Equine Obesity: Concepts and Mechanisms

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor of Philosophy

by

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September 2015

Abstract

Equine Obesity: Concepts and Mechanisms. Philippa K Morrison

Obesity in the UK leisure population of horses and ponies is a growing problem with major welfare implications. To date, research into the associations between obesity and metabolic disease such as insulin dysregulation and laminitis remain ongoing. To improve our understanding of obesity in this species, the current thesis was designed to address several related objectives ranging from psychological aspects of obesity to the role of key determinants of energy balance in the setting of obesity.

Implementing dietary restriction to reverse obesity requires an owner to correctly recognise obesity in their animal, knowledge of which is lacking for the horse. A two-tier internet-based questionnaire was created and distributed through UK equine-based forums. Tier 1 utilised lateral photographic images of horses and ponies and demonstrated that only 11% of respondents (n = 546 total) correctly identified all overweight animals from a panel of 12 images. When assessing the suitability of horses and ponies for taking part in a range of activities, respondents considered it more appropriate for each animal to carry more weight/condition for competing in affiliated showing classes. Tier 2 (n = 177 responses) provided information regarding current management practices of horse-owners in the UK.

The ability to quantify internal adiposity in live animals requires imaging technology which is not yet available for the horse. A semi-quantitative regional adipose-depot specific scoring system (EQUIFAT) was developed and tested. Associations between *ante-mortem* body condition score (BCS) and *post-mortem* EQUIFAT scores (n = 207 animals) revealed that retroperitoneal EQUIFAT score had strong positive associations with BCS, whilst omental had weaker associations and mesenteric and epicardial scores had no associations with BCS, indicating clear functional differences between regional adipose depots in the horse.

Performing in-depth molecular biology studies using abattoir-derived samples requires knowledge of the time-frame of RNA degradation. RNA was found to remain intact up to 30 minutes and 2 hours *post-mortem* for adipose tissue and skeletal muscle (n = 3 horses), respectively.

The expression of myostatin, a key regulator of skeletal muscle mass and energy balance was evaluated in lean and obese horses and ponies (n = 6/group). Myostatin gene expression was increased in skeletal muscle of obese animals, with no difference at the protein level. Circulating myostatin concentrations were increased in obese animals.

Adipocyte area was increased in adipose depots (retroperitoneal, omental, crest and tailhead) in obese animals, except for epicardial WAT. The expression of lipolytic proteins PLIN1 and HSL was reduced in retroperitoneal WAT of obese animals, with fewer differences noted between groups for other depots.

Together, findings from this thesis indicate a misperception of obesity exists among horse-owners and enthusiasts. Functional differences between regional adipose depots and altered expression of key regulators of energy balance have been identified in obese horses and ponies.

Acknowledgements

mother who was responsible for buying me my first pony and getting me well and truly hooked on horses from a young age, for that I am eternally grateful.

Finally, I could not have done this without the constant support and encouragement I have received from my husband, Richard. Not only did he up sticks with me and move to the Wirral for me to embark on this PhD, he has helped me through the rollercoaster of emotions that this journey has been. I am incredibly grateful for him for all he has done and put up with and I have promised him that normality will resume and I will return to the cooking and cleaning once I have finished my thesis!

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Chapter 1

General Introduction

1.1 Equine obesity: Implications for welfare and management

From the beginnings of equine domestication, horses and ponies have served Man by fulfilling essential roles as food or working animals, employed in diverse activities, including agriculture, mining, transport or warfare. Today, the UK population of horses and ponies is estimated at ~944,000 animals (BETA National Equestrian survey 2015). Recreational or leisure animals now account for ~60% of the National equine herd, with leisure riding being reported as the most common equestrian pursuit (Wyse *et al.*, 2008; BETA National Equestrian survey 2015). This relatively recent change of use has been accompanied by a significant decrease in individual animal workloads but has also served to uncouple animal management from traditional wisdoms in agriculture and nutrition. Not all of the welfare impacts associated with this move have been beneficial.

Horses and ponies are long–lived and under natural conditions, oscillate circannually from periods of positive to negative energy balance across successive summers and winters (Fuller *et al.*, 2001). The degree of negative energy balance experienced during the predictable winter decrease in both grazing quality and availability, in combination with increased rates of convective and conductive heat loss, is naturally attenuated by photoperiodically entrained, seasonally-adaptive physiological changes. These include a suppression of basal metabolic rate, catabolism of stored body fats and both physical and behavioural mechanisms to reduce heat loss across the body surface. By contrast, modern horse management promotes the year-round maintenance of positive energy balance by ensuring the availability of high quality forages and supplementary energy-dense feedstuffs, shelter and artificial thermal insulation in the form of rugs. For many animals, these factors have attenuated or abolished the natural winter check on body weight gain and have enhanced the deposition of body fat during summers spent grazing on cultured swards rich in non-structural carbohydrates (NSC's, (Longland and Byrd, 2006). Paradoxically, these 'improved' husbandry standards commonly culminate in the development of obesity with its attendant risks to health and performance (Argo, 2009). For many contemporary UK horses and ponies kept for leisure

purposes, it could be considered that a 'mismatch' exists between their natural biology and the manner in which we now elect to maintain them.

Epidemiological data continue to report a high prevalence of obesity in the leisure populations of horses and ponies of industrialised nations (Harker *et al.*, 2011; Wyse *et al.*, 2008). During the winter months, a time at which body condition score (BCS) might be expected to be lower than during summer months obesity prevalence in a population of leisure horses in the South West of England was ~27% and this increased to ~35% in summer (Giles *et al.*, 2014). This study reinforced earlier data which suggested that the natural, photoperiodically-entrained suppression of appetite and metabolic rate in winter was insufficient to prevent weight gain when mature ponies were housed and continued to receive *ad libitum* access to moderate quality, fibre-based feedstuffs throughout winter (Dugdale *et al.*, 2011a). More recent owner-reported data suggested that the obesity prevalence for animals used in pleasure riding, exceeded 30% (Robin *et al.*, 2015) and animals in this majority sector were identified as being more at risk of being obese than animals used in competitive events.

Obesity increases the risk of horses and ponies developing conditions which are deleterious to their health and performance. Most importantly in terms of numbers, these conditions include insulin dysregulation and laminitis (Geor, 2008) but obesity has also been associated with compromised internal organ function, impairment of athletic and reproductive performance and some strangulating forms of colic (Argo, 2009). The proportion of horses and ponies with a history of laminitis has been reported by owners to be ~15% (Ireland *et al.*, 2013; Wylie *et al.*, 2011). However, not all obese horses and ponies will develop laminitis and many have normal insulin/carbohydrate dynamics. The precise mechanisms which link insulin dysregulation, laminitis and obesity remain to be elucidated and it continues to be an area for further research.

Currently, controlled weight loss management is the mainstay of therapies to correct obesity and diminish the risk of obesity-associated disease. Strategies to promote weight-loss include dietary restriction and increased physical activity

through exercise where possible. However, it is clear that rates of weight loss are highly variable between animals even when dietary provision is robustly controlled. This has been demonstrated in a group of 12 overweight horses and ponies, for which daily dry matter (DM) intakes of forage were restricted to 1.25% body mass for 16 weeks (Argo *et al*., 2012). Rates of weight loss varied considerably, to the extent that the total weight lost by the most weight-loss sensitive animal was 3-fold that of the most weight-loss resistant animal. Despite consideration of outset body mass, BCS, body fat content and indices of insulin resistance, random effects linear regression modelling indicated that 65% of the measured variation in weight-loss responsiveness could be attributed to individual animal identity. Whilst weight-loss management is crucial in improving the welfare of obese animals, the initiation of weight-loss first requires that owners are able to recognise obesity in their animal.

1.2 Psychological basis for obesity

The psychology of obesity development has become a major research focus in humans (Karasu, 2012). Understanding the mental processes which promote obesity development is important to direct appropriate education and corrective advice if this rise in obesity prevalence, both in humans and their companion animals is to be reversed. Evidence suggests that there is a common misperception of body weight both among adults (Johnson *et al*., 2014; Wetmore and Mokdad, 2012) and among parents in the estimation of their child's weight status (Carnell *et al*., 2005; Jones *et al*., 2011). It is of concern that in the UK, only a quarter of parents correctly identified their child as being overweight; the same proportion of parents were reported to be only "a little worried" if their child was overweight (Jeffery *et al*., 2004). This latter finding was perhaps predictable, since parents not only had a poor perception of their child's weight but they also showed an inability to identify their own overweight state (Jeffery *et al*., 2004). However, it is the responsibility of parents to try and ensure their child remains at a healthy weight as childhood obesity increases the risk of developing obesity and associated complications in adulthood (Guo *et al*., 2002).

As alluded to earlier, the recognition of obesity is a crucial prerequisite for implementing corrective weight-loss programmes. A more accurate perception of body weight status has been associated with weight-loss attempts (Bittner Fagan *et al.*, 2008; Duncan *et al.*, 2011). The social influence on obesity has also been studied extensively. It was of interest that people who had an obese friend, had an 57% increased likelihood of becoming obese themselves, Similarly, among siblings and spouses, the likelihood of obesity was increased by ~40% if the other became obese (Christakis and Fowler, 2007). More recently, the impact of social influence was evaluated in terms of weight loss intention. This study identified that among overweight and obese young adults, having more social contact with individuals who were also actively trying to lose weight increased the intention to lose weight (Leahey *et al.*, 2011).

The likeliness of underestimating weight status was increased in children and youths who had school friends and parents with a high body mass index (BMI) (Maximova *et al.*, 2008). This would indicate that exposure to an environment with obese individuals will influence weight perception. In agreement with this, there is evidence to suggest that the increased exposure to obese body shapes which faces us in today's obese society, has led to an upward shift in what is considered 'normal' in terms of body weight (Burke *et al.*, 2010). Furthermore, a generational shift in parents perceptions of their child's weight has also been documented recently, with overweight/obese children being less likely to be perceived as overweight in a study conducted between 2005-2010 compared to one conducted between 1988-1994 (Hansen *et al.*, 2014).

An inability to accurately perceive our own weight has obvious implications for the animals in our care. Indeed, a discrepancy between owner and veterinarian assessment of BCS has been established for both dogs (Colliard *et al.*, 2006) and cats (Colliard *et al.*, 2009). More recently, 44.1% of dog owners (Courcier *et al.*, 2011), and 45.8% of cat owners (Courcier *et al.*, 2010) were found to incorrectly assess their pets body shape, with underestimation being the most common form of misperception. Additionally, the misperception for dog owners was found to

remain - even with the use of a body condition scoring chart (Eastland-Jones *et al.*, 2014). Owners of obese dogs have been found to have less interest in their dogs nutrition and exercise needs compared with owners of non-obese dogs (Kienzle *et al.*, 1998), whilst owners of overweight cats tended to treat their cat more like a human in terms of their feeding habits (Kienzle and Bergler, 2006).

With regard to horses, only a few studies have been conducted to assess owner's perceptions of obesity. In the UK, only a fair agreement was found between horse owner and expert assessment of BCS (Wyse *et al.*, 2008), whilst more recently, poor agreement was noted between the BCS assigned by the horse owner and that assigned by the researcher (Stephenson *et al.*, 2011).

In combination, these studies suggest that improving our understanding of some psychological aspects to obesity development in the horse would enable more tailored nutritional and management advice to be distributed to horse owners in an attempt to diminish obesity in our leisure population of horses and ponies.

It is worthy of consideration that in evolutionary terms, the ability to store excess energy efficiently during times of plenty was required for survival during periods of famine (the 'thrifty gene' hypothesis) (Hales and Barker, 2001). However, the so-called 'thrifty genes' are disadvantageous in today's in modern society with its sedentary lifestyles and ready access to energy-dense diets. The 'thrifty gene' hypothesis has been implicated in the widespread development of obesity and diabetes in man, and may play a similarly deleterious role for our companion species. Our ability to further understand the links between obesity and metabolic conditions requires that we first accurately quantify and measure adiposity. For the horse, our ability to quantify body fat is somewhat limited by their large body size. Currently, the definition of obesity in the horse is based on the subjective evaluation of BCS. Consequently, in addition to evaluating perceptions of obesity, an improved knowledge of equine adiposity is central to furthering our understanding of obesity and associated metabolic conditions.

1.3 Measuring adiposity

Whilst it is evident that excessive adiposity can impair health in both humans and animals, understanding associations between obesity and disease risk relies on our ability to quantify adiposity. For humans, measurement of BMI provides a useful indication of weight status, and clear associations have been made between BMI-defined obesity and mortality rates. However, BMI has clear limitations; perhaps most notably, it cannot distinguish between lean mass and adipose tissue. Furthermore, the ability of BMI to diagnose obesity as determined by body fat percentage is most markedly limited for individuals in the intermediate BMI ranges (Romero-Corral *et al.*, 2008). Additional anthropometric measurements of central obesity such as waist circumference and waist/hip ratio, both used as an index of the accumulation of abdominal fat, have been found to be useful in defining associations between adiposity and metabolic disease risk (Goh *et al.*, 2014; Pouliot *et al.*, 1994; Wei *et al.*, 1997). Nevertheless, whilst these straightforward measurements remain useful and are easy to apply in large population studies, accurate quantification of regional adiposity in subcutaneous and visceral compartments can only be obtained from whole-body imaging modalities such as computed tomography (CT) and magnetic resonance imaging (MRI).

For *Equidae,* due to their large body size, the use of sophisticated whole body imaging techniques (e.g. CT, MRI, and DEXA) is precluded at present and measurements of body adiposity are generally restricted to subjective measurements. Several different body condition scoring systems exist and are variously employed by equine researchers. However all systems employ the same basic principles which include the visual and palpable appraisal of superficial body fat cover at specific anatomical locations. The most commonly used equine BCS system is based on a 1-9 scale (where 1 = emaciated and 9 = obese). This system was initially developed specifically to evaluate the body condition of Quarter horse mares but its use has been systematically extended to assess adiposity across horses and ponies of diverse breeds and gender (Henneke *et al.*, 1983). Currently, the Kohnke modification (Kohnke, 1992) of the Henneke BCS system is employed by

our group. It involves the visual and palpable assessment of six body areas including neck, withers, shoulder, ribs, loin and tailhead, assigning a number from 1-9 for each area (Appendix A). The average of these six numbers equates to the overall BCS for that animal. Although BCS system is a subjective measure of adiposity, it has proved to be a useful tool for indicating adiposity in horses and ponies (Dugdale *et al*., 2012). Despite this, it has been found to be unreliable in monitoring early weight loss through dietary restriction in ponies, whereby ~6% loss in body mass (BM), accompanied by improvements in hyperinsulinemia did not result in alterations in BCS (Dugdale *et al*., 2010). The addition of objective, morphometric measurements such as body circumference (belly girth) may provide useful indications of changes in internal adipose tissues (visceral/retroperitoneal) during weight loss.

The assessment of fat stored within the nuchal crest (Figure 1.1) of the neck may be one of the most visually obvious fat depots in the horse and mean neck circumference has been associated with the magnitude of insulin resistance in horses (Frank *et al*., 2006). The Cresty Neck Score (CNS) was developed by Carter *et al*. (Carter *et al*., 2009a), and a score of ≥4/5 has been associated, along with other variables, with an increased risk of laminitis (Carter *et al*., 2009b). However, no difference in neck crest height or thickness was noted between laminitis-prone and control ponies with similar BCS (Bailey *et al*., 2008), indicating increased fat deposition in the neck region may only be relevant when more generalised obesity is also present. Furthermore, measurements of neck circumference were not found to be useful indicators of early weight loss in a group of native pony mares (Dugdale *et al*., 2010). Moreover, the precise mechanisms linking crest fat with metabolic abnormalities remain to be elucidated.
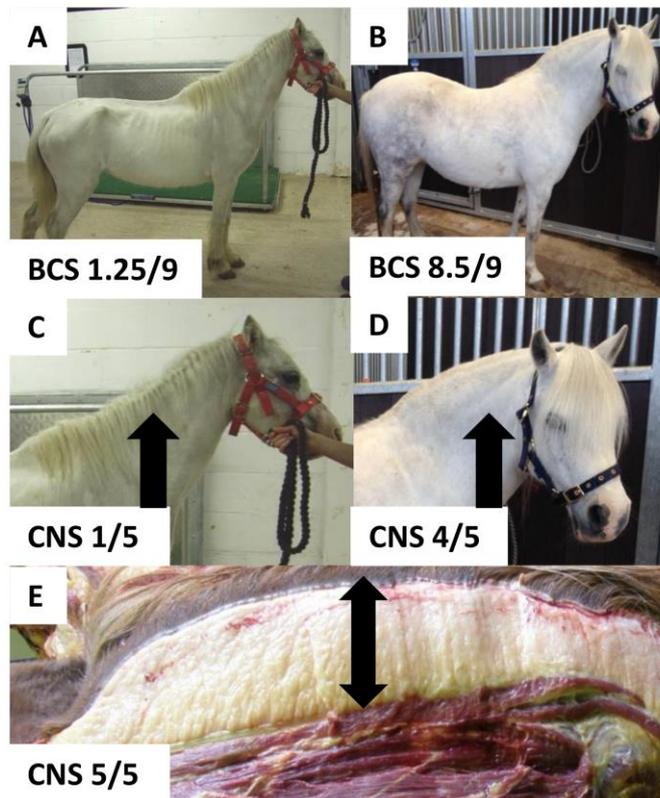
Figure 1.1: Images depicting the range in body condition score (BCS; **A** and **B**) and associated cresty neck score (CNS; **C** and **D**) in two Welsh Mountain pony mares. For context, a sagittal section through the nuchal crest of a Welsh Mountain pony mare of BCS 8.5/9 (**E**) is represented to indicate the scale of this regional adipose depot.

The validation of the deuterium oxide (D_2O) dilution technique for the determination of total body fat in horses and ponies has proved to be a useful research tool (Dugdale *et al*., 2011c). However, application of this method to describe associations between total body fat and BCS in a large group of horses and ponies identified a non-linear relationship between the two variables (Dugdale *et al*., 2012). Indeed, BCS became less reliable in estimating the body fat content of obese animals (>6.8/9), and suggests that whilst BCS is usefully accurate in estimating the body fat content of non-obese animals, it cannot predict body fat content of obese animals.

Recently, a method for estimating body fat content of horses and ponies based on objective, morphometric measurements (heart girth, belly girth, neck circumference and height) has been proposed and was found to have similar correlations to total body fat content (measured by deuterium oxide dilution) as evaluation of BCS, and this may provide a more objective tool for estimating body fat content for those inexperienced with BCS (Potter *et al*., 2015).

The most obvious problem with current measures of adiposity in horses and ponies is the inability to discriminate between regional adipose tissue depots. The contribution of visceral (omental/mesenteric) fat to the metabolic phenotype has been well characterised in humans (Fontana *et al.*, 2007; Nakamura *et al.*, 1994), however the precise contribution of regional adipose depots to insulin dysregulation and laminitis in the horse remains unclear. Until we have the ability to quantify regional adiposity in large animals *in vivo*, further work is required to establish how regional adipose tissue depots are altered in obesity.

The long-term regulation of body weight is under the control of several crucial adiposity signals that circulate in proportion to adipose tissue mass and interact with satiety signals in the brain. Perhaps the best characterised of these signals in humans are insulin and leptin (Baskin *et al.*, 1999; Havel *et al.*, 1996; Yannakoulia *et al.*, 2003), both capable of modulating the response of the brain to satiety signals (Woods and D'Alessio, 2008). Furthermore, leptin has been already been associated with body fat mass in the horse (Kearns *et al.*, 2006). For the current thesis, the molecular focus was centred upon other key pathways involved in energy homeostasis that have been implicated in states of energy imbalance in humans but have not yet been investigated in the horse. To this end, whilst adipose tissue undoubtedly plays a major role in adipose tissue dysfunction in obesity, the amount of excess energy available to deposit as fat is dependent on whole body energy requirements. As the major determinant of whole body resting energy expenditure and comprising around 40% of body mass (Dugdale *et al.*, 2011b; Webb and Weaver, 1979; Zurlo *et al.*, 1990), skeletal muscle has a considerable role to play in obesity development (Houmard *et al.*, 2011; Maltin, 2008).

1.4 Skeletal muscle: A key determinant of energy balance

For *Equidae,* the wide range in body conformation, including the shape and size of muscle mass evident between breeds of horses and ponies, is likely to reflect a combination of their original evolutionary / environmental adaptations and / or

domestic selection to fulfil diverse athletic or aesthetic roles. This diversity of selective pressures could be expected to result in wide variations in muscle mass and fibre composition between and within the breeds. Thoroughbred horses selectively bred for speed and endurance will have increased proportions of their body mass as skeletal muscle when compared to other breeds of horse (Gunn, 1987). Similarly, having increased proportions of the body as fat-free mass was beneficial to exercise performance in both Standardbred horses (Kearns *et al.*, 2002) and humans (Slater *et al.*, 2005; Stöggl *et al.*, 2010). By contrast, it could be suggested that mountain and moorland breeds of horses and ponies (Welsh Mountain, Highland, Shetland, Exmoor, Dartmoor etc.) have a greater proportion of adipose tissue to aid survival in their naturally challenging habitats. Taken together, alterations in skeletal muscle metabolism and / or between-animal differences in the mass, efficiency and composition of skeletal muscle, would be important in determining energy balance and by inference, in dictating the amount of excess energy available for storage in adipose tissue.

Skeletal muscle is comprised of distinct muscle fibre types. Muscle fibres are classified according to their contraction speed and oxidative capacity. Type I or slow-twitch fibres are oxidative and highly fatigue resistant. Type II muscles fibres are further distinguished into Type IIA fibres, which are fast-twitch, oxidative fibres and show a moderate resistance to fatigue, and Type IIB/X fibres which are fast-twitch fibres that largely rely on glycolytic metabolism to generate energy and are rapidly fatigued. Interestingly in Man, relative proportions of skeletal muscle fibre types have been associated with the propensity for obese subjects to lose weight. Human subjects who were sensitive to weight-loss through dietary restriction alone had a higher proportion of Type I fibres in the *vastus lateralis* muscle and a corresponding up-regulation of genes associated with oxidative phosphorylation in comparison to subjects who proved resistant to weight loss (Gerrits *et al.*, 2010).

The development of skeletal muscle fibres occurs during the early stages of myogenesis and motor nerve stimulation controls the activity of diverse sets of genes that are specific to the different fibre types (Chin *et al.*, 1998; Stockdale,

1997; Wu *et al.*, 2000). Fibre type composition of skeletal muscle differs between individuals and this can be partially attributed to genetic factors. The heritability of Type I fibre proportions is estimated to be around 45% in humans (Simoneau and Bouchard, 1995) and in horses; the heritability of Type I fibres is greater than for Type IIA and Type IIX fibres (Rivero and Barrey, 2001). However, skeletal muscle demonstrates post-natal plasticity. Subtle alterations in the relative proportions of fibre types may occur throughout adult life as a result of the modifying effects of disease (Gosker *et al.*, 2002) and exercise training in both humans (Aagaard *et al.*, 2011; Coggan *et al.*, 1992) and horses (Kim *et al.*, 2005; Serrano *et al.*, 2000). However, there are challenges associated with measuring fibre type in large animals such as equines due to the considerable variability of fibre types distributed both between and within individual muscles. It has been demonstrated that within the *gluteus medius* muscle of the horse, a lower percentage of type I fibres were identified at the superficial region of the muscle compared to the deeper region, highlighting the necessity to be accurate and consistent when sampling skeletal muscle for fibre typing (López-Rivero *et al.*, 1992). Therefore, although fibre type proportions are clearly of importance, it is inappropriate to attempt to study fibre type proportions via use of muscle biopsy or other sampling techniques unless the precise anatomical location of the sample sites is possible.

The relative proportions of different fibre types in human skeletal muscle have also been associated with obesity. The proportion of Type I muscle fibres was inversely related to both body fat content (Helge *et al.*, 1999; Wade *et al.*, 1990) and body mass index (BMI) (Hickey *et al.*, 1995). These findings are supported by the later observation that obese subjects had significantly lower proportions of Type I muscles fibres when compared to lean subjects (Tanner *et al.*, 2002). Further, in a 19 year follow-up study, subjects with relatively lower proportions of Type I fibres had increased weight gains, increased body fat percentages and higher BMI's relative to people who had greater proportions of Type I fibres (Karjalainen *et al.*, 2006). This decreased contribution of the highly oxidative Type I fibres in the metabolically dominant skeletal muscle mass of obese phenotypes, may offer an empirical explanation for the observation that fatty acid oxidative capacity is

decreased in obese subjects (Kelley *et al.*, 1999; Kim *et al.*, 2000).

