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# **What Causes Equine Laminitis?**

The role of impaired glucose uptake

by Martin Sillence, Katie Asplin, Christopher Pollitt and Catherine McGowan

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***Investigating the role of impaired glucose uptake in laminitis***

*Publication No. 07/158*

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# Foreword

The horse industry makes a significant economic and cultural contribution to Australia, and the maintenance of horse health and welfare is a primary concern of owners, trainers and veterinarians alike. Laminitis is a painful and devastating disease that can cripple a horse and end its productive life. There has been considerable research over recent years in attempts to better understand the pathogenesis of laminitis, but scientists have struggled to reconcile the wide range of apparently unrelated factors which can trigger the condition.

This report describes a research project that has focussed on hormone and metabolic aspects of laminitis. Initially, the research set out to prove that laminitis was caused through glucose deprivation in the horse's hoof, as a result of insulin resistance. However, when this theory was proven to be incorrect, a slight adjustment in the experimental strategy led to a major breakthrough. By following a particular line of investigation regarding a single cause of laminitis, the researchers have uncovered a mechanism which appears to be central to the disease, as it helps to unite a number of themes and explain several trigger factors.

The importance of this report is that it provides a clearer direction for horse owners, veterinarians and scientists regarding one piece of the puzzle that is laminitis. Based on this new knowledge, strategies are outlined to identify horses at risk of laminitis, to prevent laminitis from occurring, and to treat the condition early in its development. The report also highlights an opportunity for further research which will open even more avenues for more effective therapy.

This project was funded in part from industry revenue which is matched by funds provided by the Australian Government. Additional support was provided by Charles Sturt University and The University of Queensland.

This report, an addition to RIRDC's diverse range of over 1600 research publications, forms part of our Horse R&D program, which aims to support research into disease prevention, diagnosis and treatment, as well as animal breeding and genetics. Other strategic priorities include fostering industry development, environment and welfare, and developing human and animal health and safety.

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## Abbreviations

ANOVA	analysis of variance
bp	base pairs
bpm	beats per minute
BWT	body weight
°C	degrees Celsius
<sup>14</sup> C	radioactive carbon
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
Ci	Curies
CO <sub>2</sub>	carbon dioxide
DMSO	dimethylsulphoxide
dpm	disintegrations per minute
et al.	and others
g	grams
GLUT	glucose transport protein
h	hours
<sup>3</sup> H	tritium (radioactive isotope of hydrogen)
iv	intravenous
K <sub>D</sub>	equilibrium dissociation constant (binding affinity)
kg	kilograms
mg	milligrams
min	minutes
ml	millilitres
mm	millimetres
mM	millimoles per litre (10 <sup>-6</sup> moles per litre)
mMol	millimoles (10 <sup>-6</sup> moles)
mmol/L	millimols per litre (10 <sup>-6</sup> moles per litre)
mRNA	messenger ribonucleic acid
mU	milli international units (10 <sup>-3</sup> international units)
n	number of observations or animals
NaOH	Sodium hydroxide
nM	nanomoles per litre (10 <sup>-9</sup> moles per litre)
P	probability value
PCR	polymerase chain reaction
pM	picomoles per litre (10 <sup>-12</sup> moles per litre)
RNA	ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
s.e.	standard error
µL	microlitres (10 <sup>-6</sup> litres)
µm	micrometres (10 <sup>-6</sup> metres)
µM	micromoles per litre (10 <sup>-6</sup> moles per litre)
µU	micro international units (10 <sup>-6</sup> international units)
w/v	weight per volume
yrs	years

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# Executive Summary

## What the report is about

Based on ancient writings, the painful and crippling disease of laminitis is thought to have plagued horses and their owners for more than 2000 years. Part of the challenge for scientists who have tried to understand this disease is the wide range of conditions that can lead to laminitis. Among other factors, the triggers to laminitis can be dietary, drug-related, or may involve diverse medical conditions which could be described loosely as 'stressful' to the horse. Underlying factors may include a genetic component, plus a dysfunctional hormone and metabolic system.

This report describes a significant breakthrough in laminitis research. Many of the predisposing factors are explained and a number of triggers can now be understood, through the identification of a common causative agent. This report is designed to inform horse owners, clinical veterinarians and research scientists about this discovery and its immediate implications for prevention and treatment, as well as informing future research aimed at new therapeutic options.

## Background

This project was conceived after an earlier study showing that horses with Equine Cushing's Syndrome are more likely to develop laminitis and survive less than two years after diagnosis, if they also have high serum concentrations of insulin. Equine Cushing's Syndrome is a disease more often occurring in ponies and older horses, and is characterised by the excess production of cortisol. Although this is not the most common cause of laminitis, there is a likely link between this chronic disease and the short-term use of synthetic cortisol-like drugs (to treat inflammation), which can also trigger laminitis causing great concern to owners and veterinarians.

To an endocrinologist, the most obvious connection between cortisol and insulin is glucose. Following a stressful stimulus, high concentrations of cortisol act in concert with adrenaline to provide fuel for the brain to coordinate a 'flight or fight' response. This is achieved by increasing the synthesis of glucose from other energy substrates, releasing glucose from storage sites, and preventing tissues other than the brain from taking glucose out of the circulation. In this regard, cortisol and insulin oppose one another. The main role of insulin is to maintain normal blood glucose concentrations by stimulating the uptake of excess glucose by many tissues, such as skeletal muscle. To achieve this, most cells contain a specific type of glucose transport protein (GLUT4-type) which depends on insulin for its activation.

In a healthy horse, a short-term increase in glucose caused by stress, exercise, or a meal, would be followed by an increase in insulin, so that blood glucose concentrations do not rise too high, the brain is not overloaded with glucose, and the tissues are able to take up this vital energy source (Figure 1a). However, when the glucose transport proteins are overworked (e.g. through chronically elevated cortisol concentrations or chronic overfeeding), they become less responsive to insulin, such that glucose intolerance/insulin resistance develops. The defining indication of this condition is that individuals have much higher insulin concentrations than normal following a glucose challenge (Figure 1b).

This project was based on the theory that insulin resistance is the factor that predisposes horses to laminitis, and that damage to the hoof is a direct consequence of glucose deprivation. The hoof is known to have an unusually high metabolic demand for glucose, and previous work using isolated tissue had demonstrated that the lamellae become separated if the glucose supply is blocked.

## **Aims and objectives**

The aim of the project was to test the hypothesis that laminitis is caused by impaired glucose uptake in the hoof, as a result of cortisol and/or adrenaline action, coupled with insulin resistance. Establishing this fact would provide a clear direction for future research into appropriate treatments. However, before the hypothesis could be tested in tissue from horses with laminitis, it was necessary to determine the normal rate of glucose uptake and confirm the mechanisms which control this in healthy tissue. In particular, it was important to confirm that the hoof contains GLUT4-type glucose transporters that are dependent on insulin for their activation, and that hormones such as adrenaline and cortisol are able to oppose insulin action in the short-term and long-term, respectively.

## **Methods**

Samples of hoof tissue were collected from horses soon after they had been killed at a local abattoir. The samples were cut into thin slices and incubated in a warm nutrient medium allowing them to remain intact for several days. A modified form of glucose that had been synthesised with a radioactive marker was used to measure the rate of glucose uptake into the tissue, and various drugs and hormones were added to study their effects. In complementary studies, the number of  $\beta$ -type adrenaline receptors ( $\beta$ -adrenoceptors) was identified, also through the use of radio-labelled compounds.

## **Results**

- The dominant type of adrenaline receptor in the hoof was identified as the  $\beta_2$ -adrenoceptor, similar to that found in skeletal muscle. A small proportion of  $\beta$ -adrenoceptors (10%) were of the  $\beta_1$ -type, similar to those found in the heart;
- we confirmed that the  $\beta$ -adrenoceptors in lamellar tissue have a functional effect, showing that their activation with a  $\beta$ -agonist drug can reduce glucose uptake by 20 to 40%;
- we also noted that the number of  $\beta$ -adrenoceptors in lamellar tissue varies markedly between horses, and that only half the animals tested showed a glucose response to a  $\beta$ -agonist drug. This variation did not show a strong association to breed, age, or gender of the horses;
- the most significant but unexpected finding, was that hoof tissue is not dependent on insulin for glucose uptake, nor does it show a glucose response to insulin, even at high insulin concentrations;
- further studies showed that the glucose transport proteins in hoof tissue do not become saturated at low glucose concentrations (unlike typical GLUT4 transporters), but that they behave in a similar manner to the insulin-independent GLUT1 transporters usually found in the brain;
- finally, a technique was developed to measure the expression of genes for equine glucose transporters. This was used to confirm the presence of GLUT1 transporters in brain and lamellar tissue, whereas GLUT4 transporters were found in skeletal muscle, but not in lamellar tissue.

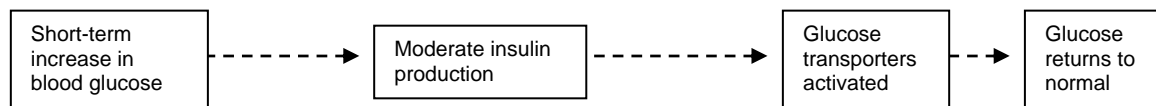
By confirming that the hoof is not dependent on insulin for glucose uptake, we had rejected the null hypothesis that glucose deprivation due to insulin resistance is the cause of laminitis.

### ***Additional findings and a new hypothesis***

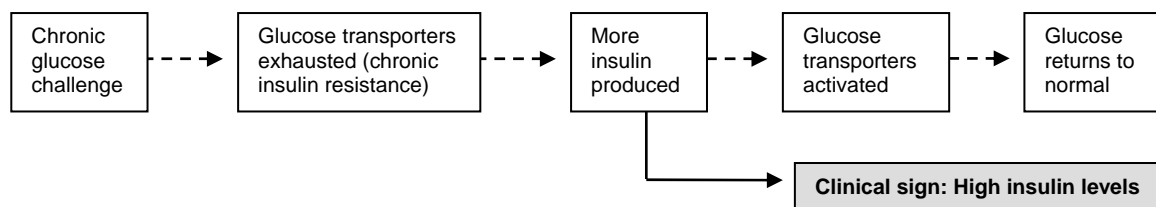
Despite eliminating impaired lamellar glucose uptake as a key trigger for laminitis, the question of how cortisol, adrenaline and insulin may interact to cause laminitis, remained. Furthermore, new evidence was published to show that high insulin concentrations are also a characteristic of ponies that are predisposed to pasture-induced laminitis, a condition in which there is an unusually high seasonal intake of non-structural carbohydrates.

By re-arranging the proposed relationship between cortisol, glucose, insulin and glucose transport, we developed a new hypothesis, which is outlined in Figure 1.

