 0.722$; fig. 1). Furthermore, there was no significant difference in mean ACTH concentration between test (treatment and control) groups ($P = 0.272$) and no significant interaction effect of time and test group on ACTH levels ($P = 0.522$). Inclusion of the outlier did not affect the significance of the above results.
4.5 Discussion

In this population of horses, the OST was not adequately reproducible. While the absolute T60 SD were low, this was still deemed unsatisfactory given the low insulin concentrations post-syrup. Values less than the lower limit of detection (i.e. with a true value of less than 2 μU/mL) were recategorised as being at the lower limit (2 μU/mL). The anticipated effect of this was an increase in the reproducibility (and thus potentially a type 1 error). Despite this, in this population, the OST does not appear adequately reproducible, as has been found elsewhere in different populations of horses.39,313

This data also supports the effect of breed on insulin dysregulation, and specifically that this group of Thoroughbreds had minimal increases in insulin concentrations in response to oral carbohydrates, similarly to Standardbreds.96,97 Given that Thoroughbreds have been shown to be affected with laminitis31 and that insulin dysregulation can be induced in this breed72,74,293, a reliable test of the enteroinsular axis is important for this breed. This may be particularly relevant given the anecdotal prevalence of laminitis in Thoroughbred broodmares, and the association of pregnancy and insulin dysregulation in this breed289.

The experimental design was not intended to compare the results of the OST and 2SIRT due to the timing of the tests and the potential for effects of season on insulin concentrations.139 These tests have been suggested to have poor agreement with each other283, although this has not been formally evaluated. The OST has also been compared to the HEC and FSIGTT and has been shown to lack agreement.321 Finally, these horses have not been confirmed as being insulin sensitive or resistant as per either ‘gold-standard’, making comparison difficult.

The cause of the poor reproducibility in the OST is unknown but likely reflects biological factors, possibly involving the enteroinsular axis.77 The source of corn syrup is the same as previously described.340 The product was thoroughly mixed and used completely during testing making precipitation of sugars unlikely. As mentioned, excessive self-exercise was not observed. Breed differences may play a role in this case. In the original study by Schuver et al340 describing the
OST, there was considerable variation in individual horse insulin responses at all time points measured. Further work by that group has found poor intrahorse repeatability of individual insulin concentrations at various time points when tested twice over 7-14 days in a population of horses considered insulin dysregulated. While the authors suggested that there was sufficient agreement when using a dichotomous outcome, assessment as a continuous variable is important for assessing risk of disease (magnitude of increase) and disease progression (improvement with management or therapeutic intervention). There may be an effect of breed on the OST. A recent study evaluated the OST with varying periods of fasting and suggested a difference between horses fasted and fed; those authors also found minimal increases in insulin concentrations in response to the corn syrup. Varied dosing (up to 0.45 mL/kg) and insulin cut-offs (normal < 30.2 μU/mL when administered at 0.25 mL/kg) have been recently described for the OST which may be more reproducible in other populations although the follow-up work has revealed only fair to poor repeatability despite using a lower cut-off or higher dose. Interestingly, all of the horses in this study would have been categorised as insulin sensitive with the new cut-offs, although not all time points were collected.

We found no significant effects of the OST on ACTH concentrations. Part of this may be related to the lack of an insulinaemic response. However, in a mixed breed population of unknown insulin dysregulation status, it has been shown that basal ACTH concentrations as part of TRH stimulation tests were not significantly affected by the OST.

An alternative to the OST is the in-feed glucose test (IFGT) although its reproducibility has not been completely investigated and there are effects of breed on markers of insulin sensitivity, although these likely reflect underlying predispositions to ID. The IFGT typically involves administering glucose in a non-glycaemic feed and measuring insulin concentrations approximately 2 hours later. There is literature supporting the reproducibility of the IFGT in ponies as well as a variant in-feed test using a pelleted pony cube mix of equivalent energy. Additionally, the interhorse variability of the IFGT appears lower than that of the OST.
Finally, glucose administered by nasogastric intubation correlates well with the HEC and FSIGTT, which may make it a better reflection of the true insulin dysregulation status of the horse.

There are several limitations of this research limiting its external validity, chiefly selection bias. However, even in ponies, the currently available data suggests the OST is not adequately reproducible. Furthermore, this study was not designed to evaluate the accuracy of testing, only the reproducibility. Given that the breeds in this study have been extensively used in studies used to characterise the OST, it is important to ascertain its validity, even if it would not be routinely employed in these breeds clinically.

Poor responses to the OST make interpreting its effects on ACTH concentrations difficult to extrapolate to animals that are insulin resistant. It had originally been planned to assess ACTH concentrations at T90 for the OST. This was not pursued due to the similar insulin responses observed at T60 and T90, and the lack of test effect on ACTH concentrations. Other time points should be reassessed in other populations given the data demonstrating differing times of peak insulin concentrations between ponies and horses.