Skeletal muscle also plays a major role in glucose uptake, with 80% of postprandial glucose disposal occurring in this tissue alone (Blaak, 2005). To this end, alterations in skeletal muscle insulin-mediated glucose uptake have been extensively studied in the context of insulin resistance in humans (Goodpaster *et al.*, 2014; Sylow *et al.*, 2014), and in horses (de Laat *et al.*, 2015; Waller *et al.*, 2011).

As discussed, alterations in skeletal muscle mass and efficiency have clear implications on whole body energy balance. With this in mind, key regulators of skeletal muscle mass have received significant attention as potential therapeutic targets for the treatment of obesity and insulin resistance. One such protein is Myostatin, a myokine secreted from skeletal muscle. Whilst perhaps it is better known as a negative regulator of skeletal muscle mass, it has also been clearly implicated in the regulation of whole body metabolism in the development of obesity.

1.5 A potential role for myostatin in equine obesity

Myostatin (also known as growth differentiation factor-8, GDF-8), is a member of the transforming growth factor β family, a group of secreted growth factors which regulate body tissue growth and differentiation (McPherron *et al.*, 1997). Since its discovery, myostatin has been well characterised as a potent negative-regulator of skeletal muscle mass (Joulia *et al.*, 2003; Whittemore *et al.*, 2003). Whilst myostatin expression may be largely genetically determined, expression levels can be modulated by both strength and endurance training in humans (Hittel *et al.*, 2010; Hulmi *et al.*, 2007). Natural mutations in the myostatin gene rendering it non-functional have resulted in the 'double muscling' phenotype observed in certain breeds of cattle (Grobet *et al.*, 1997). Furthermore, single nucleotide polymorphisms (SNPs) in the myostatin gene have been identified and associated with optimal race distance in Thoroughbred horses (Hill *et al.*, 2010) and between brachymorphic (heavy) and dolichomorphic (light) breeds of horses (Dall'Olio *et al.*,

2010). In addition to this, a promoter variant and intronic SNP of the myostatin gene were each significantly associated with greater proportions of Type IIB and lower proportions of Type I skeletal muscle fibre proportions in Quarter horses (Petersen *et al.,* 2013). Whilst it is not known whether these genetic variants result in direct changes in myostatin expression, the magnitude of myostatin expression has been shown to be significantly greater in murine muscles largely composed of fast twitch fibres compared to those predominantly slow twitch muscles (Kawada *et al.,* 2001). Cloning and sequencing the equine myostatin gene revealed a high degree of homology with other species, including that of bovine, mouse and human (Hosoyama *et al*., 2002).

Myostatin is synthesised as a 376 amino acid precursor protein, which is subsequently cleaved twice to reveal the active form of the protein. The first cleavage removes the signal peptide from the precursor protein to leave the N-terminal propeptide domain and the C-terminal domain. The final cleavage produces the N-terminal propeptide domain (myostatin propeptide) and mature myostatin, comprised of a disulphide-linked dimer of C-terminal domain (Huang *et al*., 2011). Mature myostatin is bound noncovalently to its propeptide and circulates in serum as an inactive complex (Hill *et al*., 2002). Members of the bone morphogenetic protein-1/tolloid (BMP-1/TLD) family of metalloproteinases have been implicated in the activation of myostatin *in vivo* (Wolfman *et al.,* 2003). Upon activation, myostatin binds selectively to the activin type II receptor kinase (ActRIIB), causing phosphorylation of the type I receptor, ALK5. Initiation of an intracellular signalling cascade follows, involving both SMAD and non-SMAD related pathways (Figure 1.2) allowing myostatin to regulate the expression of several genes involved in muscle mass and metabolism.
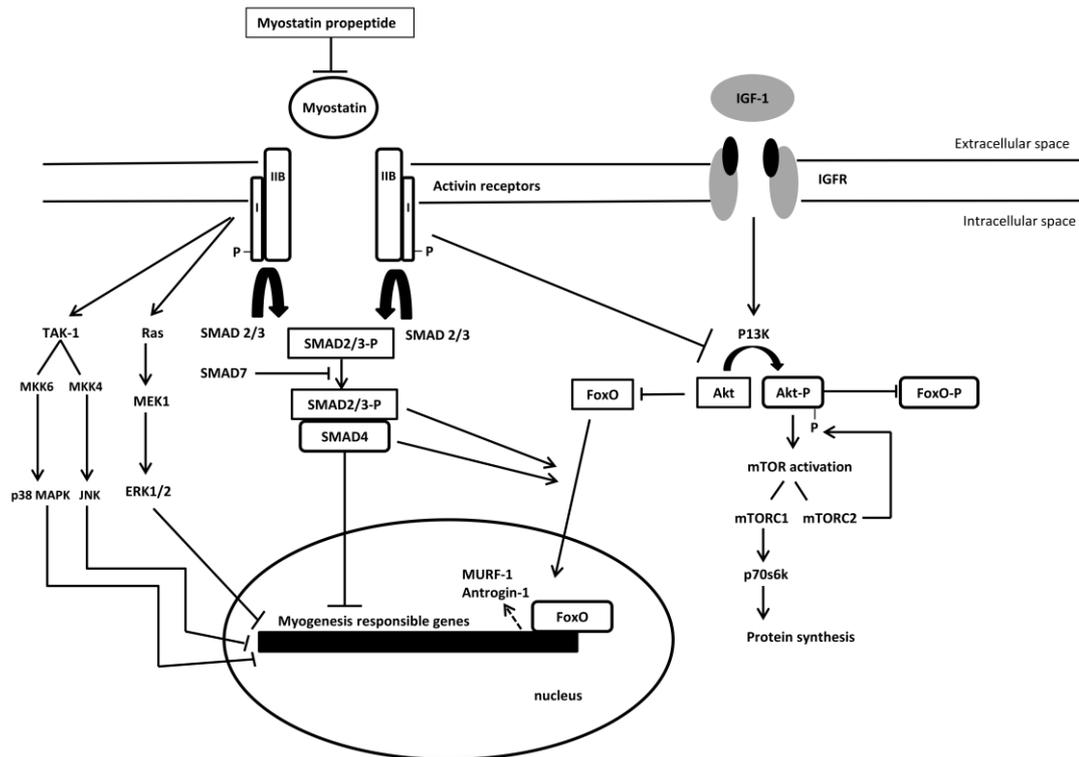
Figure 1.2: An overview of the myostatin signalling pathway. Abbreviations: *IGF-1* insulin-like growth factor 1, *IGFR* insulin-like growth factor 1 receptor, *P13K* phosphoinositide 3-kinase, *AKT/PKB* protein kinase B, *FoxO* forkhead box O, *mTOR* mechanistic target of rapamycin, *mTORC1/C2* mechanistic target of rapamycin complex 1/2, *p70s6k* ribosomal p-70-s6 kinase, *MURF-1* muscle ring-finger protein-1, *SMAD* cytoplasmic signalling molecules, *ActRIB/ActRIIB* activin type I/2 receptor kinase, *MEK1/MAP2K1* mitogen-activated protein 2 kinase, *ERK1/2* extracellular signal-related kinase 1/2, *TAK-1* transforming growth factor beta-activated kinase 1, *MKK4/6* MAP kinase kinase 4/6, *p38 MAPK* p38 mitogen-activated protein kinase, *JNK* c-JUN N-terminal kinase.

Early studies demonstrated that despite having similar planes of food intake in relation to body mass, myostatin knock-out (KO) mice possessed significant increases in muscle mass in comparison to their wild-type littermates, and they were also found to have significantly decreased fat accumulation (70% reduction in mean total body fat mass) (McPherron and Lee, 2002). Whilst it could be suggested that the absence of myostatin from birth reduced the capacity for adipose tissue accrual during development, myostatin KO mice challenged with a high-fat di*et al*so demonstrate significantly decreased gains in body fat compared to wild-type mice (Dilger *et al.*, 2010; Hamrick *et al.*, 2006). A summary of the key literature

investigating the role of myostatin in obesity is summarised for clarity in Table 1.1. In addition to a lack of body fat, loss of myostatin function in mice has also been found to confer additional metabolic benefits by improving insulin sensitivities (Choi *et al.*, 2011; Guo *et al.*, 2009). This may be unsurprising since, as discussed earlier, the majority of glucose uptake occurs in skeletal muscle, therefore it is biological probable that increased skeletal muscle mass would result in improvements in glucose metabolism and insulin dynamics over that observed in obesity. Conversely, a lack of adipose tissue might be protective against insulin resistance through a reduction in the secretion of pro-inflammatory cytokines that have been linked to the development of insulin resistance in other species (Gregor and Hotamisligil, 2011). In agreement with this, loss of function in myostatin in mice, resulting in a protection against whole-body insulin resistance is considered to be due to reductions in plasma tumor necrosis factor-α (TNF- α) levels (Wilkes *et al.*, 2009). Furthermore, improvements in insulin sensitivity through a lack of myostatin signalling is associated with up-regulation of the AMP-activated protein kinase (AMPK) signalling pathway in myostatin-null mice (Zhang *et al.*, 2011), whilst circulating myostatin in plasma has been shown to have an inverse relationship with insulin sensitivity in human subjects (Hittel *et al.*, 2010). In this context, myostatin has been suggested as a biomarker of insulin sensitivity and insulin resistance in a recent review (Park *et al.*, 2015).

More recent studies have investigated the degree by which blocking myostatin function can reduce fat mass gain in mature mice fed high fat diets. Blocking the action of myostatin in mature mice in combination with high fat feeding for 22 weeks resulted in these mice gaining only half the weight of control mice during the first 8 weeks, after which time the rate of weight gain was similar (Burgess *et al.*, 2011). Overall, cumulative weight gain remained significantly lower for myostatin deficient mice, despite increased gains in lean body mass combined with similar gains in intra-abdominal adipose tissue. However, as only intra-abdominal adipose tissue was evaluated, it could be suggested that other adipose depots were correspondingly reduced to account for the cumulative reduced weight loss. Similarly, no differences in fat gain were found between mature, myostatin

depleted mice and control mice fed a high fat diet (McPherron *et al.*, 2012). However, mature, myostatin depleted mice in the latter study had significantly greater gains in lean body mass, accounting for increased body weights of these mice from weeks 8-12 of the 12 weeks high-fat feeding. This is in contrast to the decreased cumulative weight gain reported in the former study and may be due to different study designs as the mice used in the latter study were already obese prior to blocking myostatin function. Taken together, this could suggest that blocking myostatin function may be ineffective in reducing body fat content in mice that are already obese, however it is generally accepted that blocking myostatin function in mature normal weight mice does confer protection against diet-induced obesity (Akpan *et al.*, 2009; Zhang *et al.*, 2012).

Attempts have been made to account for the reasoning behind the resistance to diet-induced obesity in myostatin deficient mice. It has been suggested that absence of myostatin indirectly reduces the build-up of fat due to the increased energy requirements brought about by the relatively greater muscle mass in these mice, in turn reducing the amount of excess energy available to deposit as fat (LeBrasseur, 2012). However, myostatin can be detected at low levels in adipose tissue and a direct association between myostatin and adipose tissue has been demonstrated, whereby myostatin treatment inhibits pre-adipocyte differentiation *in vitro* (Guo *et al.*, 2008; Hirai *et al.*, 2007; Kim *et al.*, 2001). Interestingly, in addition to protecting against diet-induced obesity, inactivation of myostatin was found to result in an up-regulation of genes involved in lipolysis and fatty acid oxidation, in combination with enhancing brown adipose tissue formation in white adipose tissue (Zhang *et al.*, 2012). This finding generated considerable interest after the discovery of functional brown fat in adult humans (Cypess *et al.*, 2009) led to the theory that formation of brown fat could provide a therapeutic treatment for obesity. Recently, myostatin has been found to inhibit the differentiation of brown fat by inhibiting the expression of key brown fat genes including uncoupling protein-1 UCP1) and PRDM16 (Braga *et al.*, 2013), whilst myostatin KO in mice induces the browning of WAT in mice through activation of the AMPK/PGC1-α/FNDC5 pathway (Shan *et al.*, 2013).

Table 1.1: A summary of the key studies involving myostatin and obesity. Abbreviations: *SNP* single nucleotide polymorphism, *MSTN* Myostatin, *KO* knock-out, *BM* body mass, *LBM* lean body mass, *BMI* body mass index, *HFD* high fat diet, *WT* wild type, *VL vastus lateralis*, *RA rectus abdominis*, *SC* subcutaneous, *TA tibialis anterior*

STUDY	MAJOR FINDINGS	AUTHOR
HUMAN	MSTN secretion and expression increased in skeletal muscle cells derived from extremely obese relative to lean healthy human subjects and positively correlated to severity of insulin resistance.	Hittel *et al*. (2009)
	MSTN expression decreased in quadriceps muscles around 18 months following biliopancreatic division.	Milan *et al*. (2004)
	SNP (rs3791783). AA genotype linked with increased susceptibility to obesity in a population of Chinese North Han people.	Pan *et al*. (2012)
	MSTN gene expression decreased around 1 year following gastric bypass surgery in VL muscle and increased expression of MSTN associated with obesity in RA muscle of cross-sectional group (lean vs. morbidly obese).	Park *et al*. (2006)
	SNP's associated with obesity, abdominal obesity and low LBM in population of non-diabetic Asian Indians in North India. Subjects with Thr/Thr genotype of A55T polymorphism high risk for high % body fat, truncal subcutaneous adiposity and low LBM. Subjects with R/R genotype of K153R polymorphism high risk for obesity, abdominal obesity and low LBM.	Bhatt *et al.* (2012)
	Serum MSTN increased in diabetic compared to non-diabetics and serum MSTN decreased with increasing components of metabolic syndrome	Han *et al*. (2014)
	Serum MSTN increased in overweight patients compared to normal weight controls and was positively correlated with BMI	Zhu *et al*. (2014)
OBESE MICE	MSTN gene expression increased in SC and visceral fat and TA muscle in obese mice vs. WT mice. In response to 1 month HFD (60% kcal fat), MSTN gene expression increased in TA muscle.	Allen *et al.* (2008)
MSTN KO MICE NO HFD	At 6-8 wks age, MSTN KO mice had increased energy expenditure, decreased total body fat mass and decreased % fat mass compared to WT mice.	Choi *et al.* (2011)
	12 wk old MSTN KO mice decreased retroperitoneal, epididymal, parametrial & inguinal fat pad weights vs. WT mice.	Lin *et al*. (2002)
	KO mice found to have 70% less body fat vs. WT mice	McPherron & Lee (2002)
MSTN KO MICE + HFD	Diet = 60% kcal fat for 4 weeks. Decreased body fat content in MSTN KO mice: decreased weights of gonadal & retroperitoneal fat pads. Incorporation of MSTN mutation with mice with genetic obesity (leptin db/db) did not alter obese state.	Dilger *et al.* (2010)
	Diet = 45% kcal fat for 10 weeks. MSTN KO HFD mice gained less weight than WT HFD mice. Improved insulin sensitivity in	Guo *et al.* (2009)

	KO HFD mice. Blocking MSTN function specifically in adipose tissue resulted in no effect on body composition or weight gain on standard or HFD. Blocking MSTN function specifically in skeletal muscle resulted in increased lean mass & decreased fat mass on standard and HFD.	
	Diet = 45% kcal fat for 8 weeks. Fat mass and % body fat decreased in KO mice vs. WT HFD mice.	Hamrick *et al.* (2006)
POST-DEVELOPMENTAL MSTN INHIBITION + HFD	Diet = 45% kcal fat for 10 weeks. 4 weeks treatment to block MSTN in combination with HFD resulted in BM and lean mass increased in mature MSTN null mice vs. vehicle treated mice. 10 week treatment in combination with HFD resulted in increased lean mass and decreased fat mass in mature MSTN null mice vs. vehicle treated mice.	Akpan *et al.* (2009)
	Diet = 60% kcal fat for 22 weeks. Decreased cumulative weight gain in mature MSTN KO mice vs. control HFD mice. No difference in epididymal and retroperitoneal fat pad weights.	Burgess *et al.* (2011)
	Diet = 60% kcal fat 12 weeks prior to MSTN inhibition treatment then further 12 weeks on HFD in combination with treatment. No difference in fat gain vs. vehicle treated mice. Increased gains in lean mass vs. vehicle treated mice 4-12 weeks after MSTN function blocked.	McPherron *et al.* (2012)
MSTN KO MICE + HFD POST-DEVELOPMENTAL MSTN INHIBITON + HFD	Diet = 45% kcal fat for 12 weeks. MSTN KO mice on HFD increased energy intake but BW similar to KO mice on chow diet. Up-regulation of genes associated with fatty acid oxidation and lipolysis in MSTN KO mice in peripheral tissues, along with up-regulation of uncoupling protein 1, 2, & 3 and other genes involved in BAT specification and thermogenesis in WAT. Post-developmental blocking of MSTN in combination with HFD resulted in lesser gains in WAT mass vs. vehicle treated HFD mice & resulted in similar gene expression profile to MSTN KO mice + HFD.	Zhang *et al.* (2012)

With regards to obesity in humans, myostatin gene expression was found to be significantly reduced in skeletal muscle in women following weight loss induced by gastric bypass surgery (Park *et al*., 2006) and biliopancreatic division (Milan *et al*., 2004). Furthermore, extremely obese women were found to have a significantly increased secretion and expression of myostatin in skeletal muscle samples which was positively correlated to the severity of insulin resistance (Hittel *et al*., 2009). This finding fits into the association between skeletal muscle fibre type, myostatin expression and obesity as myostatin expression is greater in Type II fibres, whilst reduced proportions of Type I fibres predisposes to obesity. It could therefore be suggested that the increased expression of myostatin in obese humans is due to lower proportions of Type I fibres. More recently, elevated circulating myostatin

concentrations have been identified in overweight human subjects (Zhu *et al.*, 2014). Conversely, serum myostatin levels were found to be reduced in individuals with the metabolic syndrome (Han *et al.*, 2014). Differences in the method for measuring myostatin may attribute the differing results, as different ELISA kits were used to measure circulating myostatin levels, with the latter study measuring full length myostatin peptide. Furthermore, a SNP in the human myostatin gene has been recently linked with increased susceptibility to obesity in a group of Chinese North Han subjects (Pan *et al.*, 2012), and two further SNPs have been associated with increased adiposity in Asian Indians (Bhatt *et al.*, 2012).

Taken together, data presented here clearly suggests that although definitive mechanisms for myostatin signalling in equine obesity have yet to be established, myostatin is likely to have an important role in whole body energy homeostasis. In addition to the key role played by skeletal muscle in maintaining energy balance, maintaining the balance between fat deposition and mobilisation is also crucial in the regulation of energy balance.

1.6 Lipolysis in the regulation of energy balance

Traditionally viewed primarily as an inert storage site for excess energy, WAT is now widely acknowledged as a highly metabolically active tissue, specialised in the storage of excess energy as triglycerides in intracellular lipid droplets within adipocytes (Brown, 2001). Under basal conditions, adipose tissue regulates the balance between triacylglycerol (TAG) synthesis (lipogenesis) and TAG breakdown (lipolysis). The ability of adipose tissue to store TAG enables it to provide the body with energy during periods of negative energy balance, such as during fasting or prolonged physical exercise. The complete hydrolysis of TAG results in the production of three molecules of free fatty acids (FFA) and one molecule of glycerol which are released into the circulation to be metabolised by other organs.

Adipose tissue is innervated by sympathetic and sensory nerve fibres which are able to regulate lipolysis. The main controlling factors of mammalian lipolysis are

the activity of the autonomic nervous system and the hormonal influences of insulin. The ratio between lipolytic β- and antilipolytic α_2-adrenoceptors will ultimately determine the net outcome of catecholamine-induced lipolysis. In the postprandial state, elevated circulating insulin facilitates glucose uptake into tissues, whilst concomitantly suppressing lipolysis. Insulin's inhibitory actions on both basal and catecholamine-induced lipolysis are considered to occur through the phosphorylation and activation of phosphodiesterase 3B, which in turn catalyses the breakdown of cAMP into inactive 5'-AMP, thereby reducing protein kinase A (PKA) activation. The actions of the catecholamines, adrenaline and noradrenaline exert their lipolytic functions through binding to adrenergic receptors (β_1- and β_2- in humans) located on the plasma membrane of adipocytes (Arner, 2005). This leads to a subsequent increase in intracellular cyclic AMP (cAMP) production, which in turn activates PKA. Hormone-sensitive lipase (HSL) appears to be the major target of activated PKA, whereby PKA-induced phosphorylation of HSL initiates its translocation from the cytosol to the surface of the lipid droplet to initiate lipolysis (Egan *et al.*, 1992). At the lipid droplet surface, the hydrolysis of triglycerides occurs through the sequential actions of three lipases. Adipose triacylglyceride lipase (ATGL) converts TAG to diacylglycerol (DAG), whilst HSL converts DAG to monoacylglycerol (MAG). Finally monoglyceride lipase (MGL) cleaves MAG into glycerol and FFA. Although both ATGL and HSL exhibit the capacity to hydrolyse triglycerides *in vitro*, ATGL appears to have higher substrate specificity for TAG than DAG (Frühbeck *et al.*, 2014), whilst only HSL demonstrates the ability to hydrolyse DAG (Haemmerle *et al.*, 2002). Furthermore, complete activation of AGTL requires its binding with the co-activator comparative gene identification 58 (CGI58). Taken together, between them, ATGL and HSL are thought to be responsible for over 95% of TAG lipase activity (Schweiger *et al.*, 2006). An overview of lipolysis is provided in Figure 1.3. Regional differences in catecholamine lipolysis have been observed in humans, with a more marked response to catecholamine stimulation in visceral adipose tissue in comparison to subcutaneous adipose tissue, whilst basal lipolytic activity is noted to be higher in subcutaneous compared to visceral depots (Arner, 1995). This is considered to be due in part to differences in the expression of

expression and function of the catecholamine receptors.

In addition to the function of the lipases, lipid-droplet associated proteins also have significant functional roles in the control of lipolysis. Lipid droplets contain a core of neutral lipid surrounded by a phospholipid monolayer which is coated with specific proteins, including members of the PAT family of proteins. Perilipin-1 (PLIN1) is the founding member of the PAT family (named after the first three members, perilipin, adipocyte differentiation-related protein (ADRP) and tail-interacting protein of 47kDa (TIP47)), all of which share sequence similarities and the ability to bind to lipid droplets. PLIN1 is expressed almost exclusively in adipocytes, and within adipocytes it is generally restricted to lipid droplets, however recently it has also been shown to localize in the endoplasmic reticulum (Skinner *et al*., 2013). In the basal state, PLIN1 is associated with CGI58, however maximal phosphorylation of PLIN1 results in the dissociation of CGI58 from the lipid droplet, allowing it to interact with ATGL in the initiation of lipolysis (Yamaguchi *et al*., 2007). In addition to HSL, PLIN1 is also phosphorylated by PKA in response to catecholamine stimulation. It has been demonstrated *in vitro* that that PKA-mediated phosphorylation of PLIN1 is not essential for HSL translocation to the lipid droplet, but it is essential for lipid droplet interactions between PLIN1 and HSL to allow lipolysis to proceed (Miyoshi *et al*., 2006).
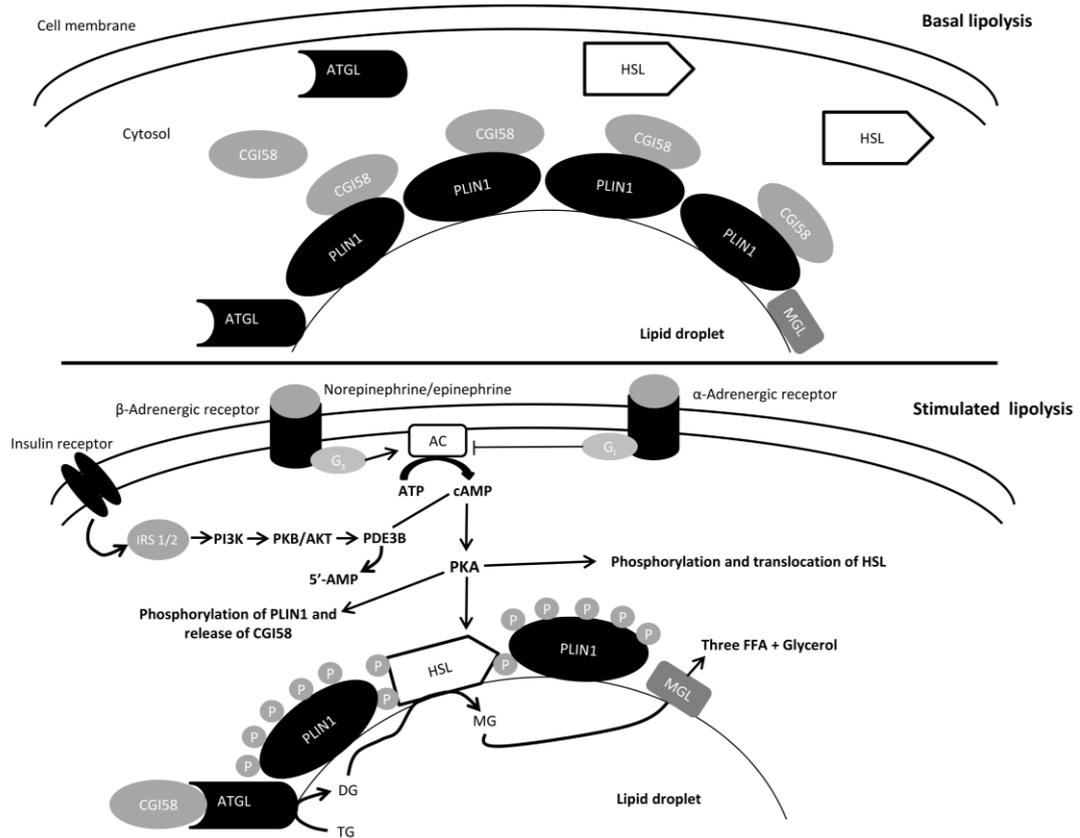
Figure 1.3: An overview of the key pathways activated during lipolysis. Abbreviations: *CGI58* comparative gene identification-58, *ATGL* adipose triglyceride lipase, *HSL* hormone sensitive lipase, *PLIN1* perilipin 1, *MGL* monoacylglycerol lipase, G_i G inhibitory subunit, G_s G stimulatory subunit, *AC* adenylyl cyclase, *ATP* adenosine triphosphate, *cAMP* cyclic adenosine monophosphate, *PKA* protein kinase A, *IRS1/2* insulin receptor substrate 1/2, *P13K* phosphoinositide 3-kinase, *PKB/AKT* protein kinase B, *PDE3B* phosphodiesterase 3B, *5'-AMP* 5 adenosine monophosphate-activated protein kinase, *TG* triacylglycerol, *DG* diacylglycerol, *MG* monoacylglycerol.