#### **a. Healthy horse + glucose challenge**



#### **b. Glucose intolerant horse**



#### **c. Glucose intolerant horse + glucose challenge**

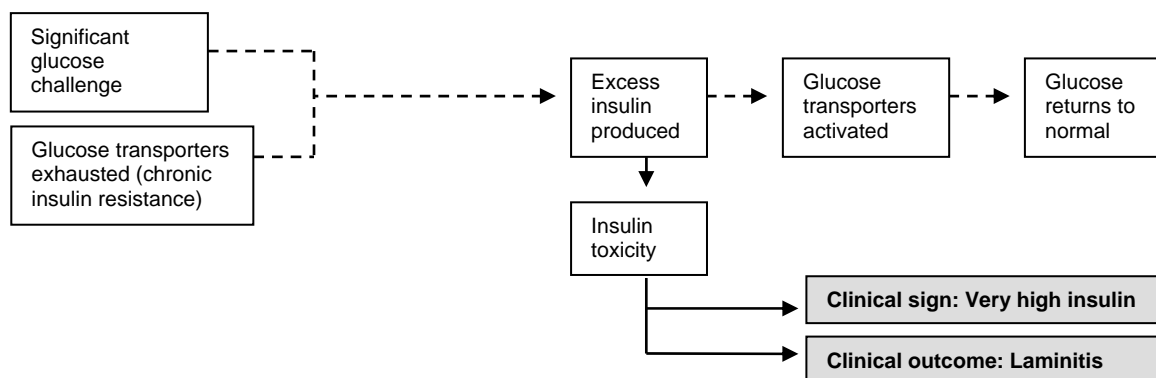


Figure 1. Proposed relationship between glucose, insulin resistance/glucose tolerance and laminitis. (a) moderate insulin production occurs in a healthy horse when glucose concentrations rise following a meal or stressful event; (b) Chronic overfeeding, Cushing’s Syndrome, overuse of corticosteroids and/or genetics can cause a horse to become intolerant to glucose (insulin-resistant); (c) A significant glucose challenge resulting from stress or the consumption of lush pasture in a glucose-intolerant horse, leads to a massive insulin response which is able to restore blood glucose concentrations, but precipitates laminitis.

According to this hypothesis, laminitis occurs as a consequence of insulin resistance, coupled with a significant glucose challenge. The underlying cause of insulin resistance may involve a number of factors including Cushing’s syndrome, excessive use of corticosteroid drugs, obesity, overfeeding, or a genetic component. However, the high concentrations of insulin occurring in these horses are not merely a sign of failing glucose transporters, but are the direct cause of laminitis. Accordingly, for this hypothesis to be correct, high concentrations of insulin would be expected to induce laminitis in any horse.

For our final experiment nine ponies were selected with no clinical signs of illness and no recorded history of laminitis. Five ponies received a constant infusion of insulin for up to 72 hours, together with an infusion of glucose to maintain normal resting glucose concentrations. The other four ponies received a saline solution and served as controls.

All the ponies given insulin developed laminitis within 58 to 72 hours, diagnosed by clinical signs and confirmed by histological examination of their hooves. None of the controls developed laminitis.

### **Implications**

We have shown that high concentrations of insulin cause laminitis. This occurs as a result of a glucose challenge, combined with glucose intolerance (insulin resistance) which may be inherited, or developed over a long period of illness, corticosteroid use, or dietary mismanagement. Insulin toxicity may not account for all cases of laminitis, but the concept unites a number of major themes and explains several known triggers. It also provides a clear pathway towards reducing laminitis by:

- identifying animals at risk through the use of insulin measurements and a glucose tolerance test;
- avoiding the use of corticosteroids or high-energy diets in such animals;
- intervening to restore glucose tolerance through diet and exercise, or with the use of glucose-sensitising agents; and
- conducting further research to discover the mechanism of insulin action, leading to new avenues for therapy.

### **Recommendations**

A simple, rapid and reliable glucose tolerance test should be employed to identify horses at risk of hyperinsulinaemia and laminitis.

It is important that all horse owners become aware of the risk associated with prolonged hyperinsulinaemia and take appropriate action to avoid this.

While some information about restoring insulin sensitivity/glucose tolerance is already available to guide horse owners, further research is needed to test novel pharmaceutical, nutraceutical and dietary approaches.

The techniques used by different laboratories to measure insulin concentrations in horses need to be standardised using a national or international reference standard, with appropriate quality controls, so that meaningful clinical comparisons can be made.

The potential of corticosteroid drugs to cause glucose intolerance should be investigated further.

Meanwhile, if corticosteroid drugs are indicated to treat inflammation or other conditions, the user should be mindful of the risks, particularly in horses that are intolerant to glucose. Monitoring insulin concentrations in horses that require corticosteroid therapy may be useful in detecting when insulin resistance becomes clinically significant.

The mechanism by which insulin causes laminitis should be investigated further, with a view to discovering new avenues for therapy.

# Introduction

Laminitis is a disease of horses and ponies where the hoof wall becomes detached from underlying tissue and in severe cases the so many laminae are affected the hooves can no longer support the animal's weight. It is a painful condition which can cripple a horse and end its productive life. Laminitis is known to occur in response to a wide range of trigger factors, including carbohydrate overload (Garner et al. 1975), overfeeding (Harkema et al. 1978) and excess dietary oligofructose (VanEps and Pollitt 2006). These dietary triggers have been the focus of much research, particularly in the area of hind-gut metabolism and the search for plant and microbial toxins (Galey et al. 1991).

Other triggers to laminitis are associated with abnormal hormone activity and may be described as endocrine or 'endocrinopathic' in origin. The principal hormones in this case are cortisol and insulin. Thus, laminitis has been associated with the excessive use of synthetic corticosteroid drugs to treat inflammation (Harkins et al. 1993, Bailey and Elliott 2007), and is commonly associated with Equine Cushing's Syndrome, where excess cortisol production occurs through the failure of central control mechanisms in the pituitary gland. In both these situations, insulin resistance can occur.

In fact, most horses that suffer Equine Cushing's Syndrome develop abnormally high insulin concentrations as a clinical sign of insulin resistance. Further, serum insulin concentrations are a powerful prognostic marker, such that animals with abnormally high concentrations of insulin as well as cortisol, are much more likely to develop laminitis and survive less than two years after diagnosis, than animals with Cushing's Syndrome and normal insulin concentrations (McGowan et al. 2004).

A connection between insulin resistance and laminitis was first proposed by Field and Jeffcott (1989) as part of their hypothesis for the pathogenesis of laminitis associated with abnormal carbohydrate metabolism. This research was largely overlooked, however, until Johnson described a syndrome of insulin resistance linked to obesity (Equine Metabolic Syndrome) in animals that were predisposed to laminitis (Johnson 2002).

More recent studies focussing on a predisposition to pasture-induced laminitis have also strongly implicated insulin. Treiber et al. (2005, 2006) made a series of measurements on a group of ponies that are particularly prone to development laminitis when placed on lush pasture. These workers describe a 'Prelaminitic Metabolic Syndrome' and among other signs, ponies with this Syndrome had twice the normal blood concentration of insulin when grazing on winter pasture. In spring, when lush pasture was available, the insulin response to this extra carbohydrate intake in the ponies that actually developed laminitis, was more than five times the normal response (Treiber et al. 2006).

Based on these observations, it seemed that insulin resistance was the clue to a common mechanism that could explain why laminitis is triggered by such seemingly diverse factors as Equine Cushing's Syndrome, anti-inflammatory drugs and energy-rich pastures. The factor which connects insulin, cortisol and carbohydrates is glucose, as this is the end-product of carbohydrate digestion in the horse, and the main role of cortisol and insulin is to control glucose metabolism.

## Glucose metabolism, cortisol and insulin resistance

Under normal circumstances, when glucose enters the circulation after a meal it is disposed of rapidly through uptake by the liver, muscles and adipose tissue. An efficient uptake system is necessary to maintain blood glucose concentrations within a narrow range; otherwise the brain (which is freely permeable to glucose) becomes starved or overloaded, potentially leading to unconsciousness. In most tissues glucose uptake relies on specific transport proteins controlled by insulin. Glucose intolerance (or insulin resistance) occurs when these transport proteins fail or become overused, such as in the case of human Type 2 diabetes, or its counterpart 'Equine Metabolic Syndrome' (Johnson 2002). The body responds to this problem by producing increasing amounts of insulin, until the insulin signal becomes strong enough to reactivate the transporters and clear the excess glucose from the blood. However, unlike humans whose pancreas will eventually fail if pressed into continually secreting large amounts of insulin, the equine pancreas appears to be inexhaustible, so that glucose intolerant horses

and ponies can produce insulin concentrations that are at least two orders of magnitude (100 times) higher than resting concentrations.

This project was conceived on the premise that the high concentrations of insulin reported in laminitis-prone ponies, and in horses with Equine Cushing's Syndrome, were primarily a sign of glucose intolerance i.e. a failure of glucose transport mechanisms. Thus, the first question to be addressed was - *How important is glucose uptake in the equine hoof?*

## Glucose and the equine hoof

It has been known for some time that healthy hoof tissue has an absolute requirement for glucose, such that when hoof explants are incubated in the absence of glucose, or in the presence of a glucose uptake inhibitor, the layers of tissue separate rapidly, as they do when laminitis occurs (Figure 2, Pass et al. 1998). Additionally, the hoof utilises glucose at an exceptionally fast rate compared with most other tissues (Wattle and Pollitt 2004), so in theory, even a small decrease in the rate of glucose uptake could be extremely damaging.