There are data demonstrating that the chemiluminescent assay used in this study gives lower concentrations than radioimmunoassays. Unfortunately, the Siemens Coat-a-Count assay is no longer available in Australia. In contrast, the chemiluminescent assay is widely available in Australia, and its use likely represents the most clinically relevant testing method.

4.6 Conclusions

In this population of horses in Western Australia, the OST is not adequately reproducible. A more sensitive test, such as the IFGT or a variant thereof, may be more appropriate. Additionally, the insulinaemic responses of Thoroughbreds and Standardbreds to enteral sugars are similarly poor, suggesting that these breeds should not be used to validate tests of the enteroinsular axis. Finally, there does not appear to be a clinically significant effect of performing the OST on endogenous ACTH concentrations, although this should be confirmed in animals with larger insulin responses.
4.7 Table 1

Agreement analysis for the OST for T60 time points. Results are expressed as insulin concentrations in μU/mL. \(^a\)Back transformed data after log transformation. For the analysis between week 1 and week 2, the median of the differenced data was 1.005 μU/mL. In ninety five percent of cases, the week 1 median would be between 70% below or 200% above the week 2 median. For the analysis between week 1 and week 3, the median of the differenced data was 0.81 μU/mL. In ninety five percent of cases, the week 1 median would be between 20% below or 300% above the week 2 median.

<table>
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<th>Upper agreement limit (95% CI)</th>
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<td>N/A</td>
<td>N/A</td>
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<tr>
<td>Week 2 and Week 3 (^a)</td>
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<td>N/A</td>
<td>N/A</td>
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<td>Week 2 and Week 3</td>
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<td>4.62</td>
<td>-10.82 (-15.25, -6.38)</td>
<td>7.66 (3.23, 12.09)</td>
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### 4.8 Table 2

Agreement analysis for the OST for T90 time points. Results are expressed as insulin concentrations in μU/mL.

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<th>Upper agreement limit (95% CI)</th>
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</thead>
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<tr>
<td>Week 1 and Week 2</td>
<td>0.11 (-2.27, 2.49)</td>
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<td>-8.48 (-12.61, -4.36)</td>
<td>8.71 (4.60, 12.83)</td>
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<tr>
<td>Week 2 and Week 3</td>
<td>-1.32 (-3.02, 0.38)</td>
<td>3.08</td>
<td>-7.47 (-10.4, -4.5)</td>
<td>4.83 (1.88, 7.78)</td>
</tr>
<tr>
<td>Week 2 and Week 3</td>
<td>-1.43 (-3.56, 0.702)</td>
<td>3.80</td>
<td>-9.14 (-12.84, -5.44)</td>
<td>6.28 (2.58, 9.98)</td>
</tr>
</tbody>
</table>
4.9 Figure 1
Comparison between basal and T60 time points in test and control groups during the oral sugar test (with outlier included) demonstrating no significant difference between groups at either test time point ($P = 0.4272$). There was no effect of time ($P = 0.722$). Columns and bars represent the mean and standard deviation, respectively.
Chapter 5. Discussion
Pasture-associated laminitis is a debilitating disease of the foot that appears to be triggered by ingested simple CHO. Recent research has revealed the important role that insulin plays in the induction of laminitis. A genetic predisposition to excessive post-prandial hyperinsulinaemia likely results in insulin-mediated laminar pathology and failure, given (in)appropriate management conditions.

Insulin responses to oral CHO can also be used to assess and define the risk of laminitis in at-risk horses. Categorisation of horses as at-risk, however, relies on correctly identifying them as insulin dysregulated. In particular, the detection of excessive post-prandial hyperinsulinaemia may remain the most important and clinically relevant method to diagnose ID. In clinical practice, horses and ponies at high risk of laminitis might be easy to identify based on signalment, body morphology and management conditions. The sensitivity of a diagnostic test affects the post-test probability of that horse having that condition. Thus, in horses with a lower pre-test probability, more sensitive tests are required to correctly recognize ID in these horses. These tests should be satisfactorily reproducible such that changes over time accurately reflect disease state, and hopefully laminitis risk, rather than random variability or systematic bias. Furthermore, sensitive tests might be useful in detecting insulin dysregulation prior to corticosteroid administration in otherwise healthy horses, and thus be part of a risk-benefit analysis prior to therapy.