It is clear from the evidence present here that the control of adipose tissue lipolysis is a tightly regulated physiological process, mediated by several key factors. It is unsurprising that states of altered energy balance such as observed in obesity will result in marked alterations in lipolysis and lipid-droplet associated protein functions, which may contribute to the pathogenesis of metabolic abnormalities associated with obesity.

1.7 Lipolysis in obesity

Over-nutrition results in the excessive accumulation of triglycerides esterified from fatty acids and stored within lipid droplets in adipocytes. Obesity is well characterised by the expansion of the fat mass through an increase in adipocyte size (hypertrophy) in a variety of species (Arner *et al*., 2010; Grant *et al*., 2011; Van de Velde *et al*., 2013). The identification of adipocyte precursor cells in adipose tissue *in vivo* in mice (Rodeheffer *et al*., 2008), which have the capacity to differentiate to increase the number of terminally differentiated adipocytes (hyperplasia), is also a means by which adipose tissue can expand during positive energy balance. Expansion of the lipid droplet to accommodate triglyceride deposition is a highly regulated process, and whilst the exact mechanisms remain to be identified, PLIN1 and its interaction with another adipocyte specific protein, fat specific protein of 27kDa (FSP27) which is a crucial factor in maintaining large unilocular lipid droplets, appear to be important in mediating lipid exchange and lipid droplet growth (Sun *et al*., 2013).

Continued expansion of adipose tissue beyond appropriate expandability limits is known to result in metabolic abnormalities in humans including ectopic lipid deposition and insulin resistance (Rutkowski *et al*., 2015). Obesity in humans is associated with increased circulating fatty acids, indicative of the elevated basal lipolytic activity noted in obese human subjects. This may be a direct effect of increased fat cell size, as fat cell size is strongly correlated with levels of basal lipolysis (Engfeldt and Arner, 1987). Increased circulating fatty acid concentrations may result in ectopic lipid deposition in other tissues such as liver and skeletal muscle which can be associated with insulin resistance in humans (Boden and Shulman, 2002). Furthermore, increased inflammation associated with obesity in Man results in the release of the circulating inflammatory factor, TNF-α from adipose tissue, which has known lipolytic effects in human adipocytes (Zhang *et al*., 2002). These effects are considered to be due to increased phosphorylation of PLIN1 by PKA as a result of upstream elevations in intracellular cAMP through activation of the extracellular signal-related kinase pathway (ERK) (Zhang *et al*.,

2002). It remains to be elucidated whether this could apply for the horse. To date, data surrounding a role of inflammation in equine obesity have been contradictory. In a single trial, increasing BCS was associated with an increase in the blood gene expression of interleukin-1 (IL-1) and serum TNF-α concentrations in a group of light breed mares (Vick *et al.*, 2007). However, a more recent study of Thoroughbred geldings failed to detect any associations between BCS and serum TNF-α concentrations (Suagee *et al.*, 2011). In addition to alterations in basal lipolysis, obesity is associated with a blunted response to catecholamine-stimulated lipolysis, however this may be depot-specific in humans, with the lipolytic effect of catecholamine's being decreased in subcutaneous adipose tissue but increased in visceral adipose tissue (Arner, 2005).

The effect of obesity on PLIN1 expression has been extensively studied after initial studies using PLIN1 KO mouse models identified that they not only had elevated basal lipolysis, they were resistant to diet-induced obesity and had an attenuated stimulated lipolysis (Tansey *et al.*, 2001), suggesting that PLIN1 phosphorylation is required for maximally stimulated lipolysis to proceed, as alluded to earlier. Conversely, overexpression of PLIN1 in mice was also found to be protective against diet-induced obesity (Miyoshi *et al.*, 2010). This appears counter-intuitive, however the authors suggest this finding was due in part to alterations in brown adipose tissue metabolism. Similarly, another study found that PLIN1 overexpression in mice resulted in a down-regulation of FSP27, decreasing lipid droplet size and promoting a brown-fat like phenotype (Sawada *et al.*, 2010). The discrepancy between studies may indicate that PLIN1 has diverse functions and extreme alterations in PLIN1 result in marked changes in whole body metabolism in the mouse which may or may not be relevant to other species.

A summary of literature for PLIN1 and HSL in obesity is provided in Table 1.2. A reduction in the protein expression of PLIN1 in adipose tissue of obese human subjects may contribute to elevations in basal lipolysis (Ray *et al.*, 2009), although when PLIN1 was quantified in human abdominal subcutaneous adipocytes, it was found that per fat cell, PLIN1 content was unaltered in obesity (Wang *et al.*, 2003).

This would indicate that increases in fat cell size in obesity are not accompanied by relative increases in PLIN1 content, a factor which could contribute to elevations in basal lipolysis. Reduced protein expression of phosphorylated HSL and PLIN1 has been observed in epididymal and inguinal adipose depots of high-fat diet fed mice (Gaidhu *et al*., 2010). This was considered to be a contributing factor to the observed blunted response to catecholamine stimulated lipolysis. HSL haploinsufficiency and treatment with an HSL inhibitor in mice has been associated with improvements in insulin sensitivity through reductions in fatty acid uptake and an increase in glucose uptake (Girousse *et al*., 2013). However, the recent identification of HSL deficiency in humans, resultant from a frameshift mutation in the LIPE gene encoding HSL, resulted in defects in lipolysis and the development of type 2 diabetes (Albert *et al*., 2014). It has been speculated that the confounding nature of these two findings may be partly due to the fact that human heterozygote carriers of the frameshift mutation maintain functional lipolysis, whilst haploinsufficient mice have dysfunctional lipolysis, indicative of species differences (Zechner and Langin, 2014). However these findings do indicate a putative functional role for HSL in the control of insulin dynamics.

In addition, to functional differences in the expression of PLIN1 and HSL in obesity, polymorphisms in these genes have been characterised and associated with lipolytic rate (Hoffstedt *et al*., 2001), waist circumference in lean subjects (Carlsson *et al*., 2006), obesity risk (Qi *et al*., 2004), and weight-loss resistance (Corella *et al*., 2005).

In the horse, whilst studies have assessed lipid metabolism in the context of hyperlipemia (Frank *et al*., 2003; Schmidt *et al*., 2001), only one study has directly assessed adipocyte response to lipolytic stimulation (Briedenbach *et al*., 1999). That study identified that whilst rates of lipolysis were significantly greater for ponies compared with horses, the ability of insulin to inhibit lipolysis was similar between animals (Breidenbach *et al*., 1999). The authors speculate that the high rates of lipolysis in ponies may partly explain the increased susceptibility of ponies to hyperlipemia.

Table 1.2: An overview of the key PLIN1/HSL studies. Abbreviations: *KO* knock-out, *WT* wild-type, *HFD* high-fat diet

PLIN1/HSL	MAJOR FINDINGS	AUTHOR
PLIN1	PLIN1 KO mice showed elevated basal lipolysis and attenuated stimulated lipolysis. The KO mice consumed equal amounts of food than WT mice but had ~30% less accumulation of adipose tissue. Following 7 weeks of HFD (55% calories from fat) KO mice showed resistance to obesity.	Tansey *et al*. (2001)
PLIN1	Overexpression of either human or mouse PLIN1 in mice (transgenic mice). 25 weeks HFD (60% calories from fat) resulted in reduced body weight, reduced adipose tissue mass compared to WT mice and increased expression of oxidative genes from BAT. Basal and catecholamine stimulated lipolysis reduced and glucose tolerance improved in transgenic mice	Miyoshi *et al*. (2010)
PLIN1	Human subcutaneous fat cells. Basal and noradrenaline lipolysis increased and PLIN1 protein decreased in obese women compared to lean women. High rate of lipolysis associated with low PLIN1 content. Polymorphism in PLIN1 (rs891460A/G) associated with lipolysis rates.	Mottagui-Tabar *et al*. (2003)
PLIN1	Omental and subcutaneous adipose tissue from lean and obese human subjects. PLIN1 mRNA significantly lower in Sc adipose tissue from obese compared to lean subjects. PLIN1 protein relative to protein/fat cell surface area reduced in obese subjects but PLIN1 per fat cell unchanged in obese vs. lean subjects	Wang *et al*. (2003)
PLIN1 & HSL	Omental and subcutaneous adipose tissue from lean and obese women. Increased adipocyte size in obese vs. lean. PLIN1 protein reduced in both depots in obese vs. lean subjects and inverse correlation between PLIN1 protein and adipocyte size and basal lipolysis. Basal and stimulated lipolysis increased in subcutaneous vs. omental fat. HSL mRNA increased in obese vs. lean subjects, HSL protein reduced in both depots in obese subjects.	Ray *et al*. (2009)
PLIN1	Polymorphisms in PLIN1 (rs2289487 and rs894160) identified and associated with a lower risk of obesity in women	Qi *et al*. (2004)
PLIN1	Subjects (n = 48) with 11482A polymorphism found to be resistant to weight loss following a 1 year low energy diet	Corrella *et al*. (2005)
HSL	Polymorphism in HSL gene: increased frequency of allele 5 in HSLi6 polymorphism in obese and NIDDM subjects compared to lean subjects	Hoffstedt *et al*. (2001)
HSL	HSL haploinsufficiency in mice associated with reduced lipolytic capacity, increased fatty acid turnover and improve glucose metabolism and insulin sensitivity, with no change in fat mass. In humans, lipolytic rate positively correlated with indexes of insulin resistance	Girousse *et al*. (2013)
HSL	HSL deficiency in humans associated with dyslipidaemia, hepatic steatosis, systemic insulin resistance and subjects had type 2 diabetes	Albert *et al*. (2014)
HSL & PLIN1	Mice fed HFD (60% calories from fat) for 8 weeks. Phosphorylated HSL & PLIN1 protein content reduced in subcutaneous and visceral fat in HFD mice compared with WT mice. Basal lipolysis increased but epinephrine-stimulated lipolysis blunted in HFD fed mice compared to WT mice.	Gaidhu *et al*. (2010)

Taken together, evidence presented here demonstrates that PLIN1 and HSL are closely associated proteins that have crucial functional roles in adipose tissue lipolysis and glucose homeostasis. The expression of these proteins has been shown to be altered in an obese state in humans and rodents which appears to be associated with alterations in basal and catecholamine-stimulated lipolysis. Although research surrounding PLIN1 and HSL in human metabolic disease remains ongoing, the function of these proteins in the setting of equine obesity remains unknown.

In conclusion, there is a clear need for further research to improve our understanding of equine obesity. Hypotheses to be tested in this thesis were formed on the basis of the literature reviewed above and are fully described in Chapter 2.

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Chapter 2

Thesis in context: Rationales, hypotheses, aims and objectives

Overarching aims of this thesis were,

> 'To explore some novel concepts and mechanisms which might underpin the obese state in the horse and pony, with a view to improving our understanding and exploring previously unconsidered therapeutic directions'.

These **aims were addressed through a number of related objectives** which encompassed studies, extending from the exploration of the owner's ability to correctly identify obesity in their animals, to focused molecular approaches to evaluate the obesogenic role of targeted cellular pathways. Intermediate objectives were introduced to overcome knowledge gaps related to requirements to quantify regional adipose tissue reserves in the horse and to determine the timeframe for post-mortem tissue sampling for molecular studies.

Objectives were delivered through the following studies which are reported in 5 separate Chapters (Chapters 3 to 7).

Hypotheses and Objectives

Chapter 3: Perceptions of obesity and management practices in a UK leisure-based population of horse owners and enthusiasts.

Rationale: On the combined basis of current information regarding perceptions of obesity in man and other companion species and the strength of unsupported but widespread anecdotal data for the horse, the following hypotheses were addressed.

> **Hypothesis 3a:** That professional horse keepers would be more able to identify equine obesity than amateur owners who maintain their animals for leisure purposes alone and that this would be reflected in animal management practices.

> **Hypothesis 3b:** Those perceptions of ideal body condition (adiposity) would be

dependent on the specific intended use of an animal across the spectrum of non-racing equine pursuits.

Two key objectives were addressed.

Objective 3a: To evaluate horse-enthusiasts perceptions of obesity and the influence that the equestrian discipline for which the horse is used may have on perceptions and to stratify responses in accordance with the professional or amateur status of the owners/keepers.

Objective 3b: To collect and collate more detailed information in order to characterise current animal care and management practices for horses and ponies in the UK.

These objectives were achieved through the development of an internet-based, two-tier questionnaire. A brief pictorial questionnaire was designed to survey horse owner and enthusiast's perceptions of obesity in horses (Tier 1). Respondents had the option to participate in a more detailed management-focused questionnaire (Tier 2).

Chapter 4: EQUIFAT: a novel scoring system for the semi-quantitative evaluation of regional adipose tissues in *Equidae*.

Rationale: Given the literature discussed in Chapter 1, it is evident that body fatness is most readily estimated for living horses by the use of subjective body condition scoring (BCS) systems. Studies of adiposity in human subjects clearly indicate that the impact of adipose tissue expansion on health is associated with the specific anatomical distribution of excessive fat deposition. Molecular studies describing the cellular activity of adipose tissues in horses are beginning to identify comparable between-depot differences in gene and protein expression. Internal fat depots cannot be quantified in living horses and the exploration of depot differences and any associations with health or metabolic disease are largely dependent on *post-mortem* tissue sampling methods. Most commonly, data and

samples with respect to regional adiposity and specific disease are collected within an abattoir or *post-mortem* room or surgical setting. . Further, BCS systems to assess whole body 'adiposity' have not been validated for post-mortem application. Before embarking on detailed molecular studies of adipose and skeletal muscle in horses of known *ante-mortem* BCS, the following hypotheses were addressed.

> **Hypothesis 4a:** That the population of animals presented for slaughter at a UK abattoir would reflect the distribution of phenotypes recorded in epidemiological surveys of horses and ponies living in the UK.

> **Hypothesis 4b:** That the discrete adipose depots studied will demonstrate a spectrum of fat accumulation that can be succinctly described and used as the basis of a robust 5 point semi-subjective scoring system to evaluate specific fat depots *post-mortem*.

> **Hypothesis 4c:** That discrete adipose tissues within the equine body are functionally divergent and that these differences may be reflected in different degrees of association with *ante-mortem* BCS.

From these hypotheses, 4 key objectives were formed.

> **Objective 4a:** To collect and collate phenotypic data to describe the population of horses presented at a UK abattoir on seven random dates within an 18 month period.

> **Objective 4b:** To collect anatomically defined photographic images and measures of specific regionally-discrete adipose tissues and use these data to develop region-specific, text and photographic descriptors as the basis of the EQUIFAT, 5 point equine fat scoring system.

> **Objective 4c:** To robustly validate the EQUIFAT system for field use by testing within and between observer evaluations and whether this was improved by the ability to use 'half scores'.

> **Objective 4d:** To use data collected across a wider population of horses and

ponies to determine any associations between *ante-mortem* BCS and EQUIFAT scores for the discrete *post-mortem* adipose depots surveyed.

Objectives were achieved using sub-sets of abattoir-derived data and photographic images of specific adipose depots in combination with ante-mortem phenotypic descriptors for the individual animals.

Chapter 5: Post-mortem stability of RNA in skeletal muscle and adipose tissue and the tissue-specific expression of myostatin, perilipin and associated factors in the horse

Rationale: Conducting molecular biology studies requires knowledge of the appropriate time-frame to collect *post-mortem* tissues in order to extract intact RNA and protein. Whilst studies have assessed this for other species, no studies have yet addressed this for skeletal muscle and adipose tissue from the horse. In addition to this, establishing the tissue-specific distribution of key proteins of interest is an important prerequisite to detailed studies. From the perspective of the literature and the intended studies, the following hypotheses were formed.

> **Hypotheses 5a:** That intact RNA suitable for molecular biology studies can be extracted from skeletal muscle and adipose tissue samples obtained from horses *post-mortem.*

> **Hypotheses 5b:** That extracted RNA is more stable when harvested from skeletal muscles than from adipose tissues.

> **Hypotheses 5c:** That the myostatin gene and protein is predominantly expressed by skeletal muscles, whilst PLIN1 gene and protein would predominate in adipose tissues.

To test these hypotheses, the following objectives were addressed.

> **Objective 5a:** To describe the time-frame for RNA degradation in skeletal muscle and adipose tissues sampled *post-mortem.*

Objective 5b: To characterise the expression of myostatin, ActRIIB, follistatin and PLIN1 RNA across a range of tissues.

Tissue sample used to address these hypotheses were collected from thoroughbred horses at the point of slaughter, and in accordance with 'best practice' as defined in Chapter 3.

Chapter 6: Preliminary investigation into a potential role for myostatin and its receptor (ActRIIB) in lean and obese horses and ponies

Rationale: Not all horses or ponies maintained under common management become obese. This variation in the proclivity of animals to become obese has major implications for animal management. Understanding the physiological basis for this variation might allow the identification and targeted nutritional management of obesity prone animals. At the inception of this project, work in other species had implicated the myokine, myostatin as a key regulator of whole body energy homeostasis. Myostatin had already been associated with differences in equine athletic performance and was considered a major component of 'cross talking' pathways between the major labile energy reserves, adipose and skeletal muscle tissues. The literature reviewed in Chapter 1 made myostatin a key candidate in the determination of energy balance. However, a role for myostatin in equine obesity has yet to be defined. On this basis, we tested the following hypothesis,

Hypothesis 6: That myostatin gene and protein expression in skeletal muscles and circulating myostatin concentrations in blood serum will be altered in obese horses and ponies.

Which was addressed by,

Objective 6a: To evaluate the gene and protein expression of myostatin and its receptor, ActRIIB in 4 discrete skeletal muscles in lean and obese horses and ponies.

Objective 6b: To assess the circulating concentration of myostatin in blood serum in the same group of animals.

Tissue and blood samples collected to address these objectives were harvested from lean and obese horses and ponies at the point of slaughter, and in accordance with 'best practice' as defined in Chapter 5.

Chapter 7: **Expression of PLIN1 and hormone-sensitive lipase in adipose tissues from lean and obese horses and ponies**

Rationale: Lipolysis is a tightly regulated process in adipose tissues that relies on the coordinated actions of several key proteins associated with the surface of the lipid droplets within adipocytes. Two of these proteins, HSL and PLIN1 have been well characterised in murine and human studies and have both been implicated to play important roles in obesity development. To date, no studies have yet addressed the expression of these proteins in equine obesity. Mobilisation of lipids from the lipid droplets within adipocytes effects changes in adipocyte size. Morphological changes in adipocytes in discrete adipose tissue depots in the obese and lean state are known for other species but this information is absent for the horse.

Hypotheses 7a: Adipocyte cross-sectional areas are uniformly increased in all adipose tissue depots in the obese state.

Hypotheses 7b: Changes in cell size distributions within specific adipose tissue depots are associated with corresponding changes in the expression of the key lipolytic proteins, HSL and PLIN1.

Hypotheses 7c: Lipid:protein ratios in adipose tissue homogenates are associated with mean adipocyte size and that the cellular protein distribution between lipid and cytosolic cell fractions will alter correspondingly.

These hypotheses were tested by addressing the following objectives

> **Objective 7a:** To characterise adipocyte area across a range of adipose depots from lean and obese horses and ponies.

> **Objective 7b**: To extract protein from the cytosolic (internatant) and lipid associated (fat cake) fractions of adipose tissue and to evaluate the protein expression of HSL and PLIN1 in these fractions across a range of adipose depots in lean and obese horses and ponies.

> **Objective 7c:** To evaluate associations between lipid:protein ratio and corresponding adipocyte area for each adipose depot.

Tissue samples used to address these hypotheses were collected from lean and obese horses and ponies at the point of slaughter, and in accordance with 'best practice' as defined in Chapter 5.

Chapter 3

Perceptions of obesity and management practices in a UK leisure-based population of horse-owners and enthusiasts

3.1 Abstract

The high prevalence of obesity in the leisure population of horses and ponies in the UK is partly attributable to reduced workloads, improved husbandry techniques and the increased availability of energy-dense diets. An inability to accurately recognise obesity will further exacerbate this and preclude the initiation of weight-loss management. Currently, knowledge of human perceptions of equine obesity is lacking. A two-tier, internet-based questionnaire was developed to assess horse owner and enthusiasts' perception of obesity using lateral photographic images of horses and ponies (Tier 1). Tasks included: Asserting their involvement in the sector; identifying overweight animals; scoring the suitability of animals for participation in different equestrian activities on the basis of body weight. There was an option to partake in a detailed questionnaire at the end of Tier 1. Information regarding animal management practices employed by horse owners were collected (Tier 2). The questionnaire was distributed through UK-based equine forums. Tier 1: Of 546 respondents, 98% were female. Amateur involvements dominated professionals (81%:19%). Key findings included that, only 11% correctly identified all overweight animals presented (6/12), but between 37 and 98% correctly identified individual overweight animals. Professional status did not alter an owner's ability to identify overweight animals. On assessing the weight/condition suitability of a sport horse, a cob and a pony for different disciplines, respondents rated each animal significantly lower (towards underweight; $p < 0.01$) for the showing discipline compared to other disciplines (eventing, dressage etc.). Tier 2: In total there were 177 responses. Horse/pony information gathered included owner-reported obesity prevalence (4.5%), seasonal changes in horse/pony weight, seasonal management practices, exercise and feeding routines. Tier 1 provided evidence that horse owners and enthusiasts' vary in their ability to identify overweight animals by visual appearance alone. Data support our anecdotal understanding that owners consider it appropriate that horses and ponies should carry more weight when competing in showing classes. These data will aid in targeting nutritional and management advice for horse owners.

3.2 Introduction

Equine obesity has become a major health issue among horses and ponies especially in the leisure horse sector. Implementing controlled weight loss by nutritional restriction can be problematic for many horse owners and requires long term commitment. The recognition of obesity is a prerequisite for corrective management (dietary energy restriction) and commonly, treatment is instigated by a veterinarian when animals present with laminitis as opposed to being a direct response to owner concern.