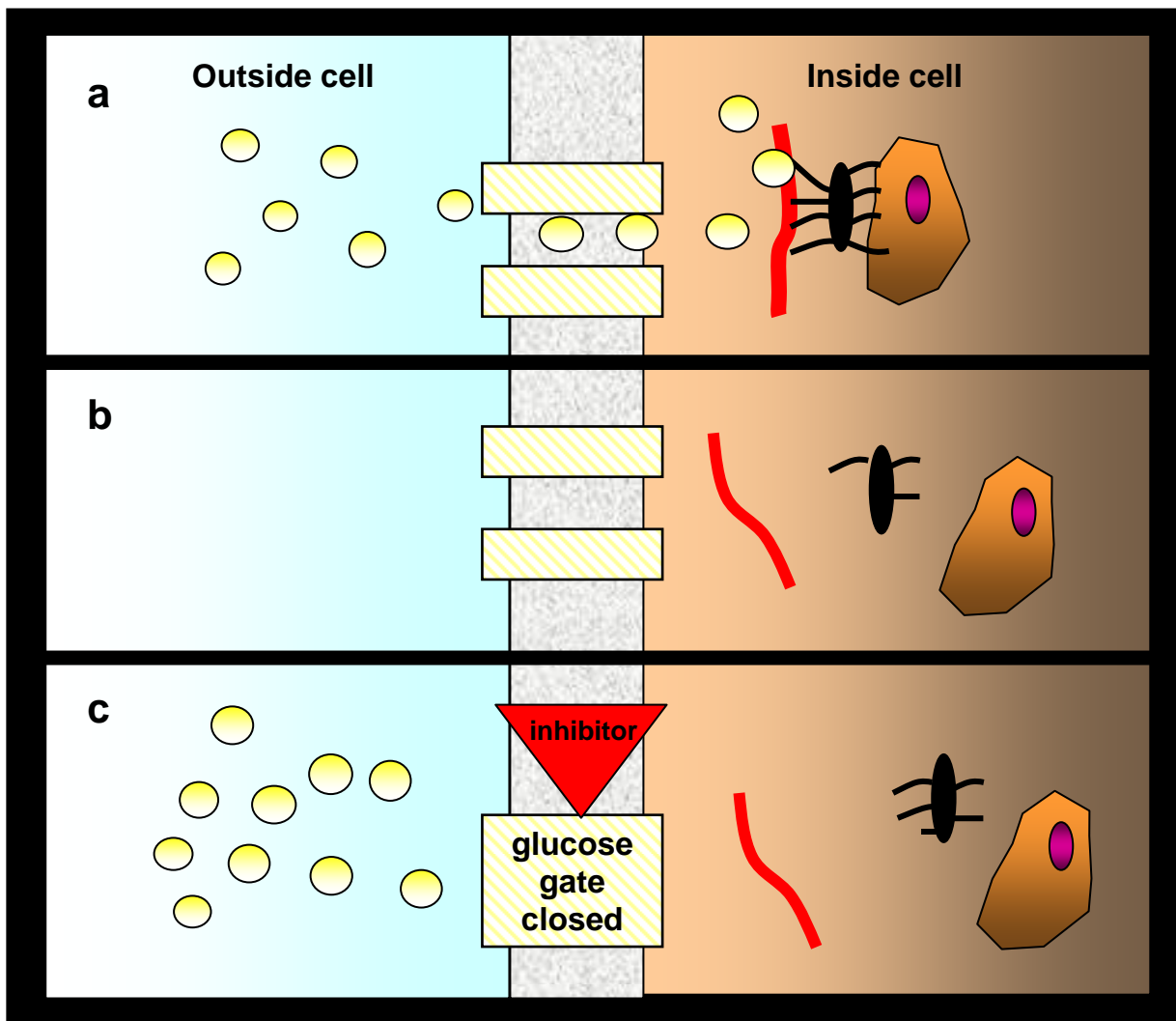


Figure 2. Schematic representation of (a) normal hoof tissue explant showing attachment between the dermis and epidermis by hemidesmosomes under conditions where there is a constant supply of glucose, (b) destruction of the hemidesmosomes and detachment of the membranes occurs when the tissue is incubated without glucose, (c) detachment of the membranes occurs when glucose is prevented from entering the cell by a glucose uptake inhibitor (Adapted from Pass et al. 1998).

Under normal circumstances, insulin stimulates the uptake of glucose by most tissues outside the brain, so that blood glucose concentrations are held constant and the tissues are supplied with energy. Under conditions of extreme stress, cortisol and adrenaline inhibit the action of insulin, providing more glucose for the brain to metabolise and allowing a rapid ‘flight or fight’ response. Thus, in the long-term, excess corticosteroids (via medication or disease states) and insulin resistance are both conditions associated with decreased glucose uptake by most tissues of the body.

Corticosteroids inhibit insulin-mediated glucose uptake by a direct mechanism that limits the movement of glucose transporters to the cell surface (Figure 3). Corticosteroids also act indirectly by causing the cell to produce more receptors for adrenaline, principally the  $\beta$ -adrenoceptors (McGraw et al. 1995, Huang et al. 1998, 2000). In turn, adrenaline blocks glucose uptake by inactivating insulin receptors (Carpene et al. 1993). In summary, cortisol can trigger a complex cascade of both short-term and long-term events that limit glucose utilisation by peripheral tissues.

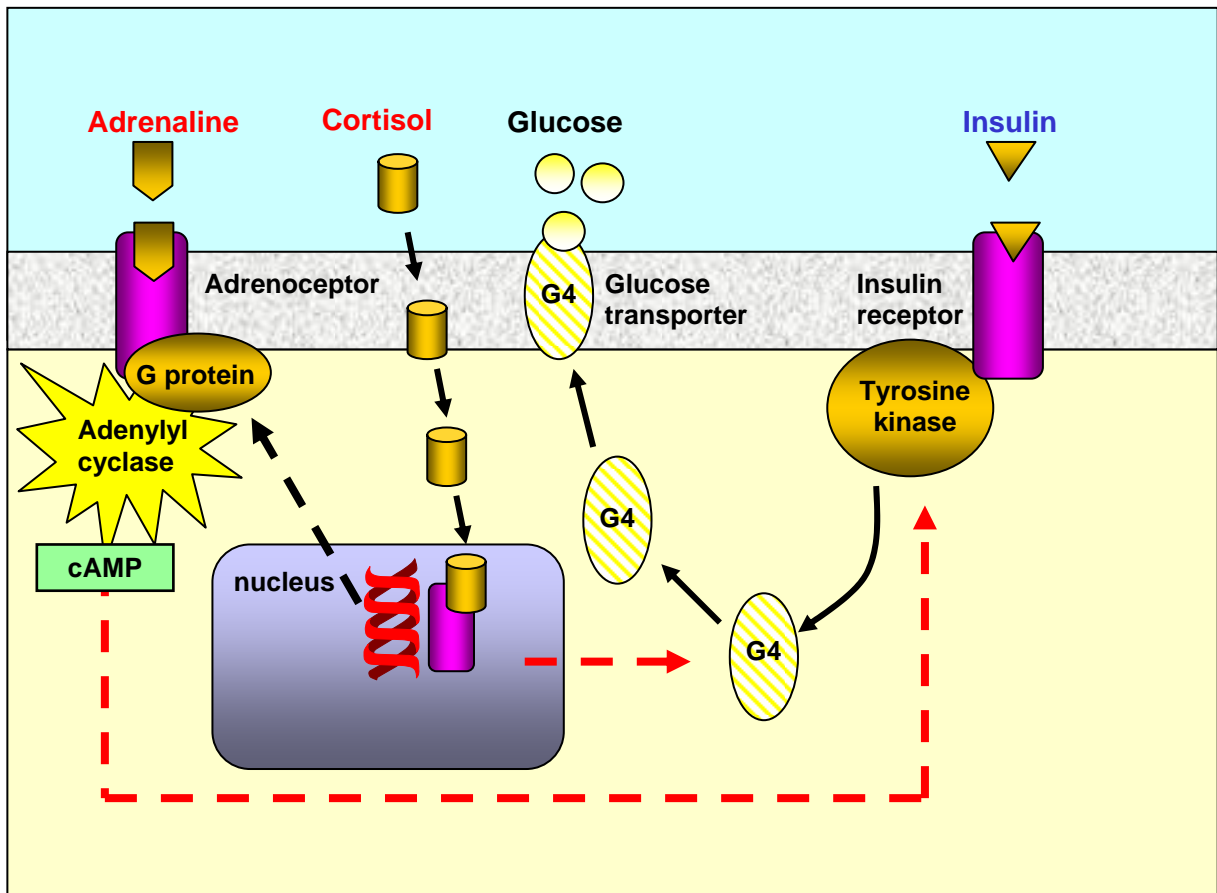


Figure 3. Schematic representation of the control of glucose uptake in mammalian cells, showing activation of the GLUT 4 (G4) transport proteins by insulin (solid black arrows), and the inhibition of this process by cortisol and adrenaline (dashed red arrows).

Our first hypothesis concerning the cause of laminitis was based on the theory that hormones such as cortisol and adrenaline may limit glucose uptake by the hoof. Under normal circumstances their effects would be counteracted by insulin, explaining why laminitis does not occur in every horse with Equine Cushing’s Syndrome, or each time a corticosteroid drug is administered. However, in horses that have become resistant to the effects of insulin, the glucose uptake would be impaired to a much greater extent, and with its high metabolic demand the hoof may soon begin to suffer glucose deprivation. The lack of this vital energy substrate would then lead to separation of the basement membrane, as reported for isolated tissues when deprived of glucose.

To test this hypothesis it was necessary to study glucose uptake by the equine hoof. While much was known about glucose transport in other species, including the discovery of many different types of

glucose transport protein (Khan and Pessin 2002), few studies had been conducted in horses. The predominant glucose transporter in the equine hoof was unknown, and the actions of insulin, cortisol and adrenaline had not been tested. Thus, before any judgements could be made about the atypical nature of glucose uptake in laminitic tissue, it was necessary to characterise the normal equine hoof.

## Objectives

The original aims of the project were as follows.

1. Identify the mechanisms that control glucose uptake in healthy lamellar tissue
2. Confirm that impaired glucose uptake is a characteristic of lamellar tissue taken from horses with clinical signs of laminitis, that has been triggered by a variety of metabolic causes
3. Determine which glucose control pathways are dysfunctional in lamellar tissue, and identify any differences in histopathology when laminitic tissue from different metabolic conditions is compared
4. Determine the extent to which glucose uptake can be increased or normalised *in vitro*, through the use of agonist and antagonist drugs that target specific control pathways

After an initial series of experiments, the results caused us to reject our initial hypothesis and to develop a new aim.

5. Determine whether laminitis is caused by insulin toxicity

## Methodology

### 1. Characterising and measuring adrenaline receptors in the hoof

Adrenaline causes its effects by binding to adrenoceptors, which are specific proteins found on the surface of most cells. When an adrenoceptor is activated, production of the second messenger cAMP is increased. It is the cAMP which can interrupt the action of insulin receptors by blocking the second messenger for insulin, tyrosine kinase (Figure 3).

There are two main types of adrenoceptor termed alpha ( $\alpha$ ) and beta ( $\beta$ ), each with various sub-types that predominate in different tissues.  $\beta$ -Adrenoceptors are known to influence glucose uptake in other species, and so these were the focus of the present study. There are three sub-types of  $\beta$ -adrenoceptor which are all involved in glucose and fatty acid metabolism.  $\beta_1$ -Adrenoceptors are found mainly in the heart and adipose tissue, whereas  $\beta_2$ -adrenoceptors tend to predominate in other important sites for glucose disposal, namely liver and skeletal muscle (Sillence et al. 1993).  $\beta_3$ -Adrenoceptors are also known to exist, but are not widely distributed in the body. Furthermore, there is a lack of highly selective drugs suitable for their study. The present research focussed on  $\beta_1$ - and  $\beta_2$ -adrenoceptors.

### Research strategy

The first aim of this study was to identify the predominant subtype of  $\beta$ -adrenoceptor in the equine hoof. This was achieved by identifying a radio-labelled drug that would bind to both equine  $\beta_1$ - and  $\beta_2$ -adrenoceptors, then studying the ability of various sub-type selective drugs to displace the radioligand. Hoof tissue was compared with other reference tissues including heart and skeletal muscle, which were all collected from horses freshly killed at an abattoir.

Once the main receptor sub-type had been identified, the next objective was to measure the normal level of expression of these receptors in tissue from healthy horses, and to identify any factors that may influence this, such as age or gender. This would allow future experiments to determine whether tissue from horses with laminitis was abnormal, as shown by an unusually high or low level of receptor expression. The significance of this research is that the number of receptors in a tissue is

important in determining the size of response, when the tissue is activated by a drug or hormone such as adrenaline.

### **Preparation of adrenoceptors from equine hoof tissue**

Hoof tissue was obtained from 27 horses aged between 2 and 30 years that had been killed at a commercial abattoir. The forelegs were removed and cut into small explants using a band-saw (Figure 4). These explants were then trimmed of excess tissue, leaving the hoof wall and connective tissue intact. The explants were then frozen rapidly using liquid nitrogen and transported to the laboratory for storage at  $-80^{\circ}\text{C}$ .