In this population of horses, the OST was found to not be adequately reproducible. While the absolute T60 SD were low, this was still deemed unsatisfactory given the low insulin concentrations post-syrup. Values less than the lower limit of detection (i.e. with a true value of less than 2 μU/mL) were recategorised as being at the lower limit (2 μU/mL). The anticipated effect of this was an increase in the reproducibility (and thus potentially a type 1 error). Despite this, in this population, the OST does not appear adequately reproducible, as has been found elsewhere in different populations of horses. While the test may be appropriately sensitive in horses with a high pre-test probability, this may not be true for all populations of interest.
The 2SIRT was also found to not be adequately reproducible. It was decided to use continuous data in case there were horses that had similar results centred around the cutoff, thus increasing the chances of finding the test reproducible. Indeed, some horses crossed categories each week or had ratios equal to 0.5. Even allowing for this, there was insufficient agreement between weeks. This is disappointing given the practical and straightforward nature of the test, which provides results quickly and with minimal equipment.

No significant effects of the 2SIRT or OST on ACTH concentrations were detected. Any observed effects are unlikely to lead to recategorisation of horses as false positives in the majority of horses given the bias, using regionally appropriate reference intervals. From a clinical perspective, while T0 sampling might more accurately represent the true disease status of the horse, post-test sampling is likely appropriate.

The results of the OST testing are consistent with minimal increases in insulin concentrations identified elsewhere in the literature \(^{39,283}\). There is likely a breed effect involved \(^{40}\), although insensitivity of the test is also suspected. The exact dose of sugar within the corn syrup used in the OST is unknown but was originally thought to be approximately 0.15g/kg of syrup as it was originally described \(^{340}\). Recent work has shown that the concentrations are much lower than this, with a higher dose resulting in 160.3 mg/kg bwt of sugars \(^{343}\). This may explain the poorer insulin responses observed in this study and the literature in comparison with those obtained using an IFGT, which has a dose of sugar approximately six times higher. The IFGT may also be a more flexible test for several reasons. Peak insulin concentrations in one study were found between two and four hours post-feed ingestion \(^{99}\) while in another, 90 minute insulin concentrations were not significantly different to two hour concentrations \(^{345}\). This may allow for more varied testing time points. This is in contrast with the OST where insulin concentrations were not different from baseline at four hours post-Karo syrup administration \(^{267}\). In addition, the IFGT, as well as being more practical in Australia (with regards to sugar source and test timing), has been shown to be reproducible in a group of ponies of unknown insulin dysregulation status \(^{345}\). That same study demonstrated that a commercially available pony pellet administered at approximately 0.3%...
bodyweight lead to similar insulin responses to glucose, which may make the test even more practical. When the same dose of glucose is administered by nasogastric tube \(^{282}\), insulin dynamics appear to better correlate with data from the HEC and FSIGTT than the OST \(^{39,321}\).

Recent literature has also described a modified IFGT using nasogastric intubation as a delivery method for the glucose \(^{361}\). This paper described higher cut-off values to separate horses with and without ID. The authors used a different insulin assay to that used in many other studies describing the IFGT (the Mercodia ELISA vs the Siemens Coat-A-Count RIA), however, making generalizability difficult to assess.

As previously mentioned, the degree of post-CHO hyperinsulinaemia may be able to predict the risk of laminitis in a given period \(^{91,99}\). As a patient-oriented outcome, this is likely of more use than cellular IR findings, which may not predict disease occurrence. In the Queensland study \(^{91}\), one third of ponies that had insulin concentrations of 50-84 μU/mL after 1g/kg of in-feed glucose (i.e. lower than that used to diagnose ID) developed laminitis after exposure to a high NSC feed for a relatively short period of time. Thus, cut-offs used to diagnose ID, typically defined in certain populations, should be interpreted in light of predisposing factors present in any individual case. The Queensland study above described an algorithm for detecting insulin dysregulation which correlated well with subsequent development of laminitis \(^{91}\), which is a potentially useful future diagnostic tool (Fig. 5.1). Importantly, those ponies who had post-sugar insulin concentrations of less than 50 μU/mL did not develop laminitis.

The literature suggests that glucose is the main stimulus for insulin release in horses \(^{77}\). The majority of glucose absorption occurs in the proximal small intestine and is mediated by SGLT1, GLUT2 and GLUT5 receptors \(^{362}\) – thus, these receptors may be an area of future research. It may be that differences in expression or function of these receptors accounts for interindividual differences in insulin responses, and thus laminitis risk. Given the potential role of the enteroinsular axis in hyperinsulinaemia, antagonism of GIP \(^{124}\) or GLP-1 \(^{125}\) may be an area of future potential research as a therapeutic option. Another potential therapeutic option is
inhibition of SGLT2. This cotransporter is responsible for reabsorbing the majority of filtered glucose in the nephron. Inhibition of this reabsorption has been shown to both reduce glucose and insulin responses to high NSC diets, and more importantly, to prevent laminitis associated with this diet.

![Classification and Regression Tree]

**Figure 5.1.** A classification and regression tree predicting the probability of laminitis in 37 ponies based on the retrospective serum insulin concentrations from an oral glucose screening test performed during enrolment (modified from Meier et al. 2018).