Current trends predict that by 2030, 57.8% of the global adult human population could be overweight or obese (Kelly *et al.*, 2008). Epidemiological studies have established that for man, obesity is a significant risk factor for the development of cardiovascular disease, some cancers, diabetes and premature death (Flegal *et al.*, 2013; Must *et al.*, 1999). Although a reduction in physical activity and increased availability of high-fat, calorie-dense diets and high sugar drinks have undoubtedly contributed to the increased incidence of obesity in the human population; there is evidence to suggest that under-recognition of weight status is common among both adults (Johnson *et al.*, 2014; Wetmore and Mokdad, 2012) and parents of children (Etelson *et al.*, 2003; Jones *et al.*, 2011). In agreement with this, a UK-based study (which compared two surveys of human populations taken 5 years apart), identified that whilst self-reported body weights increased, the weight at which people perceived themselves to be overweight also increased (Johnson *et al.*, 2008), further emphasising a misperception of weight status among adults. Evidently, the perception of body weight status will impact on whether or not an individual will take action to reverse weight gain. This has been demonstrated in studies showing that those with a more accurate perception of their weight status will be more likely to have tried to lose weight (Bittner Fagan *et al.*, 2008; Duncan *et al.*, 2011).

Misperception of body weight status has now manifested in animals under the care of humans. Owners have been shown to misperceive their dog's body shape (Courcier *et al.*, 2011), with underestimation being the most common form of misperception, and this misperception remains even with the use of a body

condition scoring chart (Eastland-Jones *et al.*, 2014).

With regards to *Equidae*, the growth in the UK population of leisure horses and ponies (those kept for pleasure purposes and competing at unaffiliated/riding club level competitions) has been associated with an increased incidence of obesity, which has been recently documented during the summer months in a population of leisure animals at over 35% (Giles *et al.*, 2014). This high obesity prevalence could be partly attributed to modern husbandry techniques such as increased provision of shelter and rugs, reductions in workload and year-round availability of energy-dense diets, although breed has also been established to be an important risk factor for obesity (Giles *et al.*, 2014; Robin *et al.*, 2015). Data describing the owner perceptions of obesity in horses are sparse, although there is some evidence that owners underestimate body condition score (Ireland *et al.*, 2012; Wyse *et al.*, 2008). Additionally, perceptions of appropriate or 'ideal' body weight/condition of horses and ponies may differ between owners who intend their animals to participate in the divergent equestrian disciplines. This disparity is likely to be greatest for show-ring animals where debate in the lay press has highlighted concerns that overweight animals are more likely to succeed (Horse and Hound, 2005).

The primary objective of the current study was to evaluate horse-enthusiasts perceptions of obesity and the influence that the equestrian discipline for which the horse is used may have on perceptions. A secondary objective was to gather more detailed information regarding the care and management practices of horses and ponies in the UK since these may have an effect on the high prevalence of obesity in this population. These objectives were achieved *via* the development of an internet-based two-tier questionnaire whereby a short questionnaire was designed to obtain information on horse owner and enthusiasts perceptions of obesity in horses (Tier 1), with the option for horse-owners to continue and partake in a more detailed questionnaire (Tier 2). It was hypothesised that perceptions of obesity would differ between professionals and non-professionals, and that management practices would differ between hobbyists/leisure riders and amateur competitors/professionals and depending upon whether the horse/pony was kept

for leisure (pet/companion, non-competitive riding and competing in unaffiliated competitions) or competition purposes (competing in affiliated competitions), therefore data were also analysed to identify any such differences between the populations.

3.3 Materials and Methods

Questionnaire design

The survey software, SelectSurvey (ClassApps, Missouri, USA) was used to create two internet-based surveys. The first questionnaire (Tier 1; Appendix B) comprised 16 questions and gathered information relating to the respondents geographical location, age, duration of time involved in horses, their involvement/interest in equestrian disciplines, and the horse ownership status including whether they were professionals or non-professionals (involvement in horses forms part of or entire job (professionals) or their involvement was purely for fun/enjoyment (non-professionals) and whether they own or loan a horse/pony. This was followed by a set of questions to evaluate obesity perceptions which used lateral photographic images of horses and ponies across a range of body condition scores (which had been previously assessed *in vivo* by an experienced assessor) using the Kohnke modification of the Henneke body condition score system (Kohnke, 1992). A range of breed types and coat-colours were used in compiling the image selection in order to limit bias. To determine the ability of the respondent to identify overweight and obese animals, the first question used 12 images and asked respondents to select the images of all horses/ponies they considered to be overweight (Figure 3.1).

In order to determine the impact of the animal's intended use (equestrian discipline) on obesity perceptions, lateral photographic images of a Sport horse, Welsh pony and a Cob horse were shown independently and respondents were asked to categorise the animal's weight/condition for different disciplines (e.g. Dressage, Eventing, Showing) on a five point scale (1= very underweight to 5 = very overweight). Finally for Tier 1, respondents were presented with another two sets

of images (Appendix B) and asked firstly to match pictures of horses and ponies to the correct scenario (e.g. this horse/pony needs to lose weight immediately, this horse/pony could do with gaining a little weight/condition before a busy summer competing) and secondly to rank the horses and ponies in order of increasing weight/condition.

At the conclusion of Tier 1, there was an option to take part in a more extensive questionnaire for horse-owners, comprising 65 questions (Tier 2; Appendix C). If respondents owned more than one horse/pony they were asked to answer all the questions with respect to a single animal of their choice. In brief, the questionnaire gathered horse/pony data on: basic information, health and wellbeing, use and exercise as well as nutrition and management of their horse/pony.

Figure 3.1: Percentage of respondents classifying images of horses and ponies as overweight (Tier 1). All animals were expertly assessed in vivo and assigned a body condition score from 1 (very poor) to 9 (extremely fat). The percentage of respondents classifying each image as overweight is shown. Overweight animals (BCS ≥6/9): A, C, E, G, J and L.

Data analysis

Questionnaire responses were downloaded into Microsoft Excel and exported for statistical analysis into STATA Version 13 (StataCorp, Texas). Statistical significance was set at $p < 0.05$.

For Tier 1 analysis, binary variables were created for professional status (non-professional = 0 professional = 1). For Tier 2 analysis, as respondents were all horse owners, binary variables were created for horse ownership status (hobbyist/leisure rider = 0 amateur competitor/professional =1) and for the reason the horse/pony was kept for (animals kept for leisure/pleasure purposes = 0 animals kept for affiliated competitions = 1). Chi square tests were used for the analysis of proportions where the outcome was binary. Multinomial logistic regression was employed where the outcome was more than 2 categories whilst the Wilcoxon signed-rank test and Student t tests were employed for score and continuous data respectively.

3.4 Results

Tier 1

Survey population

In total there were 546 responses to the questionnaire. The survey population is described in Table 3.1. Almost all (98%) of respondents were female and over 70% of the respondents were aged between 26 and 60 years old. The geographical distribution of responses was as follows: England 81.3%, Scotland 13%, Wales 4.2% and Northern Ireland 1.5%. Over 90% of respondents either owned or loaned a horse or pony, and over half the study population had had an active interest in horses for between 20 and 40 years with a further 15.9% having had an interest for over 40 years. For the majority of respondents (n = 441/546; 81%), their interest in horses was purely for fun/enjoyment (non-professionals), whilst 19% (n = 105/546) of respondents earned money from their involvement in horses (professionals).

Table 3.1: Respondent information for Tier 1 and Tier 2.

Respondent information	Tier 1	Tier 2
Gender		
Male	535 (98.00%)	3 (1.69%)
Female	11 (2.00%)	174 (98.31%)
Age		
Under 18	29 (5.31%)	11 (6.22%)
18-25 years	102 (18.68%)	37 (20.09%)
26-40 years	190 (34.80%)	74 (41.81%)
41-60 years	199 (36.45%)	48 (27.12%)
Over 60 years	26 (4.76%)	7 (3.95%)
Location		
England	444 (81.32%)	147 (83.05%)
Scotland	71 (13.00%)	22 (12.43%)
Wales	23 (4.21%)	5 (2.83%)
Northern Ireland	8 (1.47%)	3 (1.69%)
Professional	105 (19.23%)	
Non-professional	441 (80.77%)	
Hobbyist/leisure rider		67 (37.85%)
Amateur competitor/professional		110 (62.25%)

Ability to recognise overweight horses/ponies from photographic images

Of the twelve images presented to the respondents, six animals were classified as overweight or obese by the determination of body condition score (BCS > 6/9) by an experienced assessor *in vivo*. Only eleven percent of respondents correctly identified all six overweight animals (Figure 3.1). Three of these overweight animals were correctly identified as overweight by between 65 and 98% of the respondents. For the other three animals, a lower percentage (between 37 and 41%) of respondents correctly identified that they were overweight (Figure 3.1). For two animals that had a BCS of 5/9, (both cob-breed horses) 96% and 73% of

respondents respectively considered them to be overweight. There was no difference between professionals and non-professionals in their ability to identify the six overweight animals (Table 3.2), apart from one image (A; BCS 7/9) where a significantly greater proportion of non-professionals (80.7% vs. 71.4%; p = 0.04) correctly identified the animal as being overweight.

Weight categorisation of horses/ponies across different disciplines

Sport horse

Respondents were shown a photographic image of a horse that was evaluated *in vivo* by an experienced assessor and was assigned a BCS of 5/9 (equating to "about right"). Analysis revealed that respondents rated this horse significantly lower (towards underweight) for competing in affiliated showing classes compared to the other three disciplines (p < 0.01). Multinomial logistic regression was employed for assessing professional/non-professional differences in rating a horse's condition for partaking in a given discipline.   For mainly staying in the field and participating in affiliated one day events, there was no difference in the response between professionals and non-professionals, however significantly more professionals considered the horse to be "very underweight" for participating in affiliated showing classes compared to non-professionals (relative risk ratio (RRR) = 2.06; p = 0.04; Table 3.2). Similarly, significantly more professionals considered the horse to be "slightly underweight" for competing in affiliated dressage competitions compared to non-professionals (RRR = 1.81; p = 0.02). The difference between the expert opinion of the weight categorisation (about right) and the respondents' answers for each discipline was also evaluated. This showed that for all the disciplines apart from eventing, the horse was considered underweight in comparison to the expert opinion (p < 0.01) (Figure 3.2). There was no difference between the expert opinion and whether the respondents were professionals or non-professionals for competing in one day events or mainly staying in the field (p = 0.59 and p = 0.18, respectively), whereas for both competing in affiliated showing classes and competing in dressage competitions, the difference between the expert and respondents was significantly greater for professionals (p = 0.03 for both

activities).

Pony

Respondents were shown a photographic image of a pony that that was evaluated *in vivo* by an experienced assessor and assigned a BCS of 6.5/9 (slightly overweight). As for the sport horse, respondents rated this pony significantly lower for competing in affiliated showing classes compared to the other two disciplines (staying in the field and a busy summer of Pony Club activities and one day events) ($p < 0.01$). Overall there were no significant differences between professionals and non-professionals in how they rated the pony for each of the disciplines (Table 3.2). The difference between the expert opinion of the weight categorisation (slightly overweight) and the respondents' answers for each discipline was evaluated. Statistical analysis revealed that for showing and staying in the field, the pony was considered by respondents to be closer to 'about right' in comparison to the expert opinion ($p < 0.01$), whilst the opposite was true for Pony club activities, where it was considered to be more overweight than the expert opinion ($p < 0.01$) (Figure 3.2). There was no difference between the expert opinion, and whether the respondents were professionals or non-professionals for taking part in Pony Club activities or mainly staying in the field ($p = 0.91$ and $p = 0.11$, respectively), whereas for competing in affiliated showing classes, the difference between the expert and respondents was significantly greater for professionals ($p = 0.05$).

Cob horse

Respondents were shown a photographic image of a cob horse that was expertly evaluated *in vivo* and assigned a BCS of 6.5/9. As for the other images, respondents rated the cob significantly lower for competing in affiliated showing classes compared to the other disciplines (mainly staying in the field, competing in affiliated dressage classes and competing in affiliated one day events; $p < 0.01$). Overall there were no significant differences between professionals and non-professionals in how they rated the horse in terms of weight/condition for each discipline (Table 3.2). The difference between the expert opinion of weight

categorisation (slightly overweight) and the respondents' answers for each discipline was evaluated. For all four disciplines, the horse was considered by respondents to be closer to very overweight than the expert opinion ($p < 0.01$) (Figure 3.2). There was no difference between the expert opinion, and whether the respondents were professionals or non-professionals for any of the disciplines evaluated ($p > 0.05$).

Table 3.2: Number and percentage of professional and non-professional respondents categorising the weight/condition of a Sport horse, Pony and Cob horse for each discipline (Tier 1). * denotes where professionals are significantly different ($p < 0.05$) from corresponding non-professional response.

Weight status	Professional/Non-professional	Sport horse				Pony			Cob horse			
		Field	*Showing*	*Dressage*	*One Day Event*	*Field*	*Showing*	*Pony Club*	*Field*	*Showing*	*Dressage*	*One Day Event*
Very Overweight	**Professional**	0	0	0	0	16 (15.24)	10 (9.52)	25 (23.81)	70 (66.67)	59 (56.19)	83 (79.05)	95 (90.48)
	Non-professional	0	0	3 (0.68)	2 (0.45)	101 (22.90)	67 (15.19)	119 (26.98)	273 (61.90)	218 (49.43)	311 (70.68)	379 (85.14)
Slightly Overweight	**Professional**	1 (0.95)	1 (0.95)	3 (2.86)	14 (13.33)	54 (51.43)	39 (37.14)	67 (63.81)	30 (28.57)	31 (29.52)	18 (17.14)	9 (8.57)
	Non-professional	14 (3.17)	15 (3.40)	19 (4.31)	64 (14.51)	214 (48.53)	185 (41.95)	252 (57.14)	139 (31.52)	172 (39.00)	118 (26.82)	59 (13.38)
About Right	**Professional**	80 (76.19)	40 (38.10)	72 (68.57)	77 (73.33)	35 (33.33)	54 (51.43)	13 (12.38)	5 (4.76)	15 (14.29)	4 (3.81)	1 (0.95)
	Non-professional	347 (78.68)	186 (42.18)	338 (76.64)	320 (72.56)	126 (28.57)	178 (40.36)	68 (15.42)	29 (6.58)	51 (11.56)	9 (2.05)	3 (0.68)
Slightly Underweight	**Professional**	24 (22.86)	49 (46.67)	30 (28.57)*	13 (12.38)	0	2 (1.90)	0	0	0	0	0
	Non-professional	78 (17.69)	209 (47.39)	78 (17.69)	52 (11.79)	0	11 (2.49)	2 (0.45)	0	0	2 (0.45)	0
Very Underweight	**Professional**	0	15 (14.29)*	0	1 (0.95)	0	0	0	0	0	0	0
	Non-professional	2 (0.45)	31 (7.03)	3 (0.68)	3 (0.68)	0	0	0	0	0	0	0
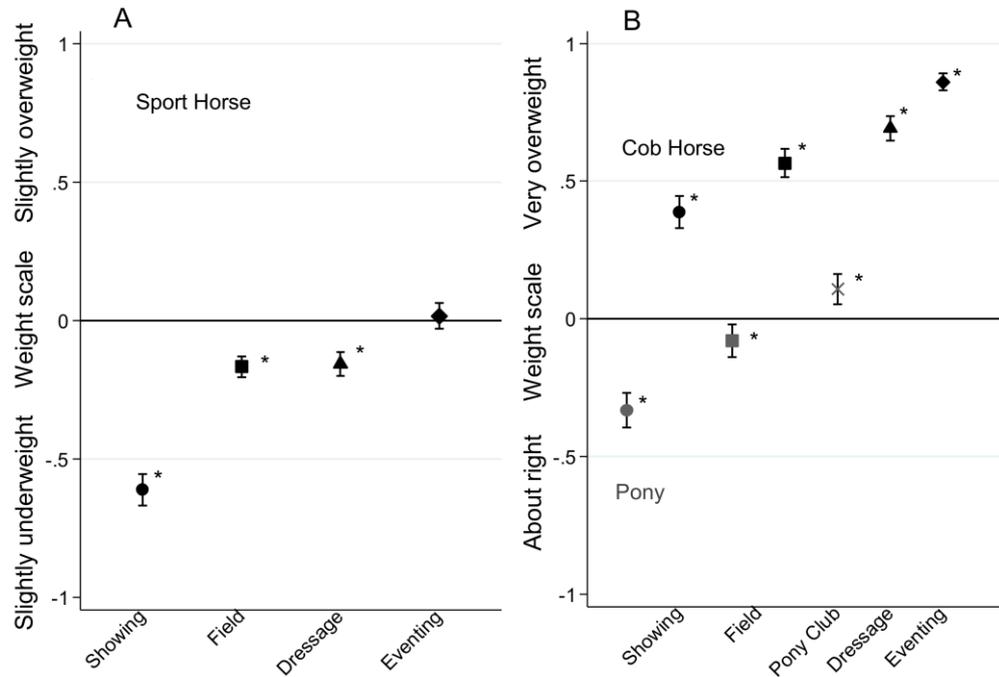
Figure 3.2: Agreement between expert and respondents in weight categorisation of a **(A)** Sport horse, **(B)** Cob horse and pony for taking part in different activities. Line at 0 denotes exact agreement ("About Right" for Sport horse and "Slightly Overweight" for Cob horse and Pony). *Denotes where respondent is significantly different from expert. Error bars = 95% confidence intervals.

Scenario and ranking

Respondents were shown four images of horses and ponies and asked to match each picture to the appropriate scenario e.g. this horse/pony is obese and needs to go on a strict diet immediately; this horse/pony could do with gaining a little bit of weight/condition before a busy summer competing etc. Over 90% of respondents correctly matched each of the four pictures with the correct scenario and chi-squared tests revealed there was no difference between professionals and non-professionals in their ability to correctly match the picture to the scenario. Lastly, respondents were asked to rank five images of horses and ponies in order of increasing weight/condition from 1-5. Over half (55%) of respondents correctly identified the very thin animal (1), with 45% of respondents ranking the horse who should have been ranked as 2 as 1. Similarly, 54.5% of respondents correctly identified the second ranked animal, with 44.3% of respondents ranking the very

thin animal (1) in second. For the three other ranks (3-5), over 98% of respondents correctly identified which horse matched each position. There was no difference between professionals and non-professionals in their ability to rank the images in order of increasing weight/condition.

Tier 2

Population Demographics

There were 177 responses to the questionnaire. The majority of respondents resided in England (83.1%), with 12.4% from Scotland, 2.8% from Wales, and 1.7% from Northern Ireland (Table 3.1). The distribution of ages and gender are outlined in Table 3.1. Almost all respondents were female (98.3%). A large proportion of respondents were aged between 26 and 40 years (41.8%), whilst a further 20.9% were aged between 18 and 25 years, 27.1% were aged between 41 and 60 years, 6.2% aged less than 18 years and 3.9% aged over 60 years. The division in the respondents   between   hobbyists/leisure   riders   and   amateur competitor/professional was 37.9% and 62.2%, respectively.   The majority of respondents owned or cared for 1 horse/pony (40.8%), 25.4% owned or cared for 2 horses/ponies, whilst 33.9% of respondents owned or cared for 3 or more horses/ponies.

Horse/pony information

If respondents owned or cared for more than 1 horse/pony they were asked to choose just one of their animals for completing the questionnaire. Table 3.3 outlines the data gathered regarding the horses and ponies. Eighty eight percent of respondents reported on management practices regarding a horse under their care with 12% reporting on a pony. Over 40% of horses/ponies were kept either at home or at a friend's premises, whilst the remaining animals were reported as being kept on a livery basis, either DIY livery (22.0%) or full/part livery (36.7%). Over half (50.9%) of the horses/ponies were aged between 4 and 10 years old, whilst a smaller proportion aged either under 4 years (2.8%) or over 21 years (6.2%) and the remaining aged between 11 and 21 years old. The majority of horses/ponies were

geldings (63.3%), with only 2 colts/stallions (1.1%) and the remaining 35.6% being mares. Almost half (44.6%) of animals were reported to be Thoroughbred/Sport horse type and 17.5% were Warmbloods. The number of Native breeds, Cobs and Irish Draught/Irish Draught type were reasonably evenly distributed at around 10% prevalence. Only 7 animals were reported to be either Arabs or a cross-breed pony. Over half the horses and ponies were kept for leisure purposes (55.4%), with the remaining 44.6% kept for competing in affiliated competitions.

Table 3.3: Horse/pony information gathered in Tier 2.

Horse/pony information	Number of respondents
Number of horses	156 (88.14%)
Number of ponies	21 (11.86%)
Age	
Under 4 years	5 (2.82%)
4-10 years	90 (50.85%)
11-16 years	49 (27.68%)
17-21 years	22 (12.43%)
Over 21 years	11 (6.21%)
Gender	
Mare	63 (35.59%)
Gelding	112 (63.28%)
Colt/stallion	2 (1.13%)
Where kept	
At home/at a friends	73 (41.24%)
DIY livery	39 (22.03%)
Full/part livery	65 (36.72%)
Breed	
Thoroughbred/Sport horse	79 (44.63%)
Warmblood	31 (17.51%)
Cob	19 (10.73%)
Irish draught/Irish draught type	16 (9.04%)
Native breed	18 (10.17%)
Arab	7 (3.95%)
Cross-breed pony	7 (3.95%)

Health and Wellbeing of horses and ponies

Owners were asked about veterinary attendance in the preceding 3 month period prior to completing the questionnaire. Over 40% (44.6%) of respondents had a veterinarian attend their horse/pony in the previous 3 months. Of these 79 veterinary visits, 36.7% of them were for routine purposes (vaccination/microchipping), with 37.5% for attending a wound or lameness, 21.3% for dental reasons, and the remaining visits for colic, skin conditions, laminitis and respiratory conditions. Thirteen animals (7.3%) were reported to have suffered from laminitis on at least one occasion in their lifetime, with the diagnosis being made by a veterinary surgeon in 69% of cases. Three respondents reported that the animal had suffered from more than one laminitic episode. There was no difference in owner-reported laminitis history between leisure and competition horses and ponies (p = 0.10), however significantly more ponies were reported to have a history of laminitis compared to horses (p < 0.01).

Respondents were asked if their horse/pony had ever been diagnosed with Equine Metabolic Syndrome (EMS). Only one respondent reported that their animal had been diagnosed with EMS which had been diagnosed by a veterinarian. Furthermore, 2.8% respondents reported that their animal had been diagnosed with pars pituitary intermedia dysfunction (PPID).Respondents were asked about how often they administer anthelminthics to their horse/pony. Over half of respondents (55.1%) reported that the decision to administer anthelminthics to their animal was made on the basis of regular faecal egg counting. Thirty five percent of respondents stated they administered anthelminthics to their horse/pony on a regular basis (every 4-12 weeks), with the remaining respondents stating they administered anthelminthics to their horse/pony according to a yard/vet approved program (4%), annually/twice annually (4.6%), or never/not known (1.1%). There was no difference in the administration of anthelmintics hobbyists and amateur competitors/professionals (keeper status; p = 0.54) or between leisure and competition horses/ponies (horse kept status; p = 0.80). However there was a strong correlation between keeper status and horse kept

status (r = 0.63) so this result was not unexpected.

Respondents were asked to rank their horse/pony on a scale from 1 (very poor) to 9 (extremely fat) based on their current condition. This scale was used as it was the same scale-range as that used for body condition scoring by our group (Kohnke, 1992). A binary variable was created whereby the horse/pony was identified as obese (≥7/9) or non-obese (< 7/9). Owner-reported obesity was found to be 4.5%, with the majority of owners scoring their animals a 5/9 which would be considered to be normal (Figure 3.3). There was no difference between keeper status (p = 0.47), and owner-reported obesity. However, significantly more leisure animals were reported to be obese compared to competition animals (8.2% vs. 0%; p < 0.01).
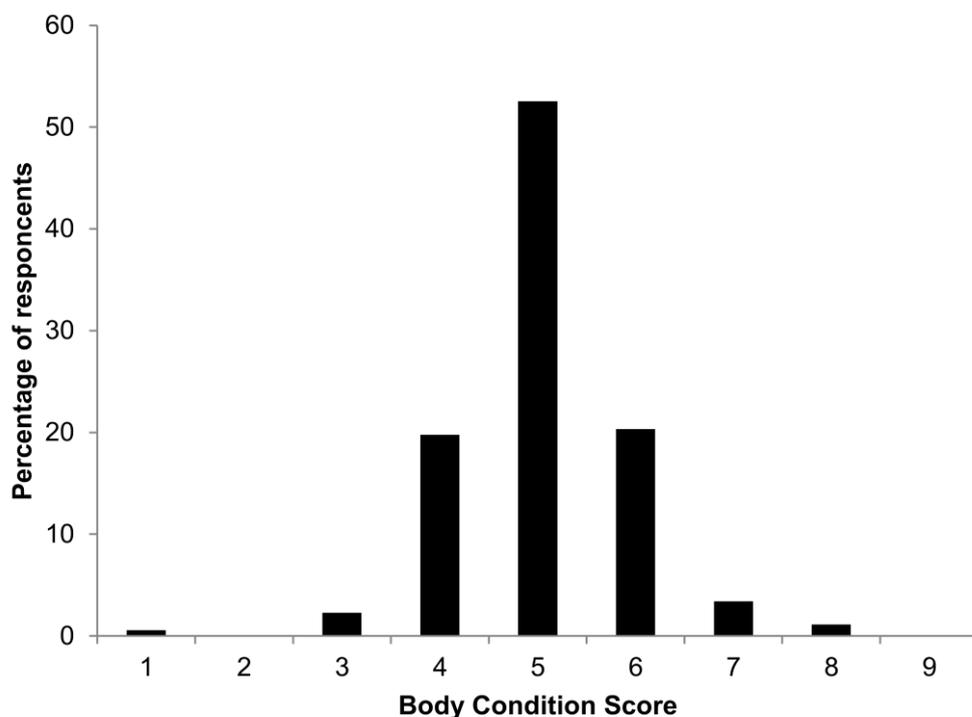
Figure 3.3: Owner-reported body condition score of their horse/pony in Tier 2.