Figure 4. Dissection of an equine hoof to obtain an explant containing the hoof wall and lamellar tissue.

The method used to isolate tissue cell membranes was derived from procedures developed by Sillence et al. (1993). Briefly, about 5g of frozen tissue was weighed and finely sliced. This tissue was then placed in to an ice-cold buffer solution and liquidised using a tissue homogeniser. The homogenate was then centrifuged for 10 min at low speed to remove any large particles. The liquid component was then removed and filtered through three layers of gauze, before being centrifuged again at medium speed for 15 min to remove remaining mitochondria, nuclei and cell debris. Using a pipette, the resulting liquid was transferred to clean tubes, and the pellet was discarded. The tubes were then topped-up with homogenate buffer and centrifuged at high speed for 30 min. This resulted in a small brown pellet containing cell membrane fragments, which was suspended in 1 mL of incubation buffer and stored at  $-80^{\circ}\text{C}$ . All operations were performed at or below  $4^{\circ}\text{C}$ .

### **Radioligand binding studies**

The number of  $\beta$ -adrenoceptors present in a known quantity of cell membrane was measured using a radioligand binding technique. A radiolabeled drug (radioligand) was used that binds to all three subtypes of  $\beta$ -adrenoceptor ( $[^3\text{H}]\text{CGP-12177}$ , specific activity of 44.5 Ci/mMol). All reagents were prepared in solutions of incubation buffer and polypropylene tubes were used throughout.

After an incubation for 90 min, the membrane-bound and free radioligand were separated by filtration over presoaked glass-fibre filter papers using a cell harvester adapted for radioligand binding studies. The test-tubes were rinsed with ice-cold incubation buffer to ensure efficient recovery of membrane-bound radioligand and to minimise non-specific binding to the filter paper. The radioligand bound to the cell membrane fragments was retained on the filter papers and these were soaked overnight in 3 mL of scintillation fluid. The tubes were then shaken thoroughly and the radioactivity in them was counted using a liquid scintillation counter.

## **2. Measuring glucose uptake**

A series of more than 20 experiments was performed to investigate the control of glucose uptake in hoof tissue collected from horses which had shown no signs of clinical disease prior to their being killed at a local abattoir.

Many explants were cut from each hoof as described earlier, but instead of being frozen, the fresh tissue was sliced immediately into sections of 5 mm x 1 mm. The slices were then incubated in a tissue

culture medium (Dulbecco's Modified Eagle Medium) containing an antibacterial agent (gentamicin) and varying concentrations of glucose, to which the radioactive marker (2-deoxy-*D*- [2,6-<sup>3</sup>H] glucose) had been added. The incubations were conducted at 37°C under an atmosphere of 95% O<sub>2</sub>/5% CO<sub>2</sub>, and the viability of the explants during this period was demonstrated using inulin [<sup>14</sup>C]carboxylic acid as a marker to measure any extracellular seepage.

The glucose uptake experiments focussed on four specific areas.

#### **a. Confirming the requirement for glucose**

To confirm that lamellar tissue has an absolute requirement for glucose, tissue was incubated for 48 hours either in the presence of glucose (25 mM) or the absence of same. After incubation the force required to separate the lamellae from the hoof wall was measured by connecting the tissue between a strain-gauge and a micromanipulator, then applying a steady increase in force until the tissue separated.

#### **b. Effect of stimulating adrenaline receptors**

The likely effects of adrenaline were investigated using the synthetic drug isoprenaline, which is a stable, potent and selective activator of β<sub>1</sub>- and β<sub>2</sub>-adrenoceptors. Incubations were conducted for various periods ranging from 10 min to 72 hours, and in the presence of various concentrations of isoprenaline.

At the end of each incubation period the explants were removed from the medium, washed in fresh cold medium to stop glucose transport and remove unbound glucose, then blotted and weighed. The tissues were then dissolved in NaOH and the amount of radioactive 2-deoxy-*D*- [2,6-<sup>3</sup>H] glucose taken up was measured using a liquid scintillation counter (LKB-Wallac 1409, Turku, Finland).

Subsequent experiments examined the effects of isoprenaline in combination with various other drugs and hormones (cortisol, β-blocker, insulin), over different incubation periods (24h, 48h, 72h) and with different concentrations of glucose in the incubation medium (9 mM, 25 mM). These experiments were performed using tissue from 12 horses.

#### **c. Effect of insulin**

A series of experiments was performed to examine the effects of insulin on glucose uptake, when present at different insulin concentrations, different glucose concentrations, and over various periods ranging from 10 min to 72 hours.

#### **d. Determining the saturation point of glucose transport proteins**

The lack of response to insulin in earlier experiments was interpreted to suggest that glucose uptake in the equine hoof may be mediated through non insulin-dependent GLUT1-type glucose transport proteins, rather than by the insulin-dependent GLUT4-type transporters found in many other tissues.

A characteristic difference between GLUT1 and GLUT4 transporters in other species is that GLUT1 transporters have the capacity to increase their rate of glucose uptake in proportion to the amount of glucose in the available medium, up to glucose concentrations of 20 mM (very high in a biological context). In contrast, GLUT 4 transporters become saturated when the glucose concentration of the medium exceeds 5 to 6 mM, so that no further increase in the rate of glucose uptake is noted at higher glucose concentrations.

Accordingly, tissue explants were incubated with various concentrations of glucose ranging from less than 1 mM to 25 mM, to determine the saturation point for glucose uptake in lamellae.

### **3. Characterising glucose transport proteins**

Tissues were obtained from horses that had been killed at a local abattoir. Samples of brain, skeletal muscle, lamellae and coronary band tissue were transported to the laboratory and processed to

extract total RNA, which was then converted to first-strand cDNA. Equine-specific primers for both GLUT-1 (287 bp) and GLUT-4 (658 bp) glucose transport proteins were designed, and these were used to amplify the products of a reverse-transcriptase polymerase chain reaction (RT-PCR) on a 2% agarose gel. The quantity of messenger RNA for each of the transport proteins expressed in the various tissues was measured by densitometry.

#### **4. Determining the effect of high insulin concentrations *in vivo***

To determine whether high concentrations of insulin could trigger laminitis in the absence of lush pasture, a high carbohydrate diet, insulin resistance, or abnormal cortisol concentrations, nine healthy ponies with no known history of laminitis were given infusions of either recombinant human insulin (Humulin R, Ely Lilly) plus glucose, or an equal volume of saline (control), for up to 72 hours. The seven geldings, one colt and one mare used for this study had a median age of 4 years (range 2 to 14 years), a mean body weight of  $258 \pm 25$  kg, and a body condition score of 3 to 4, based on a scale of 1 to 5 (Carroll and Huntington, 1988).

All solutions were infused via catheters inserted within one jugular vein. Insulin was administered to five of the ponies at a priming dose of 45 mU/kg in 50 mL of 0.9% saline, followed by a continuous infusion at a fixed rate of 6 mU/min/kg. Blood samples were collected at frequent intervals via a catheter inserted in to the contralateral jugular vein, and blood glucose concentrations were monitored progressively. An infusion of glucose (50% w/v; Baxter) was provided at a rate that was adjusted as necessary, to maintain a steady blood concentration of 5 mM glucose ( $\pm 1$  mM) throughout the experiment, which is a normal resting concentration for ponies. The four control ponies received an infusion of 0.9% saline at a fixed rate of 14.7  $\mu$ L/min/kg for 72 hours, which was the average rate of fluid infusion in the insulin-treated ponies.

In addition to monitoring blood glucose concentrations, samples were collected for the determination of serum insulin concentrations. By knowing the insulin concentration and the rate at which glucose had to be infused to maintain a steady state, it was possible to estimate the relative insulin sensitivity of each pony. Insulin concentrations were measured using a radioimmunoassay kit that had been validated for use with horse serum (DSL, Houston TX, USA).

The infusions continued for a maximum of 72 hours, but were stopped earlier than this as soon as clinical signs of laminitis were confirmed. These signs were increased digital pulses, palpably increased hoof heat over the dorsal hoof wall, weight shifting and lameness (Obel grade 2). Any pony showing these signs was given phenylbutazone (4.4 mg/kg BWT orally or IV) which rapidly relieved the clinical signs of discomfort. Heart rate, respiratory rate, rectal temperature and demeanor were also monitored at regular intervals throughout the experiment. At the end of the experiment the ponies were euthanased with pentobarbitone sodium (162.5 mg/kg BWT IV) so that histological analysis could be performed on the hooves, allowing an unequivocal diagnosis of laminitis.

Histology on lamellar tissues was performed by an investigator who was unaware of the treatments received by each pony. The explants were placed in 4% paraformaldehyde for a minimum of 24 hours, then embedded in paraffin and cut in to 5  $\mu$ m. sections. The samples were then stained using haematoxylin and eosin, and periodic acid-Schiff, before being examined microscopically. A schematic diagram of the experimental set-up is shown in Figure 5.

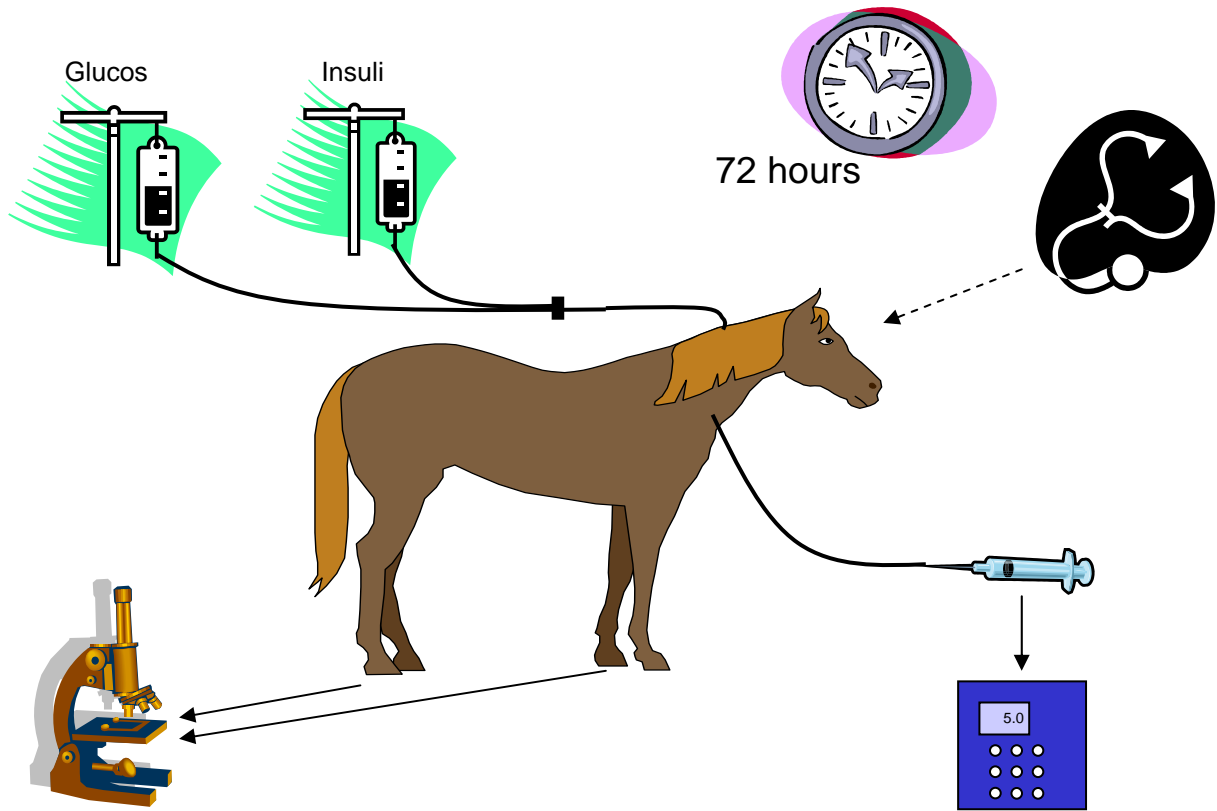


Figure 5. Experiment to determine whether a continuous infusion of insulin can induce laminitis in ponies. Each pony received insulin at a fixed infusion rate, plus glucose at a rate that was adjusted continually, allowing blood glucose concentrations to be maintained at a constant concentration of ~5 mM. Infusions lasted for up to 72 hours, during which clinical observations were made regularly. The ponies were euthanased at the end of the experiment to allow histological analysis of their hoof tissue.