Another potential area of future research involves identifying factors leading to ID in subsets of PPID-affected horses. Specifically, it is unknown why some PPID horses might have ID but others not, despite otherwise similar signalment and management (i.e. independently from EMS). Whether it relates to individual POMC expression in diseased pituitaries, post-translational processing or secretory defects, or other factors, remains to be seen.
When TRH-stimulation tests were performed in a group of clinically normal horses with a low likelihood of PPID in two geographic locations in Australia during the quiescent phase of the circannual pituitary pars intermedia cycle, the ACTH concentrations were consistent with those previously reported for normal horses as confirmed by pituitary histology \(^{180,236}\). This is of note as the reference intervals for baseline ACTH concentrations at these locations in Australia \(^{210}\) differ from other locations (in the northern hemisphere) where the TRH-stimulated ACTH concentration cut-offs have been created \(^{238}\).

The results of the TRH-stimulation tests performed at March in Perth and April in Townsville suggest these time points are within the dynamic phase of the circannual pituitary cycle as marked variation in post-TRH ACTH concentrations occurs. Although a recent abstract \(^{243}\) suggested post-TRH cut-offs during the dynamic phase of the circannual pituitary cycle, the study numbers were small. Given the marked variability in endogenous ACTH concentrations after TRH stimulation in this study, further work is required to determine the results of TRH-stimulation testing in PPID horses during the dynamic phase of the circannual pituitary cycle.

In the future, more specific ACTH assays may lead to more accurate diagnostic results, while further research into POMC post-translational handling or secretory defects may shed further light on the pathogenesis of PPID.
Chapter 6. Epilogue
It has previously been claimed that a ‘sugar conspiracy’ existed in the mid-to-late 20th century such that the sugar industry suppressed scientific research linking high sugar diets with human cardiovascular disease, and allegedly promoted low-fat diets instead 364. More recent research, however, has suggested that the rise of the low-fat diet was based on early epidemiologic studies into cardiovascular disease, in combination with unsubstantiated, but not dishonest, extrapolations in the media due to topical events 365. Further disproving inappropriate links between low-fat diet researchers and the food industry, Mark Hegsted, who was involved in research evaluating the effects of diet on cardiovascular disease, was fired by the Reagan administration for upsetting the US beef industry by claiming that the American diet was too high in protein 365. At the same time, John Yudkin discovered that sugar was linked to increased odds of cardiovascular disease, albeit based on small numbers 366.

A sugar industry-sponsored review of dietary factors related to cardiovascular disease did emphasise fat as a cause of heart disease 367, but only because Hegsted did not believe there was, as yet, sufficient evidence to incriminate sugars 365. It should be noted that Yudkin himself, one of the only researchers investigating the link between sugar and cardiovascular disease, also received funding from industries that stood to benefit from his theories 365. Yudkin and Hegsted became rivals, with Yudkin ultimately losing when a working party of the British Medical Research Council found little evidence supporting his ideas 368. Hegsted also edited the US senate committee’s 1977 recommendations for dietary goals, emphasizing a low fat diet 365.

Yudkin also had another vocal critic in Ancel Keys 369, an epidemiologist who showed, through an early example of ‘big data’, the correlation between high saturated fat intake and cardiovascular disease deaths 370. However, as this Seven Countries study continued into five decades, multiple follow-up papers have made different conclusions.
While the evidence on dietary risk factors of cardiovascular disease is incomplete, it seems that the ‘sugar conspiracy’ actually reflects insufficient available evidence, together with personal rivalries, more than industry sabotage and unethical behaviour.

It is interesting to speculate on the implications for equine laminitis had the link between sugar intake and cardiovascular disease been further emphasized 50 years ago, given the implications of high NSC diets on equine foot health.
Chapter 7. References


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Chapter 8. Appendices

8.1 ACTH concentrations (pg/mL) before and 10 minutes after TRH administration in Perth

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### 8.2 ACTH concentrations (pg/mL) before and 30 minutes after TRH administration in Townsville

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### 8.3 Percentage increases in ACTH concentrations (pg/mL) after TRH administration in Perth and Townsville

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### 8.5 Repeatability of the T30 timepoint glucose concentrations (mmol/L) as part of the 2SIRT

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### 8.8 Insulin concentrations (µU/mL) during the OST in treatment horses

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### 8.10 Comparison of 2SIRT and OST by insulin dysregulation categorisation

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</table>
‘Alle Ding sind Gift und nichts ohn’ Gift; allein die Dosis macht, das ein Ding kein Gift ist

All things are poison and nothing without poison; only the dose makes that a thing is no poison’

*Paracelsus (1493-1541)*

(like sugar)

‘Ah, there’s nothing more exciting than science. You get all the fun of sitting still, being quiet, writing down numbers, paying attention…..Science has it all.’

*Principal Seymour Skinner, The Simpsons (1987-)*

‘til next time