Owners were asked to rank their horse/pony's weight/condition, on a 5-point scale, between the seasons. The response for each season is shown in Figure 3.4, with a response of 2 equalling "about right", 3 equals "slightly overweight" and 1 equals "slightly underweight". Owners reported their horse/pony to be more overweight in summer compared to the other seasons ($p < 0.01$). Associations between the outcome variable "owner reported condition" during winter and summer and the reason the horse was kept (leisure vs. competition) were assessed by multinomial logistic regression. No difference was found between leisure and competition horses/ ponies and their owner-reported weight in winter, however leisure horses/ponies were found to be more likely to be slightly overweight than competition horses/ponies in spring (relative risk ratio (RRR) 2.4; $p = 0.03$), summer (RRR = 3.9; $p < 0.01$) and autumn (RRR = 3.6; $p < 0.01$).
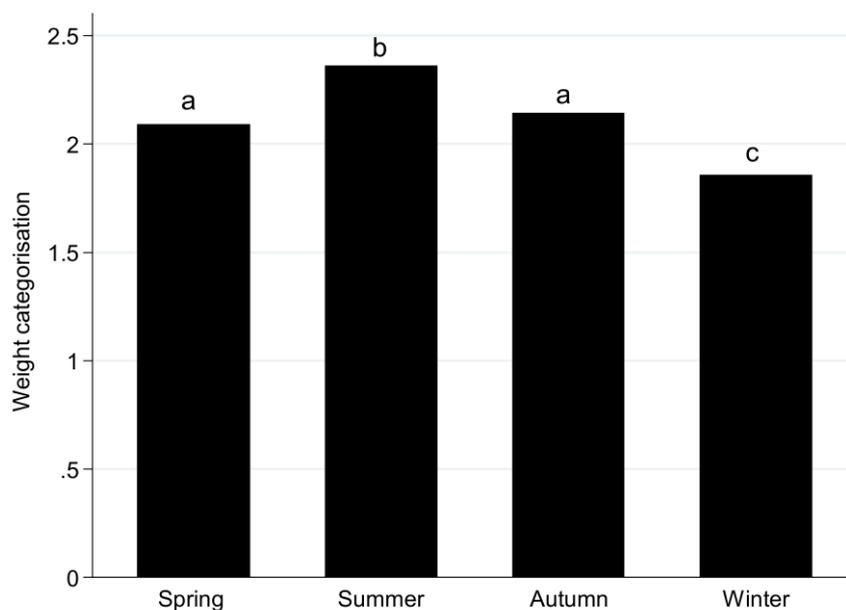
Figure 3.4: Owner-reported weight/condition of their horse/pony in the different seasons in Tier 2. The mean response was calculated for each season, whereby a value of 3 equates to slightly overweight, 2 = about right, 1 = slightly underweight and 0 = very underweight. Different lowercase letters indicate significant differences between seasons ($p < 0.05$).

Following this, respondents were asked to rank a selection of animal maintenance requirements or tasks in the order in which they perceived to be important to them, from 1 being the most important to 6 being the least important (Table 3.4). More than half of respondents (59.1%) perceived that maintaining their horse/pony at a healthy weight/condition was the most important of the options given (ranked number 1), followed by having their horse/pony's feet regularly trimmed/shod (31% ranked as number 1). Grooming their horse/pony on a daily basis was considered to be the least important scenario (52.1% ranked as number 6). There was no difference in ranking between hobbyists and amateur competitors/professionals for any of the scenarios (p > 0.05).

Table 3.4: Number and percentage of respondents ranking each maintenance requirement in order of importance from 1 (most important) to 6 (least important).

Maintenance requirement	Importance, 1 = most important, 6 = least important					
	1	**2**	**3**	**4**	**5**	**6**
Maintaining horse/pony at a healthy weight/condition	101 (59.06%)	50 (29.24%)	13 (7.60%)	5 (2.92%)	2 (1.17%)	0
Having your horse/pony's feet regularly trimmed/shod	53 (30.99%)	75 (43.86%)	24 (14.04%)	13 (7.60%)	4 (2.34%)	2 (1.17%)
Having your horse/pony's teeth regularly checked	3 (1.75%)	14 (8.19%)	61 (35.67%)	50 (29.24%)	27 (15.79%)	16 (9.36%)
Having your horse/pony's back regularly checked	1 (0.58%)	9 (5.26%)	14 (8.19%)	34 (19.88%)	59 (34.50%)	54 (31.58%)
Picking your horse/pony's feet out on a daily basis	9 (5.26%)	21 (12.28%)	48 (28.07%)	43 (25.15%)	40 (23.29%)	10 (5.85%)
Grooming your horse/pony on a daily basis	4 (2.34%)	2 (1.17%)	11 (6.43%)	26 (15.20%)	39 (22.81%)	89 (52.05%)

Use and exercise of horse/pony

Respondents were asked to assess how fit they considered their horse/pony. As expected, more competition horses/ponies were reported to be very/extremely fit compared to leisure horse/ponies (RRR 1.6; $p < 0.01$). Following this, respondents were asked how many hours they rode per week in the different seasons. The average number of hours ridden in a month was significantly greater for both amateur competitors/professionals compared to hobbyists ($p < 0.01$) and for leisure compared to competition horses/ponies ($p = 0.01$; Figure 3.5).
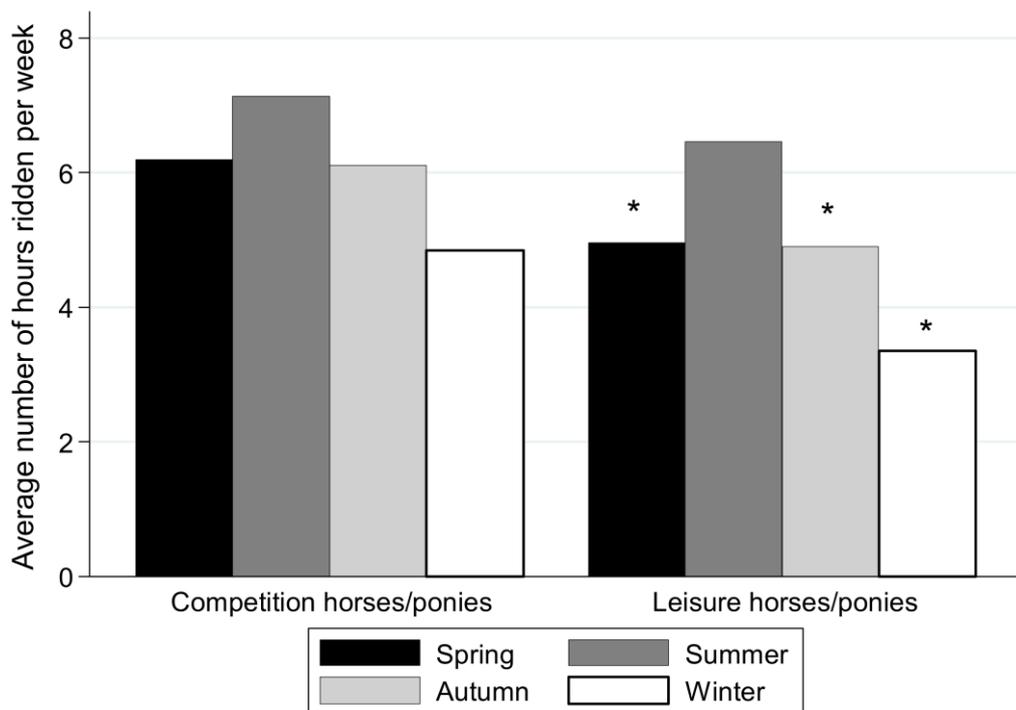
Figure 3.5: Owner-reported average number of hours ridden per week in each season for leisure and competition horses/ponies. *Denotes significant difference from corresponding competition horse/pony value ($p < 0.05$).

Seasonal Management and Feed Provision

Questions were asked about the daily routine of the respondent's horses/ponies during the summer and winter. Horses and ponies kept for leisure purposes were more likely to be kept outside at grass during the summer compared to competition

animals (61.2% vs. 41.3%; p < 0.01). There was no difference in the proportion of leisure and competition animals kept outside at grass during the winter months (p = 0.13). There was no difference in the type of grazing (restricted / unrestricted) offered during summer (p = 0.08) or winter (p = 0.92) between leisure and competition animals. Significantly more (60% vs. 41.79%; p = 0.02) amateur competitors/professionals compared with hobbyists allowed their horse/pony unrestricted grazing during the summer but this was not the case during the winter (p = 0.57). Only 5.7% of respondents stated they used grazing muzzles during the summer, with 0.6% of respondents using them during the winter months. There was no difference in the manner and provision of hay in the stable (*ad libitum* vs. restricted) between leisure and competition horses/ponies irrespective of season (p = 0.53 summer; p = 0.28 winter). A significantly greater number of competition horses/ponies were fed some form of complementary feed in addition to forage (e.g. competition mix, pasture mix etc.) compared with leisure horses/ponies irrespective of season (summer: 57.5% vs. 31.6%; p < 0.01 winter 63.8% vs. 43.9%; p < 0.01). Furthermore, a greater proportion of competition horses were fed 'straight' feeds (oats, bran etc.) and competition mix (a high energy coarse mix compounded to nutritionally support animals in hard work) compared to leisure animals (p < 0.01) during the summer months. In the winter months, a greater proportion of competition horses/ponies were found to be fed conditioning mix (designed to provide controlled levels of cereal starch, sugar and protein for the maintenance of muscle tone and to promote condition) , competition mix (p < 0.01) and a nutrient balancer (p = 0.04). Oral supplement feeding (nutraceutical food substance that may have postulated therapeutic functional benefits) was found to be more common during the summer months (63.1% fed some form of supplement) compared to winter (54%). In both summer and winter months, competition horses/ponies were more likely to be fed an electrolyte supplement than leisure horses/ponies (p < 0.01).

3.5 Discussion

The current study was designed to further our understanding on perceptions of obesity in horses and ponies and gather wider information regarding current management practices of horses and ponies in the UK.

Tier 1 has clearly demonstrated that horse owners and enthusiasts vary in their ability to identify overweight horses and ponies from photographic images. Evidence from human epidemiology studies suggest that the increased exposure to larger body sizes we are experiencing in this obesity epidemic has led to a shift in what we perceive to be normal in terms of body weight (Burke *et al.*, 2010). This concept is supported by a recent study in which exposure to photographs of obese males resulted in an overweight male being perceived to be of a healthier weight in comparison to those respondents who were initially exposed to photographs of a healthy weight male (Robinson and Kirkham, 2014). Furthermore, the exposure to obesity also resulted in respondents believing that an overweight person did not need to lose weight (Robinson and Kirkham, 2014). A follow-up study added to these findings that obesity exposure led to an increased acceptability of obesity by shifting visual preference towards a preference of increased body size (Robinson and Christiansen, 2014). Taken together, these data would suggest that exposure to obesity has normalised our perceptions of obesity and led to an increased acceptance of larger body sizes. Whilst this has yet to be evaluated for the horse, Figure 3.6 depicts a champion Hunter horse from 1935 and one from 2008, and clear differences in body shape and size are evident which may be the result of a shift in what is perceived to be normal in terms of body condition over the years.
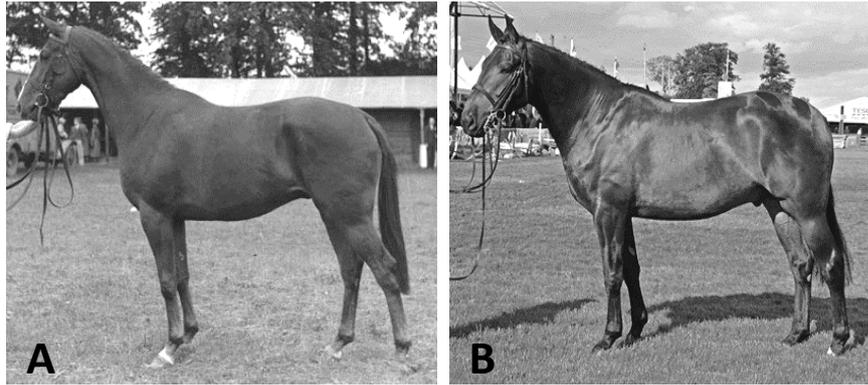
Figure 3.6: Champion Hunter horses **A**) from 1935 and **B**) from 2008 (**A**; reprinted with permission from the Museum of English Rural Life, University of Reading; **B** picture supplied courtesy of The Scottish Farmer)

Data from the current study identified that only 11% of respondents correctly identified all six overweight horses and ponies from photographic images. Interestingly, for three of the images; between 65% and 98% of the respondents correctly identified these horses/ponies as overweight. The animals in these images appeared to have more 'visually apparent' regional adiposity including a more defined 'cresty neck' compared to the other three overweight animals pictured, whereby less than half of the respondents correctly identified them as being overweight. Respondents may have been more drawn to these images due to the attention surrounding crest fat and its potential role in the aetiology of obesity and laminitis. A cresty neck score was developed and shown to be positively associated with insulin resistance and risk of laminitis (Carter *et al.*, 2009). Furthermore, expression of some inflammatory-related genes have been shown to be greater in crest fat compared to other adipose depots (Bruynsteen *et al.*, 2013; Burns *et al.*, 2010), although it is unknown whether this difference is related to tissue heterogeneity or whether this difference is translated into differences at the protein level. More recently a higher cresty neck score has been shown during winter months in comparison to summer, with breed being the strongest risk factor for cresty neck score in both seasons (Giles *et al.*, 2015). Increased regional adiposity in areas including the crest has also received attention due to its role in the diagnosis of Equine Metabolic Syndrome (EMS), a condition comprised of several factors including regional adiposity, insulin dysregulation and laminitis (Frank *et al.*, 2010). Although not directly linked, there is evidence to support an

association between crest fat and insulin dysregulation and laminitis risk, however this may be confounded by breed and more work is required to establish a direct mechanism by which crest fat may function in obesity and associated disease risk.

Additionally, for two of the images in this question, over 70% of respondents considered them to be overweight when in fact they were expertly assessed to have a normal body condition. Both of these horses were cob horse breeds and although cob type breeds have been shown to be a risk factor for obesity (Giles *et al.*, 2014; Robin *et al.*, 2015), there appears to be a misperception between the natural body shape of this breed and increased adiposity. Similarly, a study was conducted in the USA to assess owner perception of body weight and body condition score of draught and Warmblood horses, and they identified that almost half of owners considered draft horses to be overweight, whilst only 2% of Warmbloods were considered overweight (Hansen *et al.*, 2015), which also suggests that there is a misperception of obesity between different breeds. This may be an area which warrants further investigation.

Results from the discipline question in Tier 1 fell in line with anecdotal observations. For competing in showing classes, respondents deemed it more acceptable for horses and ponies, regardless of breed or body size, to carry more weight than for competing in other disciplines. As for the overweight question, the image of the cob horse used in this question was consistently considered to be more overweight than the expertly assessed response and again may reflect the misperception between the natural body shape and body fatness.

On the whole, there were limited differences between professionals and non-professionals in their responses to any of the questions, although professionals were twice as likely to consider the sport horse to be very underweight for competing in showing classes compared to non-professionals.

The BCS ranking question in Tier 1 revealed some discrepancy between animals ranked first and second (most and second-most underweight) by respondents in terms of weight/condition. This may be due in part to the images used in terms of

animal coat colour and environmental lighting. The pony that should have been correctly ranked first (most underweight, BCS 1.25/9) was light grey in colour. This colouration may have inadvertently obscured visual appraisal of anatomical landmarks such as hip bones and tailhead. Conversely, the horse that should have been correctly ranked in second (BCS 3/9) was chestnut and the rib outline was more apparent. The relative visual prominence this animals ribs may have led respondents to consider this animal to be the leanest in terms of weight/condition.

The geographical distribution of responses for both Tier 1 and Tier 2 questionnaires appear similar to a survey conducted using veterinary-registered horse owners, with the majority of the survey population residing in England (Wylie *et al*., 2013). The equine demographics from Tier 2 in the current study also appear to be in agreement with recently published data, with Thoroughbred/Thoroughbred types accounting for the majority of the survey population (Hotchkiss *et al*., 2007; Wylie *et al*., 2013), and a larger proportion of geldings was reported compared to mares; although the percentage of geldings in the current study was higher than previously published data (Wylie *et al*., 2013). Only 11% of the current study population comprised ponies, with the remaining 89% being horses, a lower proportion of ponies than has been reported in a similar previous survey (Wylie *et al*., 2013). This may be due to a large proportion of adult respondents who may be more likely to own a horse than younger respondents.

The prevalence of owner-reported laminitis history in the current study (7.34%) was lower than has been reported previously, where a prevalence of 15% was identified (Ireland *et al*., 2013). However, in agreement with that study, significantly more ponies than horses were reported to have had a history of laminitis. It could be suggested that the lower incidence of laminitis in the current study may be due to a lower proportion of ponies in the current study, which are considered to be at greater risk for the development of this condition (Alford *et al*., 2001; Geor, 2008). Owner-reported prevalence of PPID was found to be 2.82%, identical to owner-reported prevalence in a previous study (Ireland *et al*., 2013).

Owner-reported prevalence of obesity in the current study (4.52%) was markedly

lower than previous studies. A recent study demonstrated an owner-reported obesity prevalence of 30% (Robin *et al.*, 2015). There were a lower proportion of native ponies and a higher proportion of competition animals in the current study compared to that of Robin *et al.* (Robin *et al.*, 2015) which may partly explain the low owner-reported obesity rate as both native ponies and leisure animals are known to be at increased risk factors of obesity (Giles *et al.*, 2014; Robin *et al.*, 2015). Notably, there were significant differences for owner-reported body weight/condition of their animals across the seasons in the current study. For spring, summer and autumn, animals kept for leisure purposes were significantly more likely to be slightly overweight compared to animals kept for affiliated competitions. No differences were observed for winter and it is unclear whether this is due to a weight gain in competition animals or a natural winter weight loss for leisure animals. Seasonal variation in body condition is an evolutionary conserved adaptation to aid survival during winter months when food availability is scarce by increasing fat deposition during high availability of food during the summer months. However, it has been shown that for ponies with *ad libitum* access to a fibre-based diet, this seasonal mechanism is insufficient in preventing weight gain during the winter months (Dugdale *et al.*, 2011).

The daily routines during summer and winter months were reported, with over 50% of animals spending 24 hours at pasture during the summer, which reduced down to 21% during the winter months, a finding which is consistent with other published data (Hotchkiss *et al.*, 2007; Wylie *et al.*, 2013). The majority of animals were fed some form of concentrate feedstuff (energy providing complementary feed), although fewer animals received concentrate feeds during the summer months when energy requirements from grazing would be expected to be sufficient. As expected, significantly more competition animals received some form of concentrate feed compared to leisure animals in both summer and winter. Furthermore, an electrolyte-based supplement was fed more commonly to competition animals compared to leisure animals in both summer and winter, which is to be expected due to the increased workloads experienced by competition animals. The percentage of animals receiving electrolyte supplements

was greater than in other published data (Wylie *et al.*, 2013), but this is likely due to the higher proportion of competition animals in the current study.

There are some limitations to the current study. As with any questionnaires, there is a risk of responder bias whereby those with an interest in the subject are more likely to participate. Although all the images used in Tier 1 were of the same side of the horse/pony and different colours of horses and ponies were used to limit bias, certain colours against different backgrounds may have made anatomical landmarks more difficult to distinguish.

3.6 Conclusion

In conclusion, this study has demonstrated a limit in horse owners and enthusiast's ability to identify overweight animals from photographic images. Additionally, it has been clearly demonstrated that perceptions of weight/condition alter depending on the activity the horse/pony is intended for, with increased weight/condition deemed to be more appropriate for competing in showing classes. To the author's knowledge this is the first study to document this finding. These data will enable the provision of more targeted nutritional advice to horse owners. Tier 2 has gathered valuable data regarding management practices and obesity prevalence. Seasonal changes in weight, exercise, and feeding practises were identified and may form the basis for further epidemiological studies.

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Chapter 4

EQUIFAT: A novel scoring system for the semi-quantitative evaluation of regional adipose tissue in *Equidae*

4.1 Abstract

Anatomically distinct adipose tissues represent variable risks to metabolic health in man and some other mammals. Quantitative-imaging of internal adipose depots is problematic in large animals and associations between regional adiposity and health are poorly understood. This study aimed to develop and test a semi-quantitative system (EQUIFAT) which could be applied to regional adipose tissues. Anatomically-defined, photographic images of adipose depots (omental, mesenteric, epicardial, rump) were collected from 38 animals immediately *post-mortem*. Images were ranked and depot-specific descriptors were developed (1 = no fat visible; 5 = excessive fat present). Nuchal-crest and ventro-abdominal-retroperitoneal adipose depot depths (cm) were transformed to categorical 5 point scores.

The repeatability and reliability of EQUIFAT was independently tested by 24 observers. When half scores were permitted, inter-observer agreement was substantial (average κ_w: mesenteric, 0.79; omental, 0.79; rump 0.61) or moderate (average κ_w; epicardial, 0.60). Intra-observer repeatability was tested by 8 observers on 2 occasions. Kappa analysis indicated perfect (omental and mesenteric) and substantial agreement (epicardial and rump) between attempts.

A further 207 animals were evaluated *ante-mortem* (age, height, breed-type, gender, BCS) and again immediately *post-mortem* (EQUIFAT scores, carcass weight). Multivariable, random effect linear regression models were fitted (breed as random effect; BCS as outcome variable). Only height, carcass weight, omental and retroperitoneal EQUIFAT scores remained as explanatory variables in the final model.

The EQUIFAT scores developed here indicate functional differences between regional adipose depots and can be applied in surgical and *post-mortem* situations to describe associations between adiposity and disease risk.

4.2 Introduction

Adipose tissue is an active endocrine organ, secreting chemical messengers collectively termed adipokines into the circulation to mediate communication with other organs. White adipose tissue (WAT) is distributed in anatomically discrete depots throughout the body where it performs diverse functional roles. Specific depots range in function from those primarily providing structural support and local protection to the more readily recognized role of WAT as a dynamic reserve of metabolic energy and water (Trayhurn *et al*., 2011; Wronska and Kmiec, 2012). The precise distribution of adipose tissues between depots within an individual, or 'fat patterning', has been related to disease risk in a number of domestic species and in man (Catalano *et al*., 2010; Lottati *et al*., 2009). For example, increased visceral (abdominal) WAT deposition measured by computed tomography (CT) has been clearly characterised as a risk factor for the development of cardiovascular and metabolic disease in man (Fox *et al*., 2007; Goodpaster *et al*., 2005).