## 5. Statistical analysis

The data collected from  $\beta$ -adrenoceptor characterisation studies were analysed using the iterative non-linear curve-fitting program Ligand (Munson and Rodbard 1979). Effects of age, gender and other variables on the number of receptors present in a fixed mass of hoof tissue were sought using Analysis of Variance (ANOVA) and multiple regression analysis.

Comparisons of the rate of glucose uptake in tissue explants in the presence or absence of drugs were made using a Student's t-test. The presence or absence of specific glucose transport proteins was assessed empirically and not subjected to statistical analysis. Data from the insulin infusion experiments in ponies were analysed using Fisher's exact test.

All results are presented as means  $\pm$  standard error.

# Results

## 1. Characterising and measuring adrenaline receptors in the hoof

The radioligand [ $^3\text{H}$ ]-CGP12177A was found to be suitable for labelling equine  $\beta$ -adrenoceptors, binding to  $\beta_1$ -adrenoceptors in heart ventricle and  $\beta_2$ -adrenoceptors in skeletal muscle with a similar affinity ( $K_D$   $394 \pm 0.13$  and  $338 \pm 0.03$  pM, respectively).

The radioligand could be displaced by the non-selective antagonist drug propranolol (Figure 6), which was used to measure the level of non-specific (non-receptor) binding in the tissues. The level of non-specific binding was approximately 20% in most assays.

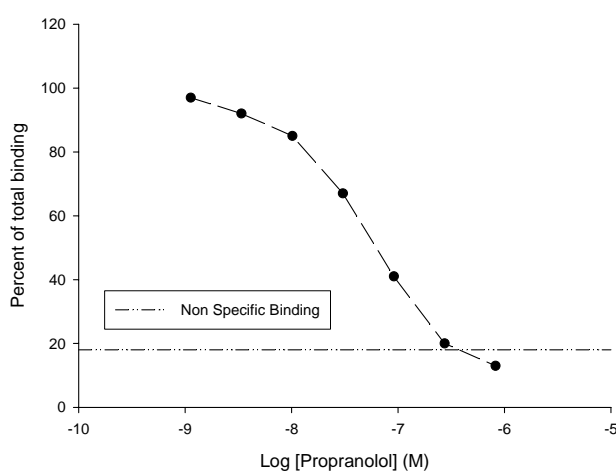


Figure 6. Displacement of the radioligand [ $^3\text{H}$ ]CGP-12177 from  $\beta$ -adrenoceptors in equine heart ventricle by the non-selective receptor antagonist propranolol.

An antagonist drug that binds selectively to  $\beta_2$ -adrenoceptors in many species was tested in heart and skeletal muscle. ICI118551 was found to have a high affinity for binding to equine  $\beta_2$ -adrenoceptors in skeletal muscle ( $K_D$   $80 \pm 5.6$  nM), but also had a reasonably high affinity for  $\beta_1$ -adrenoceptors in heart ventricle ( $K_D$   $467 \pm 36$  nM). Therefore, it was not suitable for use in discriminating between these subtypes in a tissue that may contain a mixture of both  $\beta_1$ - and  $\beta_2$ -adrenoceptors.

A second antagonist drug that is highly selective for  $\beta_1$ -adrenoceptors in many species was found to show a similar level of selectivity in equine tissue. CGP20712A bound to  $\beta_1$ -adrenoceptors with a  $K_D$  of 11 nM and to  $\beta_2$ -adrenoceptors with  $K_D$  of more than 100,000 nM. Thus, CGP20712A was the drug of choice to differentiate between these two receptor sub-types in equine hoof tissue.

Competitive displacement studies carried in equine hoof lamellae showed that  $90.0 \pm 2.6\%$  of the  $\beta$ -adrenoceptors present were of the  $\beta_2$ -sub-type and that the remainder were of the  $\beta_1$ -sub-type.

### Variation in receptor concentrations in different horses

The total number of  $\beta$ -adrenoceptors in lamellar tissue from different horses varied markedly. There was no evidence that this variation was associated with body condition score ( $P = 0.99$ ) or breed ( $P = 0.67$ ), with only a trend towards a decline with age (Figure 7,  $P < 0.1$ ) and a possible effect of gender ( $P = 0.10$ ).

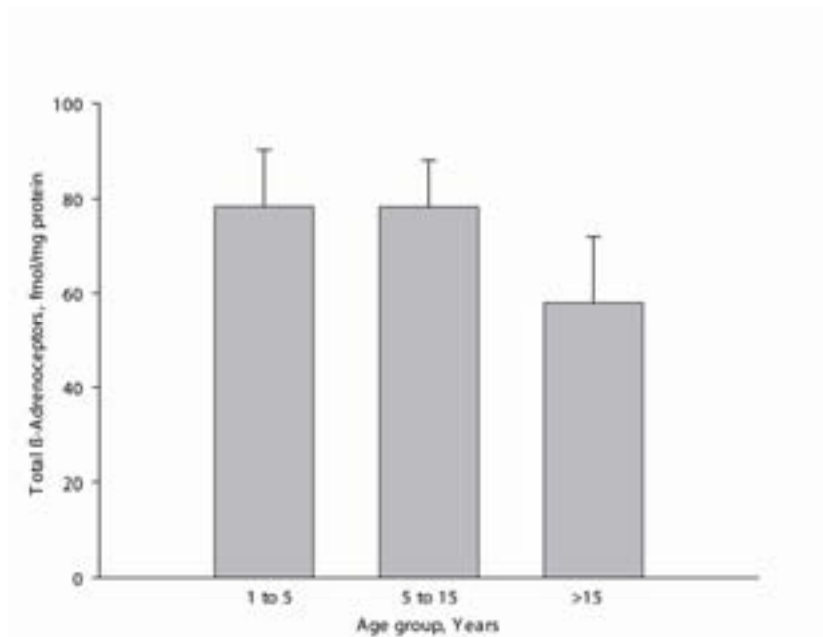


Figure 7. Mean (+ se)  $\beta$ -adrenoceptor density in equine lamellar tissue in ten horses aged 1 to 5 yrs, seven horses aged 5 to 15 yrs and three horses over 15 yrs of age ( $P < 0.1$ , ANOVA).

When the total  $\beta$ -adrenoceptor population was apportioned between  $\beta_1$ - and  $\beta_2$ -adrenoceptor sub-types, there was no significant effect of age, a trend towards an effect of gender on  $\beta_2$ -adrenoceptors (Figure 8,  $P < 0.1$ ) and a significant effect of breed on the number of  $\beta_1$ -adrenoceptors (Figure 9,  $P < 0.05$ ). The Quarterhorses appeared to have a higher density  $\beta_1$ -adrenoceptors than other breeds, but this observation should be interpreted with caution as there were only three horses in this group.

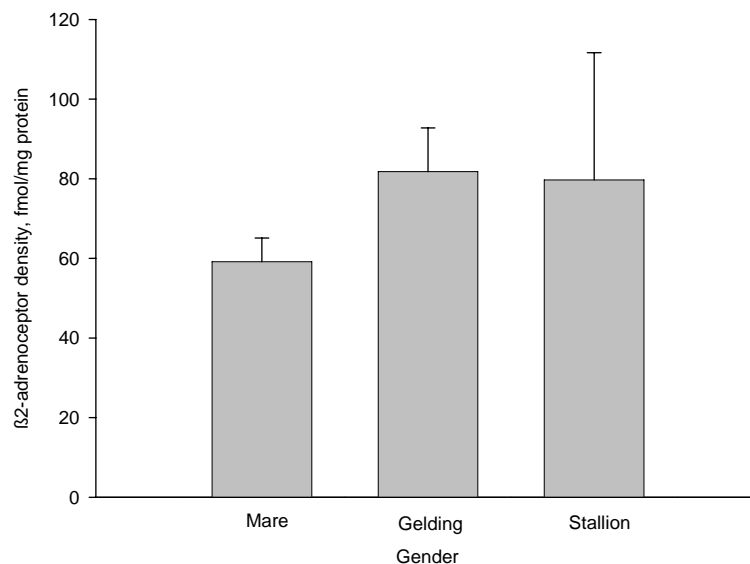


Figure 8. Mean (+ se)  $\beta_2$ -adrenoceptor density in equine lamellar tissue in 12 mares, 6 geldings and 3 stallions ( $P = 0.1$ , ANOVA).

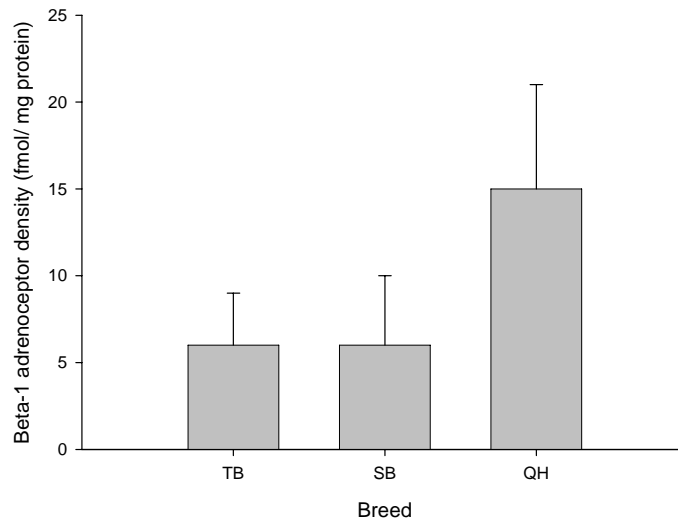


Figure 9. Mean (+ se)  $\beta_1$ -adrenoceptor density in equine lamellar tissue in seven Thoroughbreds, six Standardbreds and three Quarterhorses ( $P < 0.05$ , ANOVA).

## 2. Measuring glucose uptake

### a. Confirming the requirement for glucose

Initial studies confirmed that hoof tissue has an absolute requirement for glucose, such that tissue incubated for 48 hours in the presence of glucose (25 mM) required a force of  $1775 \pm 71$  g to become separated ( $n = 79$  explants), whereas tissue incubated for the same period without glucose separated at a force of only  $417 \pm 36$  g ( $n = 49$  explants).

### b. Effect of stimulating adrenaline receptors

The first experiments demonstrated that isoprenaline inhibits glucose uptake in lamellar tissue in a concentration-dependant manner, with a maximum decrease of 20% at an isoprenaline concentration of 200 nM (Figure 10).

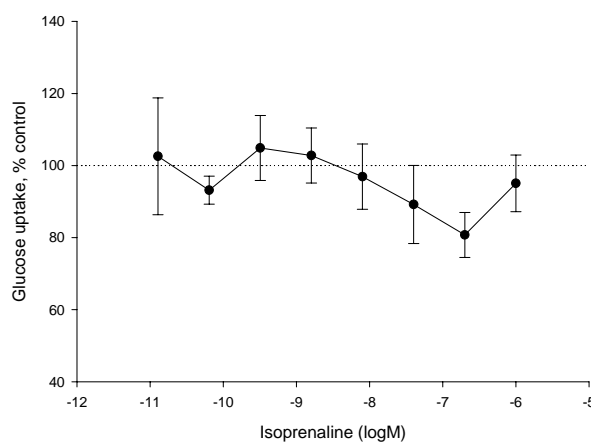


Figure 10. Dose-dependent inhibition of glucose uptake in equine lamellar explants by the  $\beta$ -adrenoceptor agonist isoprenaline.

Unexpectedly, in subsequent experiments we found that isoprenaline causes a marked significant decrease in glucose uptake in approximately 50% of the horses examined, with little or no effect in the remaining horses. We attempted to identify the factors that may determine whether a horse is isoprenaline-responsive or not, by examining for an association with breed, age, body condition and gender, but no conclusive evidence was obtained.

Because the isoprenaline response was not consistent in every horse, experiments to examine the interaction between isoprenaline and other hormones *in vitro* were confounded. Nevertheless, we did confirm that the isoprenaline response was authentic, and that it occurred at both moderate and high glucose concentrations (Figure 11).

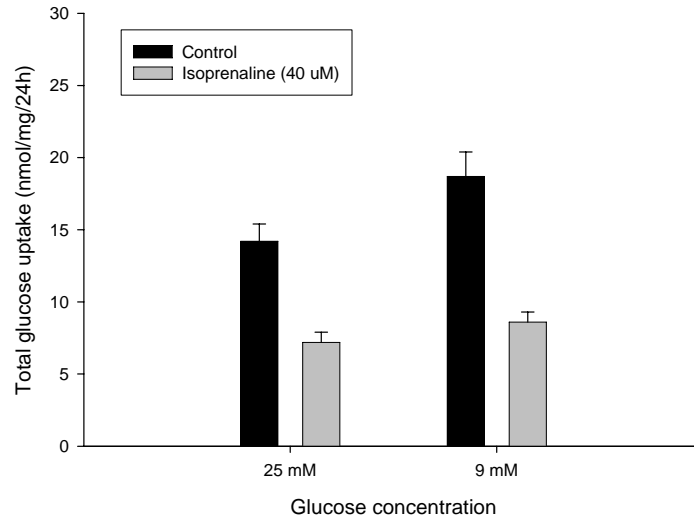


Figure 11. Inhibition of glucose uptake in explants of healthy hoof tissue by the  $\beta$ -adrenoceptor agonist isoprenaline.

### c. Effect of insulin

None of the experiments showed a positive response to insulin in terms of glucose uptake. For example, basal glucose uptake in the absence of insulin was  $550 \pm 86.0$  and  $627.6 \pm 150.5$  dpm/mg tissue following incubation for 60 min and 24 h respectively. In the presence of insulin ( $300 \mu\text{U/mL}$ ) glucose uptake was  $594.4 \pm 118.9$  and  $408.1 \pm 40.1$  dpm/mg tissue over the same periods, and this was not significantly different to basal values (Figure 12).

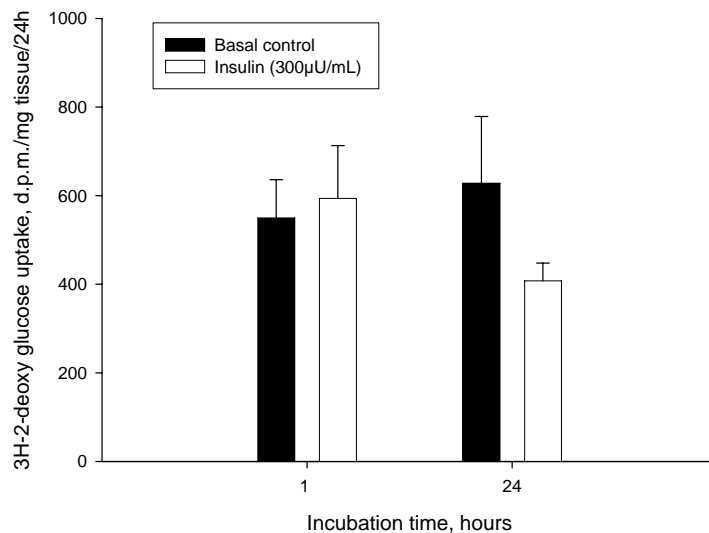


Figure 12. Uptake of (<sup>3</sup>H)2-deoxy glucose by explants of healthy hoof tissue from 6 horses in the presence or absence of insulin.

#### d. Determining the saturation point of glucose transport proteins

Figure 13 shows the results of a glucose saturation experiment, and illustrates that the glucose transport proteins in equine lamellae do not become saturated until extracellular glucose concentrations reach 18 to 20 mM. The figure also shows that glucose uptake decreases at glucose concentrations higher than 20 mM, suggesting a gluco-toxic effect.

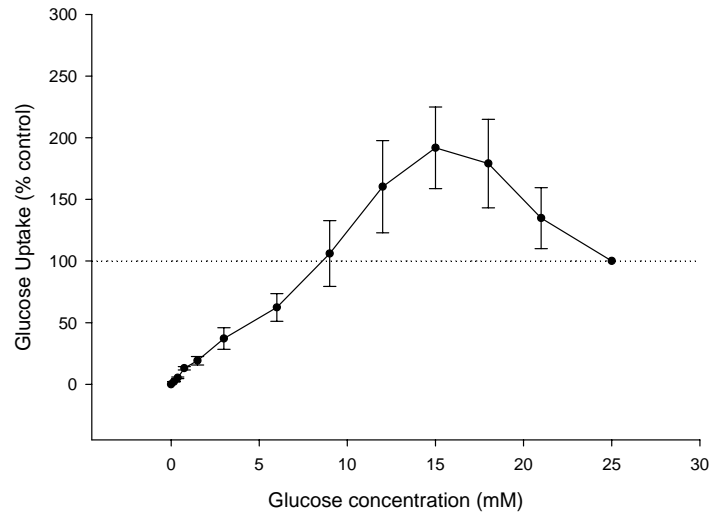


Figure 13. Relative glucose uptake over 48 h in lamellar tissue from 4 horses incubated with varying concentrations of glucose.

### 3. Characterising glucose transport proteins

The technique to measure GLUT-1 transport proteins was developed using tissue from equine brain, which showed an abundance of mRNA for these proteins as expected (Figure 14).

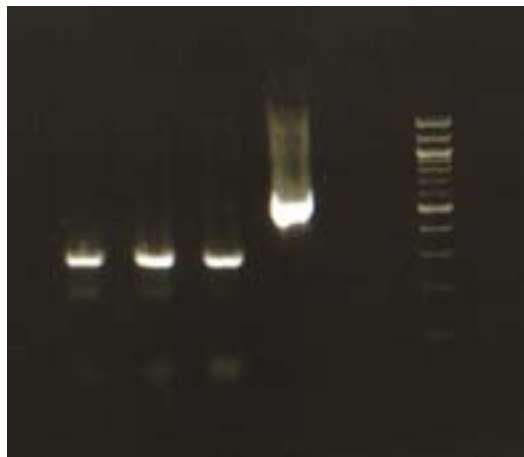


Figure 14. Expression of GLUT1-type glucose transport proteins in equine brain detected using RT-PCR. From left to right the five lanes represent (1) GLUT-1 primers, 10% DMSO; (2) GLUT-1 primers, 5% DMSO; (3) GLUT-1 primers, no additives; (4)  $\beta$ -Actin primers (housekeeping gene), no additives; (5) DNA ladder. PCR products were of the expected size for both GLUT1 (287 bp) and  $\beta$ -Actin (548 bp) primers.

Similarly, equine skeletal muscle was used as a positive control tissue for GLUT4 transporters. A comparison of skeletal muscle, lamellar tissue and tissue from the coronary band in the hoof is shown in Figure 15.

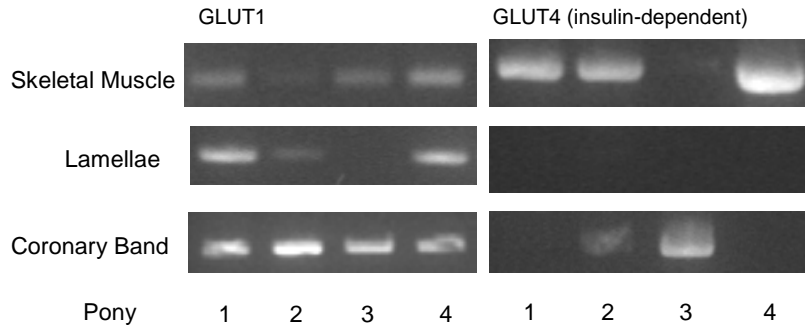


Figure 15. Expression of mRNA for GLUT1 and GLUT 4- type (insulin-dependent) glucose transport proteins measured using RT-PCR in tissues from four healthy ponies. The PCR products were of the expected size for GLUT1 (287 bp) and GLUT4 (548 bp) respectively.

The insulin-independent GLUT1 gene was expressed in coronary band and lamellar tissues with equal intensity and weakly in muscle. Conversely, the insulin-dependent GLUT4 protein was expressed intensely in skeletal muscle, weakly in coronary band tissue, and not at all in lamellar tissue. These results are consistent with glucose uptake in the hoof being mediated by GLUT1 transport proteins, independent of the actions of insulin.

#### 4. Determining the effect of high insulin concentrations *in vivo*

##### Glucose and insulin concentrations

Prior to insulin infusion, the basal concentrations of serum insulin and blood glucose were  $15.7 \pm 1.8 \mu\text{U/mL}$  and  $5.2 \pm 0.1 \text{ mM}$  respectively, and the concentrations were not significantly different between the treated and control groups.