Despite continued reports of a high prevalence of obesity in the UK population of leisure horses and ponies (Giles *et al*., 2014; Robin *et al*., 2015), relatively little is known about functional differences between discrete adipose tissue depots in this species. Whilst the exact mechanisms remain unclear, obesity has been associated with an increased risk for the development of insulin dysregulation and the common systemic condition, laminitis, which initially presents as severe foot pain (Bailey *et al*., 2008; Geor, 2008). Obesity can also have a negative impact on athletic performance and fertility (Henneke *et al*., 1983; Kearns *et al*., 2002). Understanding functional distinctions and differential health risks between the various adipose tissue depots requires a capability to evaluate these covert, internal WAT reserves. Body condition score (BCS) systems, originally intended as management tools for the assessment of flesh cover and subsequent meat yield in food animals, are now routinely applied to horses and ponies in the field to estimate 'body fatness'. Various equine BCS systems have been reported. When BCS data (using the system described in the current study) were compared to concurrently-collected data generated by the empirically validated deuterium oxide

dilution method for a mixed breed population of horses and ponies (Kohnke, 1992), BCS proved to be a robust predictor of total body fat content up to BCS 6.8/9 (Dugdale *et al.*, 2012). Although BCS systems are straightforward and useful for the assessment of 'body fatness' in *Equidae* especially when undertaken by experienced practitioners, they have clear limitations. BCS systems, for example, assess externally visible/palpable adipose tissues and cannot evaluate internal adiposity. Further, BCS system used routinely by researchers fails to predict body fat content with any accuracy in obese animals (> BCS of 6.8/9) (Dugdale *et al.*, 2012). Similarly, the ability to measure total body fat using the deuterium oxide dilution method is largely restricted to research settings and it cannot distinguish between body fat in specific anatomical regions. To quantify regional body fat distribution, powerful imaging modalities such as computed tomography (CT), dual-energy x-ray absorptiometry (DXA), and magnetic resonance imaging (MRI) have been widely applied in man (Ross *et al.*, 1992; Smith *et al.*, 2001) and smaller food and companion species (Gjerlaug-Enger *et al.*, 2012; Speakman *et al.*, 2003). The larger body size of horses has to date, prohibited the application of these methods for the quantification of regional adipose depots in living *Equidae*.

As a preliminary step towards improving our understanding of the different roles played by regional adipose depots in the horse, the present study aimed to develop and test a semi-quantitative *s*coring system for *post-mortem* evaluation of specific regional equine adipose depots. A second objective was to describe any associations between regional adiposity appraised *ante-mortem* via BCS, and *post-mortem* regional 'fat scores'.

4.3 Materials and methods

Three sequential studies were performed to address the objectives of 1) developing suitable descriptors to score 6 discrete adipose tissue depots (EQUIFAT scores) 2) testing the repeatability and reliability of these descriptors and to 3) using the system to initially evaluate any associations between these *post-mortem* 'fat scores'

and *ante-mortem* BCS. Data were derived from animals presented at a commercial UK abattoir (LJ Potters, Taunton, Somerset). Animals were slaughtered in accordance with EU legislations EC 852/2004, 853/2004 and 854/2004, for reasons unrelated to this study. All animals were in good general health.

The development of EQUIFAT scores

Anatomically defined photographic images of omental, mesenteric, epicardial and rump adipose depots were taken *post-mortem* from 38 animals between August and September 2012 (Table 4.1). The population comprised of mixed breed horses and ponies (26 horses 12 ponies; 23 mares 15 geldings) across the range of BCS (/9; (Kohnke, 1992)) as expected from a commercial abattoir setting (Mean BCS 5.0/9, SD 1.5, range 2.2-7.7/9). Photographs for each adipose depot were ranked in order of increasing 'visually-apparent' adiposity and a depot-specific 5 point scoring system, termed EQUIFAT (1 = least; 5 = greatest) was developed with detailed descriptors (Figure 4.1; Appendix D). Representative images (*n* = 5) for each score were included with the descriptors for each adipose depot to facilitate the use of the scoring system. In addition to the above subjective scores, quantitative scores were also created for nuchal crest and abdominal retroperitoneal adipose depots. Depths (± 1mm) of the nuchal crest and abdominal retroperitoneal adipose depots were recorded at their cranio-caudal midpoints on the medial aspect of the left, split carcasses. The range of recorded depths were uniformly distributed and these data were recoded as categorical scores (1 – 5) as follows: Crest: 1 = 0-2.99cm; 2 = 3-5.99cm; 3 = 6-8.99cm; 4 = 9-11.99cm; 5 = ≥12cm; Retroperitoneal: 1 = 0-1.99cm; 2 = 2-3.99cm; 3 = 4-5.99cm; 4 = 6-7.99cm; 5 = ≥8cm.

For assessment of BCS, six areas of the body (neck, withers, loin, tailhead, ribs and shoulder) are graded and each area is assigned a score from 1 (very poor) to 9 (extremely fat) based on detailed descriptors (Kohnke, 1992). The average of the six values is calculated to provide a final, overall body condition score.

Table 4.1: Population of animals used in the current study as the test population used to develop the EQUIFAT scoring system (*n* = 38) and the population used to describe associations between EQUIFAT scores and BCS (*n* = 207).

	Test population (*n* = 38)	Whole population (*n* = 207)
	Average (Range)	
BCS (/9)	4.98 (2.2 - 7.7)	5.07 (2.3 – 8.3)
Height	151cm (102-178) 26 horses; 12 ponies	154cm (92-178) 148 horses; 59 ponies
Age	10.1 years (3 – 20)	11.4 years (2-26)
Gender	15 Geldings, 23 Mares	70 Geldings, 137 Mares

Testing the repeatability and reliability of EQUIFAT scores

The constraints of the commercial setting prohibited repeatability testing at the time of *post-mortem*. Therefore, the remaining 33 photographic images (excluding 5/38 presented with the descriptors) of each depot were randomised and used to create a slideshow for each depot. In order to assess the reliability and test the agreement between observers, the EQUIFAT scoring system was tested by a total of 24 individuals (17 veterinary surgeons, 5 clinical pathologists and 2 scientific researchers).

Half of the respondents were asked to use whole numbers only (1-5) and half were given the option of using whole or half scores. Each participant was informed of the nature of the study and provided with the images and the score descriptors. They were asked to assign a number between 1 and 5 (using half or whole numbers as above) for each image on a score sheet. Participants scored the images in isolation and were blinded to each other's responses. To assess the repeatability of the scoring system, four observers from each group (those using whole scores and those allowed to use half scores) repeated the protocol at least two weeks after their first attempt.

Figure 4.1: Example EQUIFAT scoring system for mesenteric adipose depot.

Associations between EQUIFAT scores and BCS

Data for 207 animals were collected *ante-mortem* (BCS) and again immediately *post-mortem* (EQUIFAT) between August 2012 and January 2014 (Table 4.1). Information gathered *ante-mortem* included: age in years (passport), estimated withers height, breed-type, gender and BCS (/9). *Post-mortem*, carcass weight and EQUIFAT scores were recorded for omental, mesenteric, epicardial, rump, crest and retroperitoneal fats.

Data analysis

Statistical analyses were performed using STATA 12 (StataCorp, Texas). Statistical significance was set at $p < 0.05$.

Intra-observer repeatability

For the four observers who repeated the assessment in each group, the number and percentage of exact agreement, along with score differences between the observers first and second attempts was calculated. The non-parametric Wilcoxon signed-rank test was applied to test the agreement between attempts with a predicted total agreement of zero (100% agreement) for each observer. Pairwise kappa analysis using quadratic weights was then used to determine the agreement between observations. Quadratic weights assign less weight to agreement when comparative scores are further apart. Interpretation of kappa values is as follows: 0 = poor; 0.01 to 0.20 = slight; 0.21 to 0.40 = fair; 0.41 to 0.60 = moderate; 0.61 to 0.80 = substantial; 0.81 to 1.00 = almost perfect.

Inter-observer agreement

Kappa analyses were used to measure the agreement between observers for the 4 subjectively-scored adipose depots, beyond that expected by chance alone. Weighted kappa, using quadratic weights, was calculated for scores from each individual observer against those submitted by each of the other observers. The mean of these 11 weighted kappa values was recorded as the inter-observer agreement for that individual. This was repeated for each of the 4 adipose depots (omental, mesenteric, epicardial and rump fats).

Unweighted kappa was recorded for each score for each adipose depot in order to assess the repeatability of assigning appropriate scores for the level of adiposity observed.

Associations between EQUIFAT scores and BCS

In order to describe associations between fat scores and BCS, two multivariable

random effects linear regression models were fitted with BCS as the outcome variable and breed considered as a random effect. As aspects of the original BCS scoring system, crest and rump EQUIFAT scores were *a priori* excluded from these analyses. Models were fitted using a backward elimination strategy whereby a full model was built and then each variable removed in turn, a likelihood ratio test performed and the resultant P-value noted. The variable with the highest p-value was then omitted and the process repeated. This process was repeated until only variables with $p < 0.2$ remained in the model. The omitted variables were then added back in turn, starting with the lowest p-value, a likelihood ratio test performed after each addition, and the variable retained if $p < 0.2$. This process was continued until no further variables could be added, to produce the final model.

 In Model 1, all physical attributes and remaining EQUIFAT scores (omental, mesenteric, epicardial and retroperitoneal) were offered to the initial model as explanatory variables. Model 2 was fitted using only the EQUIFAT scores in order to assess the association between internal fat depots and BCS irrespective of other physical characteristics. For both models, the intra-class correlation for the random effect variable (breed) was calculated as a measure of the variance attributable to the random effect.

Predicted marginal means were calculated from regression models and displayed graphically where appropriate.

4.4 Results

Test population

A total of 207 animals presented at the abattoir were utilised in the present study. Results from thirty eight animals were employed in development and validation of the EQUIFAT system. Paired Student t tests were employed to compare baseline characteristics (BCS, age, height) of these 38 to the population ($n = 207$) from which

they were derived. The population of animals used to develop the EQUIFAT scoring system (*n* = 38) is described in Table 4.1. Figure 4.2 demonstrates there were no significant differences in attributes between the 38 test animals and the population (*n* = 207) in which they were nested.
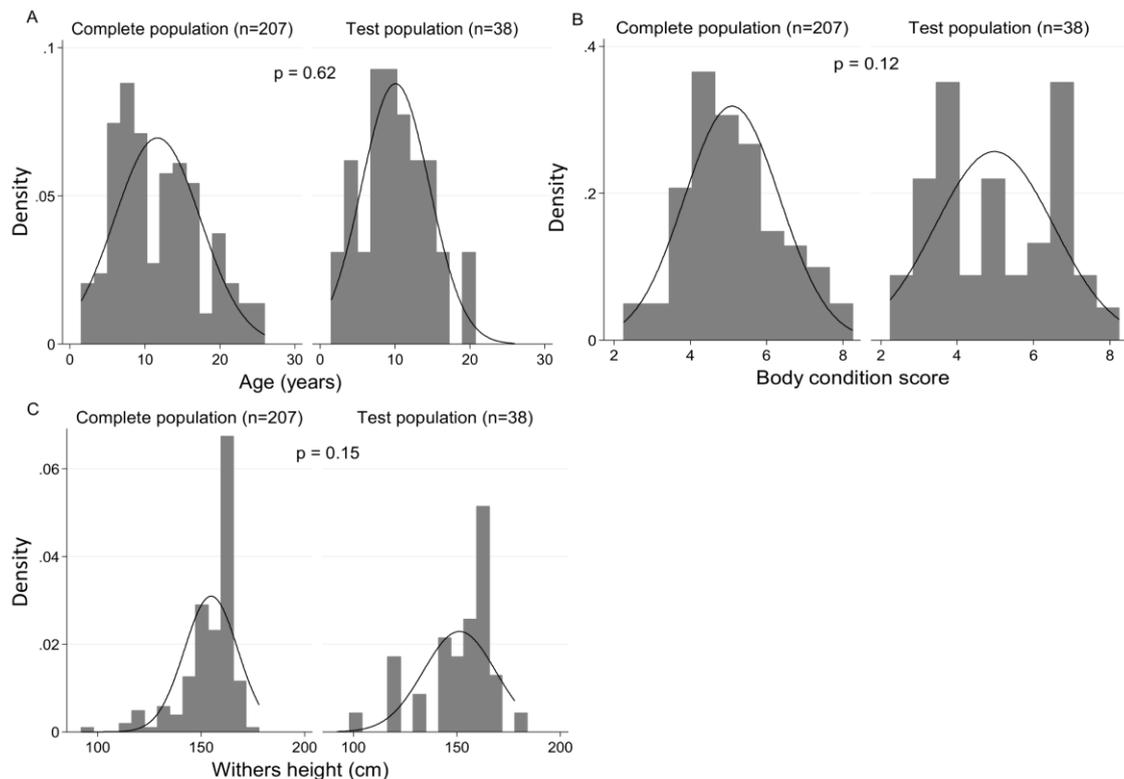
Figure 4.2: Population distributions of (A) age, (B) BCS and (C) withers height in the test animals (*n* = 38) and the population in which they were nested (*n* = 207). Paired Students T-test was used to identify any differences between the populations.

Intra-observer repeatability

Overall mean exact agreement between attempts for the four observer's using half scores was similar for all four adipose depots, ranging from 10.8 (32.6%) for rump fat to 14.8 (44.7%) for epicardial fat out of the 33 images (Table 4.2a). Mean exact agreement for the four observers using whole scores was greater than for those using half scores, with agreement ranging from 14.8 (44.7%) for epicardial fat to

20.8 (62.9%) for mesenteric fat (Table 4.2b). In order to determine if any bias was present between observers attempts, each score they assigned for the second attempt was subtracted from the equivalent score from their first attempt. Generally, all eight observers who repeated using either whole or half scores scored higher in their second attempt for each depot, most notably for rump fat, with score differences of -0.49 and -0.34 respectively. Pairwise kappa analysis was very similar between the groups of observers using half scores (Table 4.2a) and those using whole scores (Table 4.2b). There was almost perfect agreement between scores for omental and mesenteric fats and substantial agreement for epicardial and rump fats.

Inter-observer agreement

Weighted kappa analysis was employed to assess agreement within the two groups of 12 observers for each adipose depot (Table 4.3). For the four adipose depots, a weighted kappa value was generated for each observer against the 11 other observers and a mean weighted kappa was then recorded for each observer. As for the intra-observer agreement, the average kappa values obtained for each depot were very similar between those using half scores and those using whole scores (Table 4.3). For those using half scores, the overall mean weighted kappa was substantial for mesenteric (0.79; standard deviation (SD) 0.04), omental (0.79; SD 0.02) and rump (0.61; SD 0.07) fats, and moderate for epicardial fat (0.60; SD 0.07). For those using whole scores, the overall mean weighted kappa was substantial for mesenteric (0.79; SD 0.03) and omental fats (0.78; SD 0.04) and moderate for epicardial (0.54; SD 0.06) and rump fats (0.57; SD 0.08).

Table 4.2a: Intra-observer repeatability of the EQUIFAT scores for the four observers using half scores and assessing 33 images each of 4 adipose depots. A minimum of 14 days lapsed between attempts. Agreement data are presented for exact, 0.5 and 1 point differences between attempts. Kappa tests and p values for Wilcoxon sign-rank test are presented.

EQUIFAT	Observer ID	Exact agreement n/33 (%)	0.5 point difference n/33 (%)	1 point difference n/33 (%)	Mean observer difference in score between attempts	Pairwise kappa using quadratic weights	Wilcoxon signed-rank test of observer difference compared to zero p value
Mesenteric	1	13 (39.4)	19 (57.6)	1 (3.0)	0.11	0.91	0.16
	2	13 (39.4)	12 (36.3)	8 (24.2)	-0.24	0.81	0.02
	5	12 (36.4)	2 (6.1)	19 (57.5)	-0.55	0.79	< 0.001
	11	12 (36.4)	10 (30.4)	11 (33.3)	0.09	0.83	0.44
	Overall Mean	12.5 (37.9)	10.8 (32.6)	9.8 (29.5)	-0.15	0.84	
Omental	1	13 (39.4)	15 (45.5)	5 (15.1)	-0.11	0.91	0.10
	2	9 (27.3)	13 (39.4)	8 (24.2)	-0.38	0.77	<0.001
	5	19 (57.6)	3 (9.1)	11 (33.3)	-0.14	0.89	0.23
	11	14 (42.4)	9 (27.3)	9 (27.3)	0.11	0.88	0.45
	Overall Mean	13.8 (41.7)	10 (30.3)	8.3 (25.2)	-0.13	0.86	
Epicardial	1	10 (30.3)	15 (45.5)	7 (21.2)	-0.29	0.74	0.01
	2	17 (51.5)	9 (27.3)	7 (21.2)	-0.005	0.70	0.82
	5	16 (48.5)	1 (3.0)	12 (36.4)	-0.23	0.69	0.30
	11	16 (48.5)	1 (3.0)	12 (36.4)	-0.18	0.56	< 0.001
	Overall Mean	14.8 (44.7)	6.5 (19.7)	9.5 (28.8)	-0.19	0.67	
Rump	1	10 (30.3)	16 (48.5)	5 (15.1)	-0.05	0.76	0.90
	2	11 (33.33)	8 (24.2)	7 (27.3)	-0.47	0.63	0.004
	5	9 (27.3)	1 (3.0)	19 (57.6)	-0.74	0.54	< 0.001
	11	13 (39.4)	9 (27.3)	10 (30.3)	-0.09	0.78	0.75
	Overall Mean	10.8 (32.6)	8.5 (25.8)	10.3 (31.2)	-0.34	0.68	

Table 4.2b: Intra-observer repeatability of EQUIFAT for the four observers using whole scores assessing 33 images each of 4 adipose depots. A minimum of 14 days lapsed between attempts. Agreement data are presented for exact and 1 point differences between attempts. Kappa tests and p values for Wilcoxon sign-rank test are presented.

EQUIFAT	Observer ID	Exact agreement n/33 (%)	1 point difference n/33 (%)	Mean observer difference in score between attempts	Pairwise kappa using quadratic weights	Wilcoxon signed-rank test of observer difference compared to zero p value
Mesenteric	2	25 (75.8)	7 (21.2)	-0.10	0.84	0.18
	8	16 (48.5)	17 (51.5)	0.39	0.83	0.001
	9	23 (69.7)	10 (30.3)	-0.18	0.90	0.06
	11	19 (57.6)	13 (39.4)	-0.10	0.78	0.55
	Overall Mean	20.8 (62.9)	11.8 (35.6)	0.003	0.84	
Omental	2	18 (54.5)	15 (45.5)	0.33	0.85	0.004
	8	23 (69.7)	10 (30.3)	0.06	0.90	0.53
	9	19 (57.6)	14 (42.4)	-0.06	0.88	0.59
	11	25 (75.8)	8 (24.3)	-0.12	0.92	0.16
	Overall Mean	18.8 (56.8)	8.3 (25.2)	0.05	0.89	
Epicardial	2	12 (36.4)	20 (60.6)	-0.42	0.41	0.004
	8	15 (45.5)	16 (48.5)	-0.42	0.68	0.004
	9	14 (42.4)	14 (42.4)	-0.36	0.64	0.04
	11	18 (54.5)	13 (39.4)	0.27	0.72	0.06
	Overall Mean	14.8 (44.7)	9.5 (28.8)	0.10	0.61	
Rump	2	19 (57.6)	11 (33.3)	-0.45	0.72	0.001
	8	11 (33.3)	17 (51.5)	-0.57	0.61	0.006
	9	11 (33.3)	18 (54.5)	-0.79	0.58	< 0.001
	11	19 (57.6)	13 (39.4)	-0.15	0.80	0.26
	Overall Mean	15 (45.5)	14.8 (44.7)	-0.49	0.68	

Table 4.3: Inter-observer agreement of the EQUIFAT scores. Weighted kappa value was generated for each observer (*n* = 12) against each other individual observers. Mean weighted kappa are presented for each observer.

Observer ID	Half scorers				Whole scorers			
	Mesenteric fat	Omental fat	Epicardial fat	Rump fat	Mesenteric fat	Omental fat	Epicardial fat	Rump fat
	Mean κ_w for each observer against 11 other observers				Mean κ_w for each observer against 11 other observers			
1	0.83	0.82	0.69	0.67	0.82	0.78	0.61	0.65
2	0.74	0.76	0.56	0.62	0.78	0.79	0.52	0.54
3	0.81	0.81	0.62	0.59	0.74	0.79	0.54	0.64
4	0.82	0.77	0.56	0.45	0.79	0.78	0.51	0.53
5	0.78	0.79	0.67	0.68	0.79	0.72	0.40	0.53
6	0.73	0.81	0.58	0.54	0.82	0.80	0.62	0.63
7	0.79	0.81	0.67	0.70	0.81	0.82	0.56	0.59
8	0.75	0.76	0.64	0.54	0.82	0.78	0.54	0.55
9	0.77	0.79	0.65	0.62	0.80	0.82	0.57	0.39
10	0.85	0.78	0.59	0.56	0.75	0.67	0.56	0.68
11	0.82	0.82	0.62	0.57	0.72	0.80	0.51	0.53
12	0.81	0.78	0.44	0.66	0.80	0.76	0.59	0.58
Overall Mean (SD)	0.79 (0.04)	0.79 (0.02)	0.61 (0.07)	0.60 (0.07)	0.79 (0.03)	0.78 (0.04)	0.54 (0.06)	0.57 (0.08)

The application of unweighted kappa analysis to describe the repeatability of the individual scores for each depot revealed substantial agreement between observers for a score of 5 for mesenteric fat (0.70) and a score of 1 for omental fat (0.61) for those using half scores (Table 4.4a). The repeatability of the remaining scores was found to have either fair or slight agreement. For observers using whole scores there was moderate agreement for a score of 1 for mesenteric (0.41) and omental (0.49) fats and almost perfect agreement for a score of 5 for mesenteric fat (0.85), with moderate agreement for a score of 5 for omental (0.48) and epicardial fats (0.41) (Table 4.4b). The repeatability of the remaining scores was found to have either fair or slight agreement.   Lower agreement was observed for the scores between 1.5 and 4.5 for mesenteric and omental fats.

Table 4.4a: Repeatability of individual EQUIFAT scores for each depot to test for agreement between observers (n = 12) using half scores. Un-weighted kappa analyses were presented.

Score	Overall κ			
	Mesenteric fat	**Omental fat**	**Epicardial fat**	**Rump fat**
1	0.19 (Slight)	0.61 (Substantial)	0.06 (Slight)	0.20 (Slight)
1.5	0.10 (Slight)	0.09 (Slight)	0.07 (Slight)	0.18 (Slight)
2	0.24 (Fair)	0.32 (Fair)	0.14 (Slight)	0.28 (Fair)
2.5	0.12 (Slight)	0.03 (Slight)	0.06 (Slight)	0.09 (Slight)
3	0.12 (Slight)	0.21 (Fair)	0.10 (Slight)	0.10 (Slight)
3.5	0.15 (Slight)	0.10 (Slight)	0.04 (Slight)	0.03 (Slight)
4	0.29 (Fair)	0.21 (Fair)	0.20 (Slight)	0.10 (Slight)
4.5	0.25 (Fair)	0.10 (Slight)	0.08 (Slight)	0.02 (Slight)
5	0.70 (Substantial)	0.40 (Fair)	0.35 (Fair)	0.36 (Fair)

Table 4.4b: Repeatability of individual EQUIFAT scores for each depot to test for agreement between observers (*n* = 12) using whole scores. Un-weighted kappa analyses were presented.

Score	Overall κ			
	Mesenteric fat	**Omental fat**	**Epicardial fat**	**Rump fat**
1	0.41 (Moderate)	0.49 (Moderate)	0.16 (Slight)	0.17 (Slight)
2	0.31 (Fair)	0.37 (Fair)	0.20 (Slight)	0.25 (Fair)
3	0.24 (Fair)	0.29 (Fair)	0.13 (Slight)	0.17 (Slight)
4	0.51 (Moderate)	0.32 (Fair)	0.21 (Fair)	0.15 (Slight)
5	0.85 (Almost perfect)	0.48 (Moderate)	0.41 (Moderate)	0.32 (Fair)

Associations between EQUIFAT scores and BCS

The population of animals (*n* = 207) used for this part of the study are described in Table 4.1 and Figure 4.3. The animals were representative of the UK abattoir population in terms of gender, age, horse/pony split, and BCS. As outlined in the methods, both crest and rump fat scores were excluded *a priori* from analysis as they were highly correlated with two components of the original BCS system, namely "neck" and "tailhead".