During the infusions, blood glucose was also held constant at a mean value of  $5.2 \pm 0.1 \text{ mM}$ , such that there was no difference between treated and control ponies (Figure 16). However, serum insulin concentrations differed markedly between the two groups due to the insulin infusion. In control ponies the insulin concentrations averaged  $14.6 \pm 2.6 \mu\text{U/mL}$ , whereas the mean insulin concentration for treated ponies was  $1036 \pm 55.0 \mu\text{U/mL}$  (Figure 16).

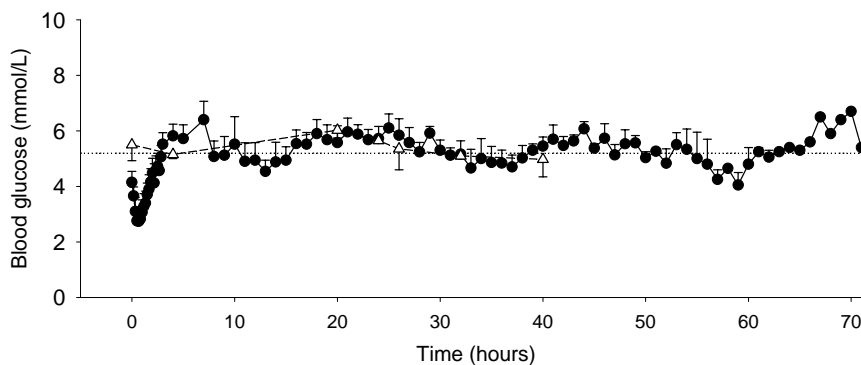


Figure 16. Concentrations of blood glucose in control ponies given an infusion of saline (open symbols, n=4) and in treated ponies infused for up to 72 h with recombinant human insulin at a fixed rate, plus glucose at a variable rate designed to maintain euglycaemia at  $\sim 5 \text{ mmol/L}$  (closed

symbols, n = 5). Values are mean + s.e for treated ponies and mean – s.e. for controls. Samples were obtained from ponies in the treated group every 5 min for the first 180 min, and at 30 min intervals thereafter. Samples were obtained from control ponies every 6 hours.

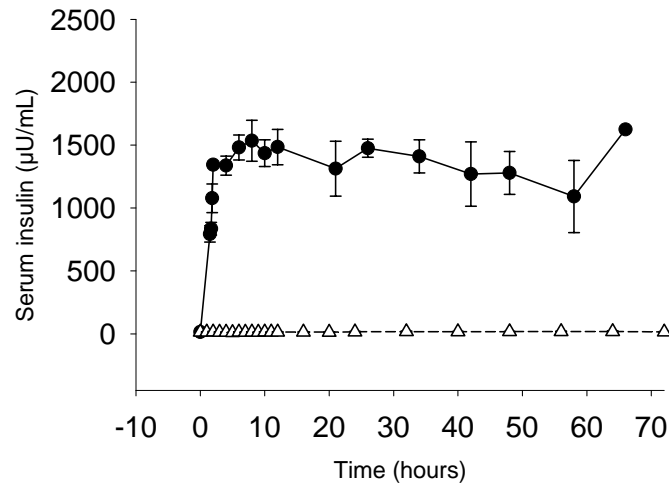


Figure 17. Serum insulin concentrations in four control ponies infused with saline (open triangles), and in five treated ponies infused with recombinant human insulin (closed circles). Values are means  $\pm$  s.e. at all time points for control ponies, and for treated ponies up to 42 hours (n = 5), 58 hours (n = 3), and 66 hours (n = 1).

The relative glucose tolerance (insulin sensitivity) of the treated ponies was calculated, based on an estimate of the glucose metabolism rate during a period when insulin and glucose concentrations were stable. The average ratio of the amount of glucose metabolised per unit of insulin infused was  $3.5 \pm 1.1$ , with a range of 2.0 to 7.4, indicating that one pony in the treated group was highly tolerant to glucose (sensitive to insulin), while another was relatively intolerant to glucose (resistant to insulin).

### Clinical observations

All five ponies in the insulin-treated group developed mild laminitis in all four hooves, whereas none of the four control ponies developed laminitis ( $P < 0.01$ ). Evidence of Obel grade 1 lameness indicative of laminitis (prominent, palpable digital pulses, heat over the dorsal hoof wall and weight shifting; Obel, 1948) were evident  $32.6 \pm 5.4$  hours after the infusions started, and the ponies progressed to Obel grade 2 lameness/laminitis by  $55.4 \pm 5.5$  hours. At this stage, mean respiratory rate had increased from  $18.7 \pm 1.0$  to  $36 \pm 3.1$  breaths/min and heart rate had increased from  $49 \pm 2.2$  to  $70 \pm 3.7$  bpm.

All the ponies remained bright and alert and were eating and drinking normally throughout the experiment. However, three out of five ponies showed signs of agitation at the onset of laminitis. Rectal temperature remained within the normal range in all ponies. In the treated ponies, signs of lameness were reduced from Obel grade 2 to Obel grade 1 by the use of non-steroidal anti-inflammatory medication. This was maintained until the ponies were euthanased.

### Histopathology

The lamellar tissue obtained from the control ponies was judged normal in all cases, whereas the tissue obtained from treated ponies was typical of laminitis. The tips of secondary epidermal lamellar were elongated and tapered, the basement membrane was disintegrated, and basal cell nuclei were rounded.

# Discussion

## 1. Adrenaline receptors in the hoof and their relevance to laminitis

Identifying and characterising  $\beta$ -adrenoceptors in the equine hoof is a small but critical step towards understanding the mechanisms that regulate hoof physiology. The current research has shown conclusively that at least two types of adrenoceptor are present in equine lamellae, in the relative proportions of 10%  $\beta_1$ -adrenoceptors and 90%  $\beta_2$ -adrenoceptors. Furthermore, we have shown that these receptors can be activated by a  $\beta$ -agonist drug, to elicit the functional response of limiting glucose uptake. This is consistent with the known effects of isoprenaline in other species and other tissues.

Although the  $\beta$ -adrenoceptors mediate many important effects of the catecholamines (adrenaline and noradrenaline), and are among the most widely studied receptors in human and animal physiology, as far as we are aware previous studies of  $\beta$ -adrenoceptors in the horse have been confined to blood cells (Abraham et al. 2001, 2002) and the cardiovascular system (Elliott and Soydan 1995, Torneke 1999, Belloli et al. 2000, Horn et al. 2002). Through the present work we have extended the characterisation of these receptors, and based on binding affinities for a range of agonist and antagonist drugs, found that the equine  $\beta_2$ -adrenoceptor is slightly different to  $\beta_2$ -adrenoceptors found in humans, cattle, sheep and rats, but almost identical to those found in the pig (Sillence et al. 2005).

Apart from identifying the presence, proportions, functional activity and ligand binding characteristics of  $\beta$ -adrenoceptors, we have discovered that individual horses show a marked degree of variation in the number of  $\beta$ -adrenoceptors present in their lamellae, and in the size of the functional response to isoprenaline. This observation could help to explain why different horses show a different intensity of physiological response to stressful conditions which result in excess catecholamine production. Specifically, it may explain why seemingly unrelated conditions such as retained placenta, pneumonia and colic, which all likely trigger a stress response, can precipitate laminitis in some horses without affecting others.

A comprehensive study of the mechanisms which regulate  $\beta$ -adrenoceptor production and responsiveness was beyond the scope of the present project. The preliminary data collected so far do not support a strong association with age, breed or gender, but may be interpreted to suggest that further investigations are warranted. In particular, it is known that  $\beta$ -adrenoceptor production can be enhanced by cortisol and synthetic corticosteroid drugs (Huang et al. 2000, Abraham et al. 2002), and there are two compelling reasons why this association should be explored further.

First, corticosteroids and catecholamines are both capable of inhibiting glucose uptake, and over the long-term they may act in concert to induce glucose intolerance. Regardless of whether glucose starvation of the hoof is a likely mechanism for laminitis, it is clear that the development of systemic glucose intolerance and resultant hyperinsulinaemia, would place a horse at risk of laminitis. Second, in an animal that was already glucose intolerant for other reasons (eg poor diet, genetics or lack of exercise), the size of the glycaemic response to a catecholamine challenge would still determine the size of the insulin response, and hence the risk of laminitis.

In summary, catecholamines have an important role in glucose metabolism, interacting with both cortisol and insulin. Understanding how their receptors are regulated and how this modulates catecholamine responsiveness, could yield important insights into the pathophysiology of laminitis.

## 2. Glucose uptake, glucose transporters and insulin

The results from a number of experiments indicated that glucose uptake in the hoof is neither dependant on insulin, nor is it influenced by the presence of insulin. This indicated that the dominant glucose transporters in the hoof are the GLUT1-type, rather than GLUT4 which are found in other important sites for glucose disposal such as skeletal muscle.

Further indirect evidence for the dominance of GLUT1 transporters was obtained by determining the glucose concentration at which lamellar glucose transporters become saturated. Thus, the ability of the tissue to continue to take up increasing amounts of glucose as the external glucose concentration rose to 20 mM, is a hallmark of GLUT1-mediated regulation. Finally, molecular studies of gene expression confirmed the presence of GLUT1 mRNA, and the absence of mRNA for GLUT4 transporters in the lamellae.

The presence of some GLUT4 mRNA in coronary band indicates that the growth of the hoof wall may be influenced by insulin to some extent, while the strong expression of GLUT1 mRNA in the same tissue suggests that there is no absolute requirement for insulin.

The predominance of GLUT1 in lamellae and the lack of effect of insulin are consistent with the hoof having such a high metabolic demand for glucose (Wattle and Pollitt 2007), similar to the brain, that the tissue can not afford to have its energy supply regulated by insulin. However, the results are not consistent with our original hypothesis that laminitis is a reflection of glucose deprivation, resulting from insulin resistance.

## 3. A new hypothesis

Having rejected the glucose deprivation hypothesis, the question remained of why horses with Equine Cushing's Syndrome are more likely to develop laminitis if they also have high circulating insulin concentrations. Furthermore, while our research was in progress another important link between hyperinsulinaemia and laminitis came to light, this time in relation to the pasture-induced laminitis in ponies (Treiber et al. 2006).

Clearly the problem was not one of insulin resistance in the classical sense, whereby impaired glucose uptake is the problem and high insulin concentrations are merely a sign. In fact, the ponies with pasture-induced laminitis had blood glucose concentrations within the normal range (Treiber et al. 2006), implying that sufficient glucose uptake had been achieved, through the excess secretion of insulin.

At this stage we began to examine the relationship between cortisol, glucose, insulin and insulin resistance from a different perspective. We realised that little was known about the effects of insulin in horses apart from its actions on glucose uptake. In common with other hormones, insulin has many actions in humans and other animals that are seldom discussed. For example, insulin can have a marked effect on blood flow (Anderson et al. 1991) and protein turnover (Millward et al. 1983). It is likely that these effects are independent of glucose transporters, and hence an individual described as 'insulin-resistant' in the context of their tolerance to glucose, may still be insulin-responsive with respect to other actions of insulin. Finally, in contrast to humans, insulin-resistant horses rarely develop pancreatic exhaustion, and are capable of producing exceptionally high serum insulin concentrations (Reeves et al. 2001, McGowan et al. 2004). This could have exceptional consequences.

The basis for our new hypothesis was that laminitis is the direct result of insulin toxicity, caused by hyperinsulinaemia. The high blood insulin concentrations were in turn a sign of glucose intolerance, coupled with a glucose challenge. Thus, ponies that are glucose-intolerant produce an exaggerated insulin response when fed lush pasture with its high content of soluble carbohydrates. Similarly, excess cortisol due to Equine Cushing's Syndrome or treatment with corticosteroid drugs, also leads to glucose mobilisation, and elicits a counter-regulatory insulin response. A modest insulin response is seen in horses that are otherwise healthy, whereas an exaggerated response is seen in horses that are intolerant to glucose. Therefore, it is glucose intolerance that predisposes horses to laminitis, and insulin that precipitates the damage to the hoof. The causes of glucose intolerance incorporate genetic,

dietary and exercise components, and may include long-term hypercortisolaemia. A schematic representation of this theory is shown in Figure 18.

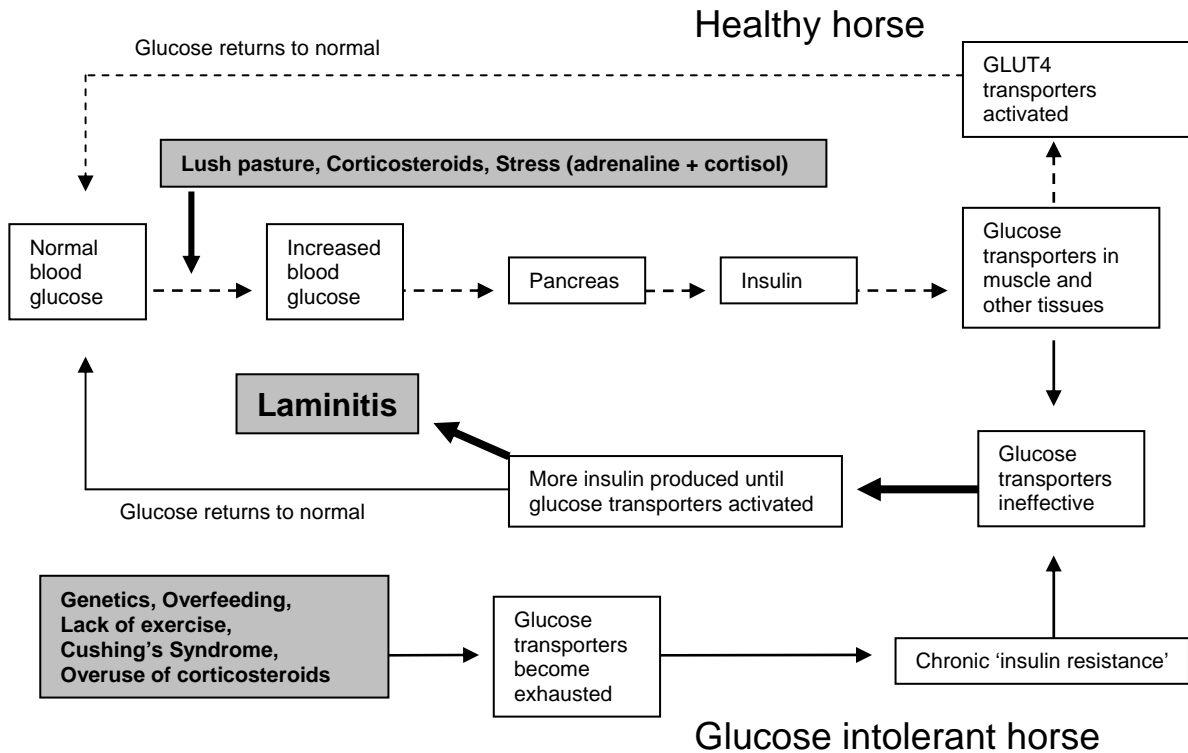


Figure 18. Proposed relationship between various triggers to laminitis and long-term factors which may predispose some animals to this disease. Dashed arrows represent the normal response of a horse to an increase in blood glucose. Solid arrows represent the response of a horse or pony that has developed glucose intolerance. In the latter case, it is believed that excessively high insulin concentrations precipitate the damage to the hoof.

The aim of our final experiment was to demonstrate that high circulating concentrations of insulin can induce laminitis without cortisol manipulation, dietary modification, or hyperglycaemia. This experiment was technically challenging, as glucose concentrations had to be monitored ‘constantly’ and the glucose infusion rate adjusted to maintain euglycaemia. There were no reports that this had ever been attempted in horses for more than 2 to 4 hours, whereas laminitis was not expected to develop for 2 to 3 days.

#### 4. Insulin and laminitis

The results of the insulin infusion experiment show unequivocally that laminitis can be induced by maintaining high concentrations of insulin, in the absence of many other factors that have confounded the interpretation of previous experiments, such as high concentrations of cortisol or glucose, obesity, glucose intolerance (insulin resistance), a genetic predisposition or history of laminitis, or any dietary modification. While such factors are important because they place an animal at risk of laminitis, we can conclude that ultimately, it is the insulin response that triggers the condition. This could be regarded as a critical breakthrough in laminitis research, as it signals a clear and rational pathway towards better diagnostics, prevention and treatment strategies for this debilitating disease.

#### 5. Improved diagnostics

An exaggerated insulin response to a glucose challenge would be a hallmark of animals at risk of laminitis, and so a modified glucose tolerance test could be used to identify candidates for preventive treatment. In fact, Bailey et al. (2007) report that ponies predisposed to laminitis can be identified by a range of physiological responses (including insulin) after feeding hay with the fructan carbohydrate inulin (3 g/kg body weight/day). To take full advantage of such diagnostic tests it would be useful to

determine the threshold for insulin toxicity in the horse, as only one insulin infusion rate was used in the present study. A reliable and fully-validated assay for equine insulin is also a requirement, and we have made good progress towards an international standard for such an assay, as part of another research project (Sillence, Munn and McGowan, unpublished data).

## **6. Preventing laminitis**

Preventive and intervention strategies for animals at risk of laminitis should focus on restoring glucose tolerance and therefore lowering baseline insulin concentrations. This is also referred to as insulin sensitivity, but the use of that term in the present context may cause some confusion, as it would seem paradoxical to sensitise an animal to a hormone, with the aim of avoiding the toxic effects of that hormone.

There has been considerable research into human Type 2 diabetes, which can inform veterinary research aimed at improving glucose tolerance in horses and ponies. Dietary modification can be recommended, with the use of feeds that have a low glycaemic index, as suggested by Johnson et al. (2004). Caloric restriction may also be appropriate in overweight horses, to achieve progressive weight loss. Physical exercise training has also been shown to help prevent insulin resistance in horses (Pratt et al. 2006) and improve glucose tolerance in ponies (Freestone et al. 1992). Finally, specific compounds could be used to restore insulin sensitivity, either of natural origin, or pharmaceutical agents.

## **7. Treating endocrine/insulin induced laminitis**

Based on our current state of knowledge about the relation between insulin and laminitis, the most obvious treatment strategy would be to lower insulin concentrations as quickly as possible without compromising the control of blood glucose, and perhaps to counteract the effects of insulin on the hoof.

The simplest approach to lowering insulin concentrations is to reduce blood glucose concentrations. In cases where the hyperglycaemia is due to diet, replacing the feed with an appropriate alternative would be effective. Providing some fatty acids (in the form of oil) as an alternative energy source could be an effective short-term measure, while the base feed should contain less than 12% non-structural carbohydrates. This could be in the form of dried forages (hay) or pasture, provided the non-structural carbohydrate content is verified. In other circumstances, access to the feed should be restricted.

Techniques to neutralise insulin, either by binding the molecule to specific antibodies or blocking the insulin receptor, are possible in theory, but would require considerable experimentation. Faster progress may be made if a better understanding is developed of the intervening stages between activation of the insulin receptors and separation of the lamellae. For example, if the effects of insulin are mediated by profound changes in blood flow to the hoof, then vasoactive drugs that have already been developed for other clinical purposes would be worth testing as potential treatments for laminitis. This could be a particularly promising area for future investigation as it is already known that insulin increases blood flow to human muscles (Steinberg et al. 1994) and opens arteriovenous anastomoses (Kihara et al. 1994). In the horse, insulin could also be responsible for the increase in blood flow to the foot that occurs after a meal (Hoffmann et al. 2001).

Whether the effects of insulin are due to changes in blood flow, localised glucose toxicity, or another mechanism, remains to be determined. However, we are confident that further studies into the mechanism of action of insulin will bring us closer to an effective short-term treatment for this form of laminitis.

# Implications

Previously, it was suggested that the risk of developing laminitis in some horses and in specific pony breeds, could be lessened by improving their glucose tolerance through diet and exercise. With new knowledge about the pivotal role of insulin in the pathogenesis of laminitis, not only can we endorse these measures and understand why they are effective, we can generalise these findings to the entire population, and state that any horse that produces excess insulin for a prolonged period, for whatever reason, is likely to develop laminitis.

This discovery creates a strong focus for strategies to identify animals at risk, countermeasures to prevent the condition, and new therapies to treat acute laminitis. Many of the tools necessary to implement these strategies are already at hand, such as a validated equine insulin assay, horse diets with a low glycaemic index, and insulin-sensitizing drugs developed for humans or companion animals. However, there is clearly scope for further research in to nutritional, pharmaceutical and nutraceutical remedies, based around the control of glucose and glucose tolerance. Additional work on the mechanism of action of insulin would increase the range of potential treatments even further.

# Recommendations

A simple, rapid and reliable glucose tolerance test should be employed to identify horses at risk of hyperinsulinaemia and laminitis.

It is important that all horse owners become aware of the risk associated with prolonged hyperinsulinaemia and take appropriate action to avoid this.

While some information about restoring insulin sensitivity/glucose tolerance is already available to guide horse owners, further research is needed to test novel pharmaceutical, nutraceutical and dietary based approaches.

The techniques used by different laboratories to measure insulin concentrations in horses need to be standardised using a national or international reference standard, with appropriate quality controls, so that meaningful clinical comparisons can be made.

The potential of corticosteroid drugs to cause glucose intolerance should be investigated further.

Meanwhile, if corticosteroid drugs are indicated to treat inflammation or other conditions, the user should be mindful of the risks, particularly in horses that are intolerant to glucose. Monitoring insulin concentrations in horses that require corticosteroid therapy may be useful in detecting when insulin resistance becomes clinically significant.

The mechanism by which insulin causes laminitis should be investigated further, with a view to discovering new avenues for therapy.

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# Appendix 1

## Publications and presentations associated with this research

Asplin K.E., Bevan B.E., McGowan C.M., Pollitt C.C. and Sillence M.N. 2005. Glucose uptake in the equine hoof. Proceedings of the Nutrition Society of Australia, Dec 2005, Melbourne Vic., Asia Pacific Journal of Clinical Nutrition 14 (Suppl.): S62.

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Asplin K.E., Sillence M.N., Pollitt C.C. and McGowan C.M. 2007. Induction of laminitis with insulin in healthy ponies. Proceedings of the British Equine Veterinary Association Congress, Sept 2007, Edinburgh UK (In Press).

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