Model 1 (Table 4.5) demonstrates there were strong positive associations between BCS and both carcass weight and retroperitoneal fat score. Withers height had a strong negative association with BCS. Age, gender, mesenteric and epicardial fat scores did not remain in the final model thereby demonstrating a lack of association with BCS. Model 2 (Table 4.5) was fitted to explore associations between the EQUIFAT scores and BCS. Variables remaining in the final model were retroperitoneal fat score and omental fat score, with neither mesenteric or epicardial fat scores remaining in the final model. In both models, the coefficient for retroperitoneal fat score was at least 3 times greater than that for omental fat. Figure 4.4 demonstrates the predicted marginal means generated from Model 1

and clearly indicate that for retroperitoneal depots, and to a lesser extent for omental fats, there was a trend for BCS to increase with each unit increase in specific fat scores.
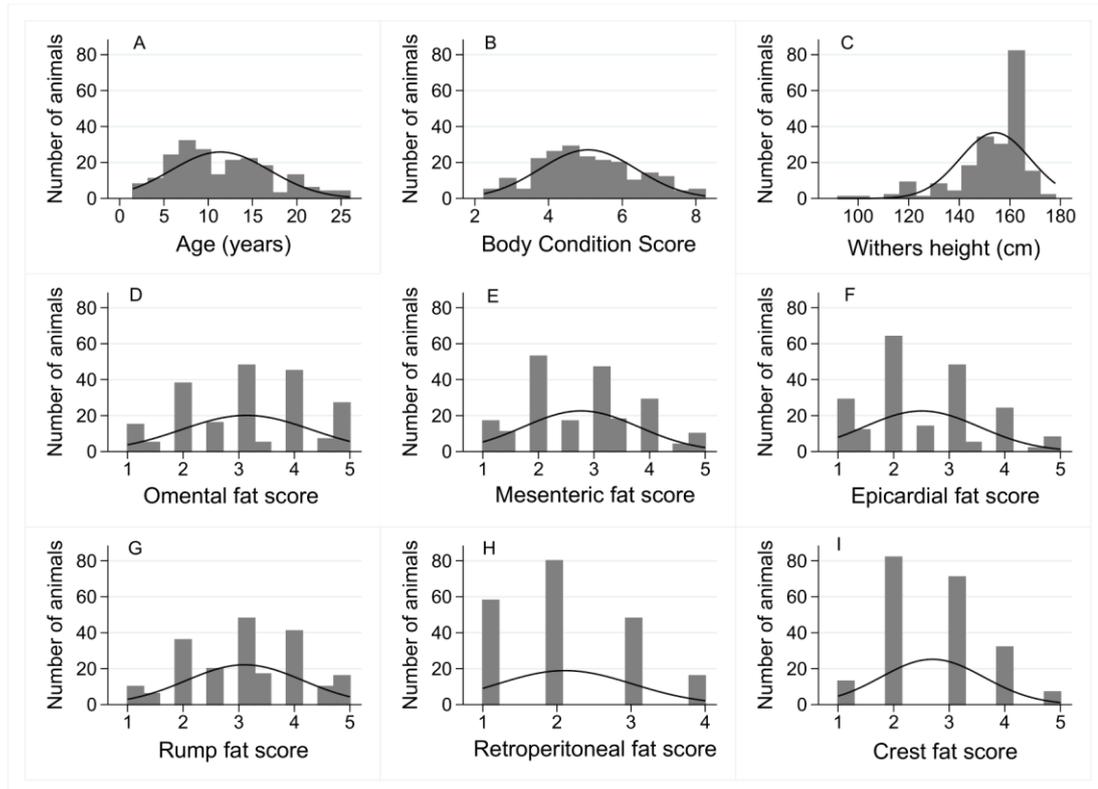
Figure 4.3: Distribution of physical attributes and EQUIFAT scores in the population of animals used to describe associations between EQUIFAT scores and BCS (n = 207). Histograms were constructed with normal distribution overlaid for **(A)** age, **(B)** BCS, **(C)** withers height, **(D)** omental fat score, **(E)** mesenteric fat score, **(F)** epicardial fat score, **(G)** rump fat score, **(H)** retroperitoneal fat score, and **(I)** crest fat score.

Table 4.5. Associations between EQUIFAT scores and BCS. Two random effects, multivariable linear regression models were built with breed as a random effect. CI, confidence interval.

Variable	Model 1 (Adj. R^2 = 0.49) Breed attributable variance = 0.23 (95% CI = 0.07 to 0.54)			Model 2 (Adj. R^2 = 0.24) Breed attributable variance = 0.31 (95% CI = 0.14 to 0.56)		
	Estimate β	95% CI	P value	Estimate β	95% CI	P value
Height (cm/10)	-0.62	-0.80 to -0.44	< 0.001			
Carcass weight (kg/10)	0.11	0.08 to 0.14	< 0.001			
Omental fat score	0.09	-0.02 to 0.21	0.10	0.16	0.03 to 0.28	0.02
Retroperitoneal fat score	0.32	0.17 to 0.47	< 0.001	0.48	0.32 to 0.64	< 0.001
Baseline	10.44	8.17 to 12.71	< 0.001	3.88	3.32 to 4.43	< 0.001
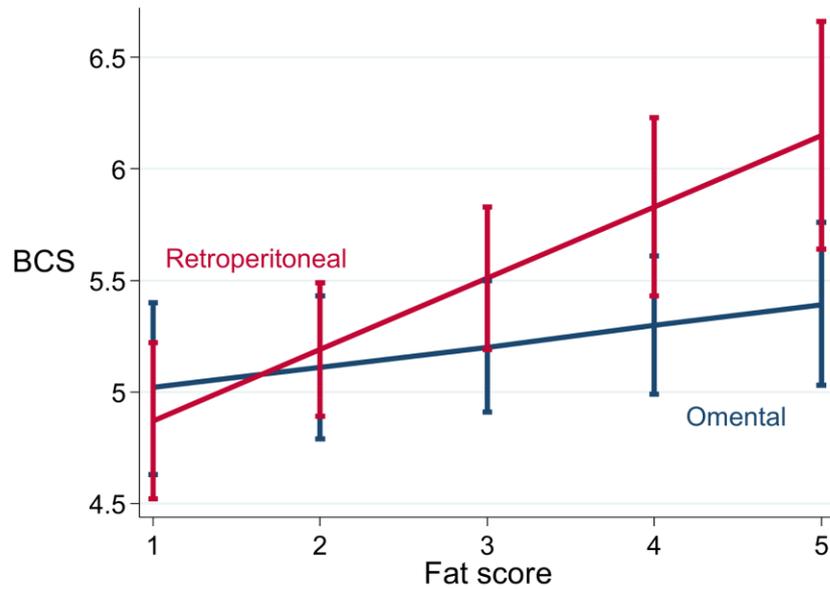
Figure 4.4: Marginal mean plots illustrating predicted changes in BCS with retroperitoneal and omental EQUIFAT scores. Error bars signify 95% confidence intervals.

4.5 Discussion

The current study firstly describes the development and testing of a novel fat scoring system for *Equidae*; the EQUIFAT scoring system, and secondly it demonstrates the application of the EQUIFAT scoring system to describe the relationship between internal adiposity and external body condition score. To the author's knowledge, there have been no previous reports of data which characterise the association between these regional superficial and internal body fat distributions and body condition score in the horse. It was noteworthy that while obesity is prevalent among horses and ponies in the UK leisure sector (Robin *et al.*, 2015), the population of animals presented for slaughter at a commercial abattoir was at variance with this. A greater proportion of animals assessed in the current study would be considered to be 'normal' or slightly underweight in terms of BCS than would be predicted had these animals been sourced from the numerically dominant leisure horse population.

In order to test the repeatability and reliability of the EQUIFAT scoring system,

kappa analysis was employed. Kappa analysis is a well-established and widely used method in numerous fields of scientific research and indicates the level of agreement either between or within observers beyond that expected by chance alone (Sim and Wright, 2005). The results from the current study demonstrate almost perfect agreement in the repeatability of omental and mesenteric fat scores and substantial agreement for epicardial and rump fat scores, irrespective of whether half scores or whole scores were used. The data suggested that the EQUIFAT scoring system was robust when used on repeated occasions, although there did appear to be some bias between observers repeated attempts to classify the same images. On the whole, observers tended to score higher on their second attempt, although the average scoring difference remained below half a score in the majority of cases which was deemed as an acceptable difference by the authors. The inter-observer agreement between observers was also found to be substantial for mesenteric and omental fats for those using both whole scores and half scores; whilst the agreement was moderate to substantial for epicardial and rump fats.

The two groups of observers in the current study were instructed either to use whole scores only or were given the option of using half scores. There were no obvious differences in agreement between the two groups and from the feedback; it appeared that the EQUIFAT scoring system was applied with more ease when the use of half scores was permitted. Therefore, it would be recommended that half scores are allowed for future use.

The second part of this study applied the EQUIFAT scoring system to a large group of animals in order to describe associations between individual depot EQUIFAT scores and BCS. Due to the lack of availability of modern imaging modalities such as CT scanning for the quantification of internal fat in the live horse, using the EQUIFAT scoring system designed in the current study at *post-mortem* allowed the investigation of associations between external 'body fatness' (BCS) and internal fat deposition.

The finding that retroperitoneal fat had a strong positive association with BCS

suggests that this intra-abdominal depot may function as a long-term storage depot. Studies on retroperitoneal WAT function in the horse are limited, although ultrasound measurements of retroperitoneal fat depths were found to be associated with percentage body fat in a group of 77 horses and ponies (Dugdale *et al.*, 2012). However, there appears to be some debate in the literature regarding whether or not retroperitoneal adipose tissues should be classed as a 'visceral fat'. In terms of venous drainage there are clear differences between peritoneal (omental and mesenteric) and retroperitoneal adipose tissues which could signify functional differences. Venous blood from peritoneal adipose tissues drains via the portal vein into the liver. Conversely, venous effluent from retroperitoneal adipose tissue depots drains into the renal circulation. Evidence from rodent studies supports the contention that retroperitoneal and peritoneal adipose tissues are physiologically distinct. For high-fat diet fed rats, exercise training decreased the response to isoproterenol-stimulated lipolysis in mesenteric but not retroperitoneal adipose tissues (Chapados *et al.*, 2008). Depot differences have been also been demonstrated in the immune cell populations of the stromal vascular fraction of omental and retroperitoneal fats in mice (Cohen *et al.*, 2013). A recent study in humans however, argues that retroperitoneal fat should be considered alongside omental and mesenteric fats to encompass the visceral depot as retroperitoneal fat was significantly correlated with metabolic syndrome and the number of metabolic abnormalities (Hung *et al.*, 2014).

The visceral adipose depot (omental and mesenteric) is more metabolically and lipolytically active in humans and it has been shown that visceral fat is preferentially mobilised over subcutaneous fat during the initial stages of a very low calorie diet; although this depot bias is lost as weight-loss progresses (Chaston and Dixon, 2008). Empirical data suggests that this may also be true for the horse. Circumferential body measures of 'belly girth' in a mixed-breed population of horses and ponies decreased during the course of a weight-loss trial, indicative of a loss of internal adiposity (Argo *et al.*, 2012). Furthermore, in the current study, omental fat score had a weaker association whilst mesenteric fat score had no association with BCS. These data suggest that, as for humans, these depots may function more as a short-

term energy reserve. Human visceral fat incubated in primary culture secretes inflammatory cytokines at a greater rate than subcutaneous fat (Fain *et al.*, 2004). For the horse, the nuchal crest may be an important source of inflammatory factors (Bruynsteen *et al.*, 2013; Burns *et al.*, 2010); although the relationship between circulating inflammatory factors and obesity is less clear in this species (Holbrook *et al.*, 2012; Vick *et al.*, 2007).

A novel observation in the current study was the lack of any association between epicardial fat score and BCS. Epicardial fat is situated between the pericardium and myocardium and is thought to function to provide energy for the heart. Of note, epicardial fat was not associated with total extracted WAT from the carcass dissection of 7 Welsh mountain ponies across a range of BCS (Dugdale *et al.*, 2011). Importantly, epicardial fat has been shown to play a key role in the pathogenesis of coronary artery disease in humans (Okada *et al.*, 2014) and an increased epicardial fat volume has been observed in patients with type 2 diabetes (Wang *et al.*, 2009). Additionally, mRNA for the brown fat marker, uncoupling protein 1 (UCP1) was expressed at higher levels in epicardial fat compared to other adipose depots (Sacks *et al.*, 2009), suggesting that this depot may have a further role in protecting the myocardium from hypothermia. Further studies may be warranted in the horse to determine whether this depot may have a role to play in insulin dysfunction or not.

The EQUIFAT scoring system was developed as an initial step towards wider applications to characterise fat patterning and clearly has broader applications in terms of furthering our understanding of regional adiposity and disease risk. The EQUIFAT system has the potential to capitalise on data readily collected during surgical interventions that require laparotomy. A relatively common cause of colic that requires laparotomy are strangulating lesions associated with the presence of a pedunculated lipoma arising from small intestine mesenteric WAT. A retrospective study conducted to assess the short-term survival rate of colic in a group of 300 horses and ponies that underwent exploratory laparotomy identified that 13% of those animals required surgical intervention due to intestinal strangulation by pedunculated lipoma, and the short-term survival rate of those 39 animals was

64.1% (Mair and Smith, 2005). Interestingly, a recent study that evaluated associations between pituitary lesions, obesity and the presence of mesenteric lipomas in insulin-resistant horses found that whilst insulin-resistant horses had a higher frequency of mesenteric lipomas, there was no association between obesity and the frequency of mesenteric lipomas (Newkirk *et al*., 2014). This finding combined with our finding in the current study that mesenteric fat scores were not associated with BCS would perhaps suggest that mesenteric fat scores as opposed to BCS may be associated with the frequency of mesenteric lipomas and may be an area for future study.

4.6 Conclusion

The current study outlines the development and testing of a novel depot-specific fat scoring system for horses and ponies 'EQUIFAT' which has been used to describe associations between regional fat depots and external BCS. The EQUIFAT scoring system proved to be robust when used on repeated occasions and on the whole there was very good agreement between observers when using the scoring system. Application of the scoring system on a large population of animals at *post-mortem* allowed associations to be made between BCS and the regional distribution of adipose tissue which demonstrated strong positive associations between BCS and retroperitoneal fat score, whilst there was no associations for mesenteric or epicardial fat scores. These associations suggest functional differences between the various adipose depots in terms of energy storage. Forward application of the EQUIFAT system would allow data collected at laparotomy or *post-mortem* to be collated with clinical findings. In combination, these methods could direct future studies towards furthering the understanding of the role played by regional adipose depots in obesity-associated pathologies such as laminitis, insulin dysregulation and pedunculated lipoma.

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Chapter 5

Post-mortem stability of RNA in skeletal muscle and adipose tissue and the tissue-specific expression of myostatin, perilipin and associated factors in the horse

5.1 Abstract

Obesity, a major concern for equine welfare, is highly prevalent in the leisure horse population. Skeletal-muscle and adipose tissues are important determinants of maintenance energy requirements. The myostatin and perilipin (PLIN1) pathways play key roles in the regulation of muscle mass and lipolysis respectively and have both been associated with obesity predisposition in other mammalian species.

High quality samples, suitable for molecular biology, are an essential prerequisite for detailed investigations of gene and protein expression. Hence, this study has evaluated a) the *post-mortem* stability of RNA extracted from skeletal-muscle and adipose-tissues collected under commercial conditions and b) the tissue-specific presence of myostatin, the myostatin receptor (activin receptor IIB, ActRIIB), follistatin and PLIN1, genes and proteins across a range of equine tissues.

Objectives were addressed using tissues from 7 Thoroughbred horses presented for slaughter at a commercial abattoir; a) samples were collected at 7 time-points from *masseter* muscle and perirenal adipose from 5 minutes to 6 hours *post-mortem*. Extracted RNA was appraised by Optical Density analysis and agarose-gel electrophoresis. b) Quantitative real time PCR and Western Blotting were used to evaluate gene and protein expression in anatomically-defined samples collected from 17 tissues (6 organs, 4 skeletal muscles and 7 discrete adipose depots).

The results indicate that, under the present collection conditions, intact, good quality RNA could be extracted from skeletal-muscle for up to 2 hours *post-mortem*. However, RNA from adipose tissue may be more susceptible to degradation/contamination and samples should be collected no later than 30 minutes *post-mortem*. The data also show that myostatin and ActRIIB genes and proteins were almost exclusively expressed in skeletal muscle. The follistatin gene showed a more diverse gene expression profile, with expression evident in several organs, adipose tissue depots and skeletal muscles. PLIN1 gene and protein were almost exclusively expressed by adipose tissue.

5.2 Introduction

Obesity, having reached epidemic proportions among horses and ponies in industrialised nations, is now considered a key concern for equine welfare (Argo *et al*., 2012; Harker *et al*., 2011). Adipose tissue and skeletal muscle can be considered as labile reserves of energy and nutrients within the body which can be used as buffers at times of negative or positive energy balance (Harris *et al*., 1986). The specific anabolic/catabolic pathways which are activated during periods of energy imbalance may be dependent on factors which regulate or modify the relative contributions of muscle or adipose tissue to whole body composition. It is widely accepted that skeletal muscle and adipose tissues engage in cross-talking pathways which ensure that they work in synergy to conserve energy balance and whole body homeostasis (Pedersen and Febbraio, 2012; Trayhurn *et al*., 2011). The cross-talk between skeletal muscle and adipose tissue is achieved through the synthesis and secretion of a variety of signalling factors and hormones respectively termed myokines and adipokines.

Muscle and adipose tissues act and interact dynamically to promote energy homeostasis but in states of active weight gain/loss, homeostasis is over-ridden and the relative contributions of these tissues to body composition are altered. Two proteins have attracted increasing interest in the regulation of tissue reserves; myostatin, which regulates reserves of metabolically active muscle (McPherron *et al*., 1997a); and PLIN1, which regulates intra-cellular lipolysis (Mottagui-Tabar *et al*., 2003).

Myostatin, a member of the transforming growth factor-beta super-family and one of the first myokines to be recognised, has been widely characterised as a potent negative regulator of skeletal muscle mass (Whittemore *et al*., 2003; Zimmers *et al*., 2002). It is secreted from skeletal muscle cells into the circulation (Brandt *et al*., 2012; McPherron *et al*., 1997b) and acts by binding to the activin type II receptor (ActRIIB), leading to a negative impact on muscle mass, while the circulating protein follistatin binds to, and inactivates myostatin (Amthor *et al*., 2004).

To date, loss-of-function mutations in the myostatin gene have been associated with a dramatic increase in skeletal muscle mass in a number of mammalian species (Grobet *et al.*, 1997; Kijas *et al.*, 2007; Mosher *et al.*, 2007). Furthermore, actions of this circulating growth factor are not restricted to muscle alone. Murine and human studies have clearly implicated myostatin in the development of obesity (Allen *et al.*, 2008; Hittel *et al.*, 2009; Zhang *et al.*, 2012). The myostatin protein has been detected in skeletal muscle from Thoroughbred and Kiso-uma horses (Hosoyama *et al.*, 2002) and polymorphisms in the equine myostatin gene have also been linked with optimal race distance in Thoroughbred horses (Hill *et al.*, 2010). However, despite these findings, the extent of expression of myostatin across a range of body tissues has yet to be established for the horse.

Perilipin (PLIN1) is a complex protein which is localised to the surface of intra-cellular lipid droplets*. In vivo*, PLIN1 can prevent lipolysis by blocking the entry of lipases to lipid droplets (Bickel *et al.*, 2009). In the presence of lipolytic stimuli, PLIN1 is phosphorylated by protein kinase A, permitting the initiation of lipolysis by the translocation of hormone sensitive lipase (HSL) to the surface of the lipid droplet. PLIN1 is pivotal in governing body fat stores. Several polymorphisms of the PLIN1 gene have been associated with obesity and weight-loss phenotypes in humans (Qi *et al.*, 2004; Ruiz *et al.*, 2011). Further, the magnitude of PLIN1 gene expression is positively associated with obesity in humans (Gjelstad *et al.*, 2012; Kern *et al.*, 2004). To date, PLIN1 expression has not been evaluated in the horse.

In order to conduct molecular and mechanistic studies, high quality samples are essential to provide RNA and proteins suitable for analysis. Collection of such samples is relatively straight forward in laboratory studies, but this is not the case for large animals such as equines, where biopsy techniques can only access superficial tissue. Hence it is critically important to develop and validate methods for the collection of high quality samples *post-mortem* prior to undertaking molecular type studies.

It is known from studies in pigs and cattle that increasing *post-mortem* interval negatively impacts on the purity and integrity of RNA (Bahar *et al.*, 2007; Fontanesi

et al., 2008). Therefore, before potential roles for the myostatin pathway and PLIN1 can be explored in the horse, it is important to first establish that good quality, intact RNA can be practically extracted from skeletal muscle and adipose tissue samples collected from the horse under commercial conditions. Further, the extent and anatomical distribution of gene and protein expression of PLIN1, myostatin and associated factors remains to be established for horses.

The aims of this study were to characterise the time-course of RNA degradation in equine *masseter* muscle and perirenal adipose tissues *post-mortem* and then to demonstrate the presence and evaluate the expression of myostatin, the myostatin receptor (ActRIIB), follistatin (an inhibitor of myostatin receptor binding) and PLIN1 across a spectrum of body tissues.

5.3 Materials and methods

Animals and tissue collection

Tissues from seven mature, Thoroughbred horses were obtained *post-mortem*. All animals were in good general health and were presented for slaughter for reasons unrelated to this study (Table 5.1). The horses were slaughtered in a commercial abattoir (LJ Potters, Taunton, Somerset) for non-research purposes in accordance with EU legislations EC 852/2004, 853/2004 and 854/2004 on several dates between March and June 2012.

Study One: Post-mortem stability of RNA extracted from masseter muscle and perirenal adipose tissue.

To evaluate the time course of RNA degradation *post-mortem*, samples were collected from the *masseter* muscle and perirenal adipose tissues of 3 animals (horses 1-3, Table 5.1) as these depots were those most rapidly accessible following exsanguination (*masseter*, ~2 minutes; perirenal adipose tissue 10-15 minutes). Tissue samples (*masseter,* around 200g; perirenal adipose tissue, around 260g) were aseptically collected onto sterile foil and maintained at ambient temperature

(~13°C). Gross tissue samples were sub-sampled (around 5g) for subsequent evaluation at 5 minutes (*masseter* muscle only), 20, 30, 40, 60 , 90, 120, 240 and 360 minutes *post-mortem* using sterile equipment. All sub-samples were macerated, snap frozen in liquid nitrogen and stored at -80°C prior to RNA extraction.

Table 5.1: Phenotypic descriptors for the 7 Thoroughbred horses used in this study.

	Horse No.	Gender	Age (Years)
Objective 1: RNA time course study	1	Gelding	11
	2	Mare	5
	3	Gelding	8
Objective 2: Across body study	4	Mare	12
	5	Gelding	8
	6	Mare	10
	7	Gelding	4

Study Two: Tissue specific gene expression of Myostatin, ActRIIB, Follistatin and PLIN1

To evaluate anatomical differences in gene and protein expression throughout the body, a total of 17 samples were collected from 4 carcasses (horses 4-7, Table 5.1). From each carcass, six body organs, plus seven anatomically-discrete adipose depots and four skeletal muscles were sampled (Table 5.2; Appendix E). Strict anatomical descriptors were used to ensure that tissue samples were collected from the same site in each animal (Table 5.2). Tissue samples were obtained as rapidly as possible *post mortem* (organs and adipose tissues, within 30 minutes; skeletal muscles within 1 hour), using sterile equipment. All samples were macerated and snap frozen in liquid nitrogen before being stored at -80°C pending RNA and protein extraction.

RNA extraction

Total RNA was extracted from all frozen tissue samples using TRIzol reagent (Invitrogen, Paisley, UK), in accordance with the manufacturers protocol. RNA concentration and purity was quantified spectrophotometrically (Eppendorf Biophotometer, Hamburg, Germany). To assess the purity of the extracted RNA, the ratio of optical density (OD) of the diluted RNA sample measured at wavelengths of 260 and 280 nm, provides an indication of any contamination of the RNA sample with RNase proteins. Reverse transcription (RT) was carried out in a 10μl final reaction volume containing 0.5μg RNA using an iScript cDNA synthesis kit (Bio-Rad Hemel Hempstead, UK). The resulting cDNA was diluted at 1:4 and used as a template for real-time PCR analysis. Visual appraisal and quantification of 28S and 18S RNA bands was conducted using agarose gel electrophoresis.

Table 5.2: Specific anatomical descriptors used to locate the tissue collection points for the 6 visceral organs, 7 regionally discrete adipose tissue depots and 4 skeletal muscles sampled from horses used in the second objective. Approximate target sample sizes are given. Where relevant, tissues were collected from the left side following carcass-splitting.

Tissue		Anatomical descriptors for sample sites
Visceral organs	Myocardium	~2cm^3 square, full thickness section, lateral wall of left ventricle midway between coronary groove and ventricle apex.
	Lung	~2cm^3 from dorsal aspect of the caudal lobe of the left lung at the intersection of the caudo-cranial and dorso-ventral midlines.
	Liver	~2cm^2 full thickness section from midway along the lateral margin of the left lobe.
	Kidney	~2cm^3, largely renal medulla, from the dorsal surface of the left kidney equidistant between the hillus and caudal pole.
	Stomach	~2cm^2 full thickness section from the body of the stomach, midway along the greater curvature adjacent to the origin of the greater omentum.
	Spleen	~2cm^3 from midpoint on the visceral surface of the intestinal lobe.
Adipose tissues	Peri-renal	~3cm^3, collected from the visceral aspect of the fat mass overlying the left kidney following evisceration.
	Ventro-abdominal	~3cm^3, collected from the left split-carcass midline at a point equidistant between xiphisternum and pubis.
	Epicardial	~2cm^3 from the coronary groove and overlying the left coronary artery
	Omental	Variable area of omentum, sufficient to harvest ~2cm^3 of adipose tissue, from a region adjoining the greater curvature of the stomach and bearing visible adipose.
	Mesenteric	Variable area sufficient to harvest ~2cm^3 of adipose tissue from the jejunum / proximal ileum mesenteries bearing visible adipose tissue.
	Crest	~3cm^3 from the left split-carcass at the deepest part of the crest, midway between wither and poll extremities.
	Tailhead	~2cm3 from the subcutaneous adipose tissue overlying the gluteal muscles of the left carcass.
Skeletal muscles	*Rectus abdominis,*	~3cm^3, collected from the left split-carcass midline at a point equidistant between xiphisternum and pubis.
	Longus colli,	~3cm^3, from its severed cranial extremity in the left split-carcass.
	Adductor	~3cm^3, collected from the centre of the exposed midline section of the muscle on the left split-carcass.
	Pectoralis transversus	~3cm^3, collected from the exposed midline section of the muscle at a point just ~10cm caudal to the thoracic inlet.

Agarose Gel Electrophoresis

To assess RNA integrity from horses 1-3 (study one), 10μg RNA from *masseter* muscle samples; 8-10μg RNA from perirenal adipose tissue samples were mixed with loading solution (containing 500μl formamide, 162μl formaldehyde and 100μl 5 X MOPS buffer) in a 1:3 dilution, then heated at 65°C for 5 minutes before being placed on ice. Two micro-litres of loading buffer (containing 1ml 50% sterile glycerol, 12μl 5% bromophenol blue, 7μl 1M NaOH and 12μl 10μg/μl ethidium bromide) was then added to the samples to make a final volume of 20μl. The samples were separated by electrophoresis through a 1% agarose gel, stained with ethidium bromide and 28S and 18S band intensities were quantified (ChemiDoc XRS+ Imaging System, BioRad).

Quantitative Real-Time PCR

The expression of myostatin and four housekeeping genes previously used in other equine studies (Ahn *et al*., 2011; Bogaert *et al*., 2006) (GAPDH, Beta-actin, HPRT1 and RPL32) was determined in tissues from horses 1-7 (studies 1 & 2), with the expression of a further three genes (ActRIIB, follistatin and PLIN1) assessed in horses 4-7 (study 2). GeNorm and Normfinder software (GenEx, Germany) was used to assess the two 'most stably' expressed genes to be used for normalisation. Gene expression was determined by quantitative real-time PCR performed in duplicate using the Stratagene Mx3005P detection system (Agilent Technologies, California USA). Primer sequences for all four housekeeping genes were obtained from previously published data (HPRT1 and RPL32, GAPDH (Bogaert *et al*., 2006), and beta-actin (Ahn *et al*., 2011)) and 100% homology was confirmed by performing a basic local alignment search tool (BLAST). Primer and Taqman probe sequences for myostatin, ActRIIB, follistatin and PLIN1 were designed using Beacon Designer (Premier Biosoft, USA). All primers were designed to be exon-spanning. All primer/probe sets were purchased from Eurogentec (Belgium) (Table 5.3).

Table 5.3: Nucleotide sequences of primers and probes used in the current study.

Gene	Primer	Sequence	Amplification efficiency
Beta-actin	Forward	GGACCTGACGGACTACCTC	97%
	Reverse	CACGCACGATTTCCCTCTC	
HPRT1	Forward	GGCAAAACAATGCAAACCTT	94.5%
	Reverse	CAAGGGCATATCCTACGACAA	
GAPDH	Forward	CAGAACATCATCCCTGCTTC	95%
	Reverse	ATGCCTGCTTCACCACCTTC	
RPL32	Forward	AGCCATCTACTCGGCGTCA	94%
	Reverse	TCCAATGCCTCTGGGTTTC	
Myostatin	Forward	GCAGTGATGGCTCTTTGGAAG	97.9%
	Reverse	GCATTAGAAGATCAGACTCTGTAGG	
	Probe	ACCACGCGACGACGGAAACAATCAT	
ActRIIB	Forward	GCCTCGCTGTTCGGTTTGAG	92.9%
	Reverse	GGCTCCCTCAAGCACCTCAG	
	Probe	ACCGCCGTGTGCCCACCTGC	
Follistatin	Forward	CAGTGACAATGCCACTTACGC	92.5%
	Reverse	GGTCTTCATCTTCCTCCTCTTCC	
	Probe	TGCCATGAAGGAAGCTGCCTGTCTCC	
PLIN1	Forward	GATCCCAGCCCTCCAGTACC	103.9%
	Reverse	GGACGCTGATGCTGTTCCTG	
	Probe	AGATCGCCTCTGAGCTGAAGGACACCATC	

Serial dilutions of pooled cDNA were used to calculate Taqman primer efficiencies. The PCR cycling conditions (using Taqman probe and primers) for the genes of interest (myostatin, ActRIIB, follistatin and PLIN1) were as follows: 10 minutes at 95°C, followed by 40 cycles of 30 seconds at 95°C, 1 minute at 55°C and 1 minute at 72°C. Cycling conditions for housekeeping genes (using SYBR green method) were as follows: 10 minutes at 95°C followed by 40 cycles of 15 seconds at 95°C and 30 seconds at 60°C and ending with, 1 minute at 95°C, 30 seconds at 55°C and 30 seconds at 95°C.

Relative gene expression was calculated using the comparative Ct method ($2^{-\Delta\Delta Ct}$)

(Livak and Schmittgen, 2001). All gene expression data were normalised to 2 internal housekeeping genes and data from the second study are presented as relative expression with respect to the myocardial tissue.

Protein Extraction and Western Blotting

Total protein was extracted from frozen tissues obtained from the horses 4-7 (study two) by homogenising around 100mg of tissue in a SHE buffer (250mM sucrose, 1mM HEPES, 0.2mM EDTA) containing both phosphatase and protease inhibitor cocktails (both Sigma, Poole, Dorset, UK). Protein concentration was determined by the BCA method and protein integrity was verified using standard silver staining of typical SDS gels (data not shown).

Forty five micrograms of protein extract were separated on 10% SDS-polyacrylamide gels under reducing conditions and proteins were transferred onto nitrocellulose membrane (Hybond-C Extra, Amersham Bioscience, Buckinghamshire, UK) by electroblotting. Membranes were stained in Ponceau S reversible stain to verify the success of protein transfer and then blocked for 1 hour in 5% BSA in Tris-buffered saline containing 0.1% Tween 20 (TBST). Commercially available primary antibodies (listed below) were used and were selected on the basis that they were listed as having equine cross-reactivity. They were added at the following concentrations: myostatin precursor (MSTN), 1:250 [ab98337 Abcam, Cambridge, UK], myostatin receptor (ACTRIIB), 1:200 [sc-25453 Santa Cruz, Dallas, Texas, USA], PLIN1, 1:200 [sc-67164 Santa Cruz], the serine/threonine Akt (AKT), 1:2500 [#9272 Cell Signalling, Danvers, MA, USA]) in blocking buffer and incubated overnight at 4°C. The myostatin antibody detected the precursor form of the protein (43kDa). The membranes were washed and then incubated for 1 hour with a secondary antibody (Cell Signalling) at appropriate concentrations. Signals were detected by chemiluminescence using a SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, US) and visualised on a Molecular Imager ChemiDoc XRS+ System (Bio-Rad).

Statistical Analysis

Statistical analyses were performed using STATA version 12.1 (StataCorp, Texas). Non-parametric, analytical methods were employed. The Friedman test for repeated measures was used to assess the effect of increasing *post-mortem* interval on myostatin gene expression in study 1. The Wilcoxon signed ranks test was used to analyse gene expression data from study 2.

5.4 Results

Study One: Post-mortem stability of RNA extracted from masseter muscle and perirenal adipose tissue.

The purity of RNA extracted from the *masseter* muscle remained relatively stable over the 6 hour time course evaluated. . The ratio of OD for RNA extracted from muscle remained in excess of 1.8, indicating minimal protein contamination (Fleige *et al*., 2006), for all horses at each time point (Table 5.4). Visual assessment of the RNA integrity confirmed that intact 28S and 18S ribosomal RNA bands were detected at all time points up to 120 minutes in all three animals (1-3). Quantification of the 28S and 18S ribosomal RNA bands demonstrated consistent 28S:18S ratios of close to 2 at all time points up to 240 minutes *post-mortem* (Table 5.4). However by 360 minutes the average ratio had reduced to 1.42 and the variation was considerably increased (Table 5.4). These results show *masseter* muscle is resilient to *post-mortem* RNA degradation, but samples should be obtained within 2 hours of death to ensure the extraction of good RNA for downstream molecular biology studies.

By contrast, in adipose tissue, the OD ratios of RNA extracted from perirenal adipose tissue were relatively lower and more variable than those obtained from *masseter* muscle (Table 5.4). Mean OD ratios ranged from 1.71 to 1.68 in samples evaluated between 20 minutes and 4 hours *post-mortem* but had decreased to 1.44 by the final test at 6 hours *post-mortem* (Table 6.4). Visual appraisal of RNA

integrity demonstrated that intact 28S and 18S ribosomal bands were visible in all horses (horses 1-3) up to 30 minutes *post-mortem*.   Quantification of the 28S and 18S ribosomal bands demonstrated the average 28S:18S ratios were 1.77 and 1.67 at 20 minutes and 30 minutes *post-mortem*, respectively, and gradually decreased down to 1.54 by 6 hours *post-mortem* (Table 5.4). This indicates that adipose tissue appears to be more susceptible to RNA degradation than skeletal muscle. However, intact RNA can be extracted from adipose tissue provided samples are collected up to 30 minutes *post-mortem* under conditions similar to those used in this study.

Expression of myostatin in masseter muscle and perirenal adipose tissue

GeNorm and Normfinder results (Table 5.5) indicated that HPRT1 and Beta-actin were the most stably expressed of the housekeeping genes evaluated.   On this basis, the mean Ct values of these genes were used for the normalisation of gene expression data. Friedman tests demonstrated no difference in myostatin expression across the time course in either *masseter* muscle (p = 0.16) or perirenal adipose tissue (p = 0.96).

Table 5.4: RNA quality assessment by spectrophotometer (A260/A280 ratio) and 28S:18S ratio (agarose gel electrophoresis and Chemi-Doc imaging and analysis) for *post-mortem* intervals from 5 to 360 minutes. *n*=3.

Tissue	*Post-mortem* interval (minutes)	Average A260/280 ratio (standard deviation)	Average 28S:18S ratio (standard deviation)
Masseter muscle	5	2.10 (0.16)	1.95 (0.23)
	20	2.05 (0.12)	1.82 (0.07)
	30	1.98 (0.04)	1.87 (0.19)
	40	2.02 (0.08)	2.01 (0.15)
	60	1.92 (0.13)	1.98 (0.15)
	90	2.06 (0.10)	1.92 (0.19)
	120	1.99 (0.01)	1.81 (0.18)
	240	1.95 (0.03)	1.85 (0.06)
	360	1.85 (0.05)	1.42 (1.07)
Perirenal adipose tissue	20	1.71 (0.41)	1.77 (0.79)
	30	1.63 (0.42)	1.67 (0.18)
	40	1.63 (0.40)	1.48 (0.19)
	60	1.65 (0.40)	1.60 (0.64)
	90	1.63 (0.36)	1.56 (0.12)
	120	1.62 (0.42)	1.53 (0.50)
	240	1.68 (0.39)	1.28 (0.57)
	360	1.44 (0.41)	1.54 (0.09)

Table 5.5: Housekeeping gene comparison using GeNorm and Normfinder analysis

Gene	Study 1		Study 2	
	GeNorm M value (ranking)	Normfinder SD (ranking)	GeNorm M value (ranking)	Normfinder SD (ranking)
HPRT1	1.41 (1)	0.41 (1)	1.20 (1)	0.25 (1)
B-ACTIN	1.41 (1)	0.41 (2)	1.55 (3)	1.46 (3)
RPL32	1.81 (2)	0.79 (3)	1.20 (1)	1.25 (2)
GAPDH	2.35 (3)	0.90 (4)	2.74 (4)	3.92 (4)

Study Two: Tissue specific gene expression of Myostatin, ActRIIB, Follistatin and PLIN1

For study two, RNA quality was assessed spectrophotometrically and was shown to be acceptable for the proposed study of gene expression in all tissues (Table 5.6). Gene expression data were normalised with respect to those for RPL32 and HPRT1. When gene expression data across the entire range of tissues sampled were assessed with GeNorm and Normfinder software, RPL32 and HPRT1 demonstrated the greatest stability of all housekeeping genes examined (Table 5.5).

Table 5.6: RNA quality as assessed by spectrophotometry; average A260/A280 ratios from various tissues throughout the body. (Study 2; *n* = 4).

Tissue	Average A260/A280 ratio (standard deviation)
Myocardium	1.91 (0.04)
Lung	1.90 (0.03)
Liver	1.86 (0.06)
Kidney	1.85 (0.02)
Stomach	1.90 (0.03)
Spleen	1.83 (0.05)
Omental fat	1.81 (0.06)
Mesenteric fat	1.81 (0.04)
Retroperitoneal fat	1.74 (0.03)
Crest fat	1.78 (0.05)
Tailhead fat	1.73 (0.10)
Perirenal fat	1.70 (0.08)
Epicardial fat	1.74 (0.06)
Rectus abdominis	1.97 (0.14)
Longus colli	1.93 (0.18)
Adductor	1.92 (0.03)
Pectoralis transversus	1.97 (0.06)

Visual appraisal of myostatin gene expression (Figure 5.1a) demonstrates considerably greater expression in skeletal muscles compared to any other tissues studied (p = 0.07 for all muscles). Although relative transcript concentrations in skeletal muscle appear varied between the specific muscles sampled, differences were not statistically significant. The anatomical distribution in the relative abundance of ActRIIB mRNA was similar to that of myostatin (Figure 5.1b), with expression of this gene being greater in the skeletal muscles when compared to all other tissues studied (p = 0.07 for all muscles relative to cardiac tissue). Similarly, ActRIIB mRNA expression was not significantly different between the four skeletal muscles evaluated.

Follistatin mRNA expression was anatomically more diverse (Figure 5.1c). Whilst no significant differences were detected, follistatin gene expression appears to be greater than cardiac tissue in lung, liver, stomach (p = 0.07), a number of adipose tissue depots (retroperitoneal, crest, tailhead, epicardial and perirenal; p = 0.07), and three of the four skeletal muscles tested (*Longus colli, Pectoralis transversus* and *Adductor,* p =0.07). Within the regional adipose tissues studied, crest, tailhead, epicardial, and perirenal samples tended to have greater follistatin expression when compared to the omental and mesenteric depots (p = 0.07)

Relative to myocardial tissue, visual appraisal suggests that PLIN1 mRNA expression tends to be greatest in all seven adipose tissue depots studied (*p* = 0.07) (Figure 5.1d). Between adipose depots, tailhead, epicardial and perirenal tended to have greater PLIN1 expression when compared to omental and mesenteric depots (p = 0.07), whilst there was a trend for retroperitoneal fat to have increased expression relative to omental (p = 0.07) and there was a trend for crest fat to have increased expression compared to mesenteric fat (p = 0.07)
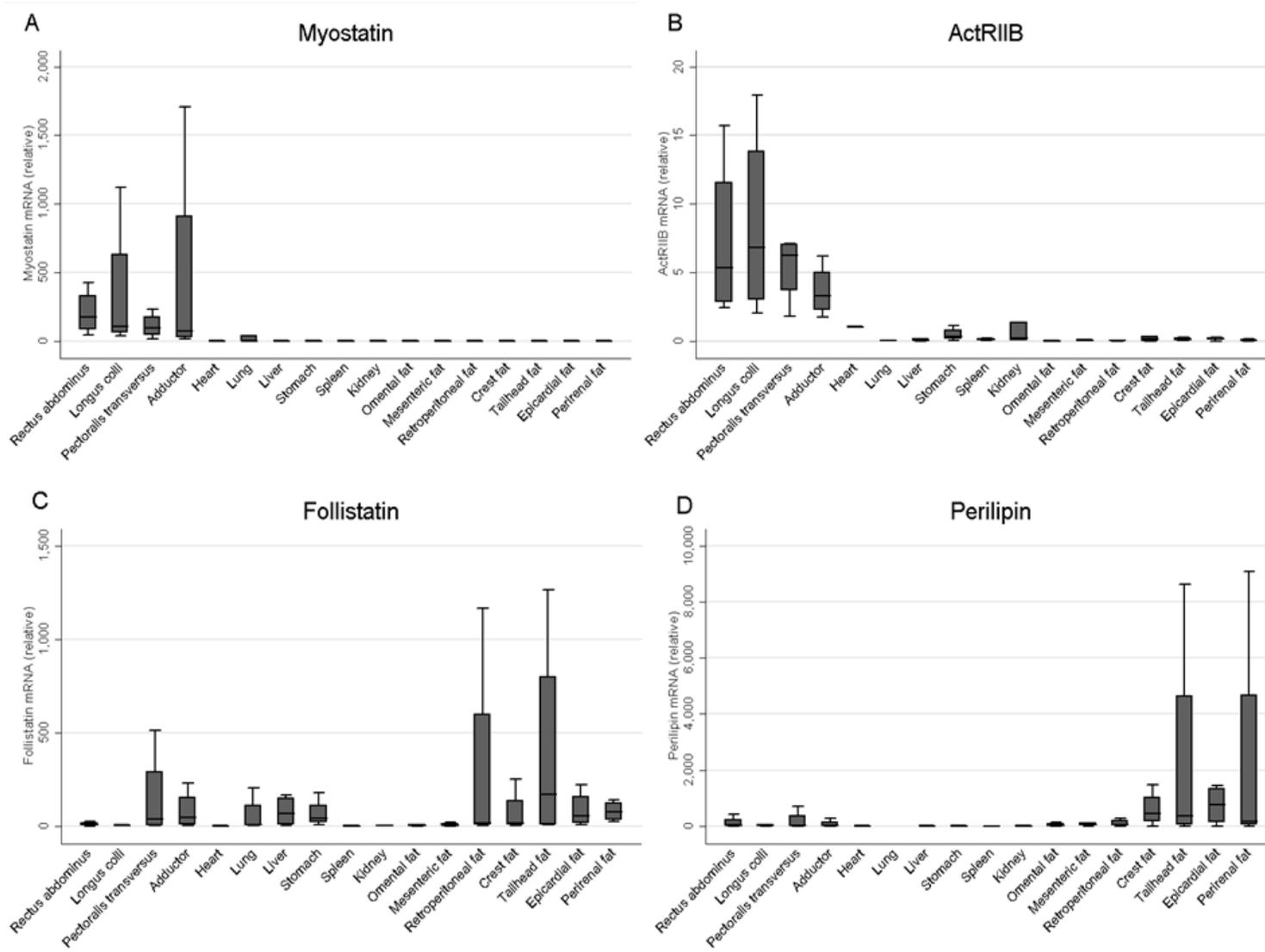
Figure 5.1: Gene expression of Myostatin, ActRIIB, Follistatin and PLIN1 across a range of equine tissues as analysed by quantitative real-time PCR. Data are presented as relative expression with respect to myocardial tissue. Relative transcript abundance is shown for **(A)** Myostatin, **(B)** ActRIIB, **(C)** Follistatin, and **(D)** PLIN1. $n = 4$.

Tissue specific protein expression of Myostatin, ActRIIB and PLIN1

Western blot analysis was used to assess protein expression of myostatin precursor protein, ActRIIB and PLIN1 across the range of tissues collected (Figure 5.2). Total AKT was used as a loading control (Koch *et al.*, 2008). Whilst there appeared to be some non-specific binding, the myostatin (43kDa) and ActRIIB (50kDa) proteins were only identified in skeletal muscle samples. PLIN1 protein (57kDa) was demonstrably present in all of the studied adipose tissue depots.
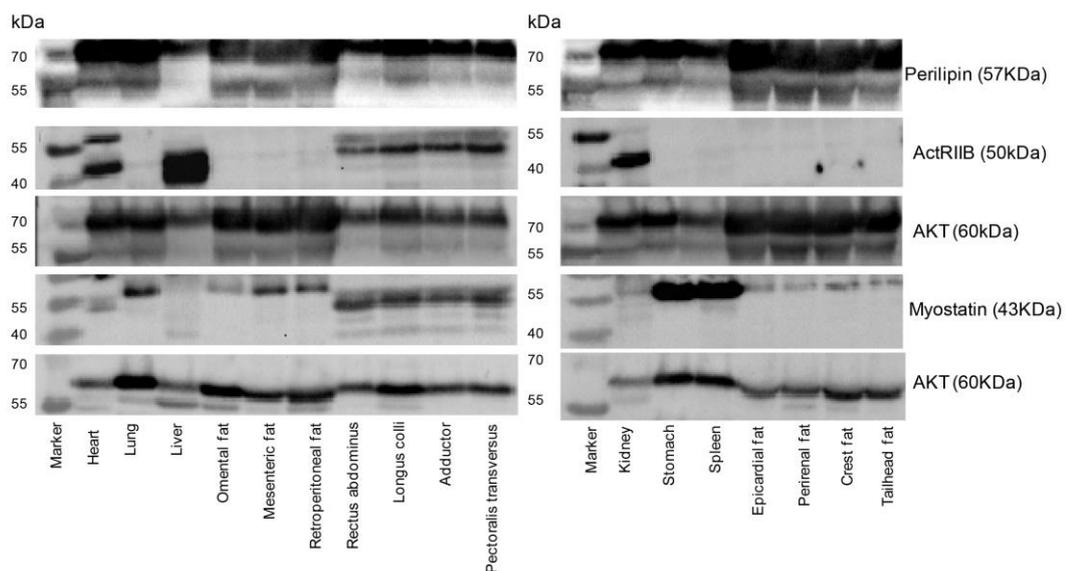
Figure 5.2: Tissue-specific protein expression of PLIN1, ActRIIB and myostatin precursor protein across a range of equine tissues assessed by Western blot with total AKT used as a loading control. Four membranes were probed for each horse (2 for myostatin and AKT, and a further 2 for ActRIIB, PLIN1 and AKT) Representative blots are shown; *n*=4. AKT loading controls are shown for each respective membrane.

5.5 Discussion

This study establishes for the first time that it is possible to obtain samples of sufficient quality for molecular studies from the collection of *post-mortem* tissues from horses in a commercial abattoir.

By characterising the time course of RNA degradation in the immediate *post-*

mortem interval, clear time constraints for the collection of *post-mortem* tissues to be used in the evaluation of gene-expression in equine skeletal muscle and adipose tissue have been described. The data indicate that while the RNA extracted from *masseter* muscle in all horses sampled was minimally contaminated with protein (OD [A260/A280] ratio > 1.8), for at least 6 hours *post-mortem,* 28S and 18S ribosomal bands were only clearly visible in all three animals for the first 2 hours following death, and average 28S:18S ratios dropped to 1.42 by 6 hours *post-mortem*. It was noteworthy that perirenal adipose tissue appeared to be more susceptible to protein contamination. Extracting good quality RNA from adipose tissue is known to be more challenging than RNA extraction from other tissues due to the naturally high lipid content in these tissues (Hemmrich *et al*., 2010; Pratt *et al*., 2013). The OD (A260/A280) ratio of adipose tissue RNA remained consistently less than that of *masseter* muscle, and whilst ribosomal RNA bands were only clearly visible up to 30 minutes *post-mortem* in all horses, average 28S:18S ratios were 1.77 and 1.67 at 20 and 30 minutes *post-mortem,* indicating RNA remained relatively intact up to 30 minutes *post-mortem.*. The gold standard 28S:18S ratio for intact RNA is 2:1. However it is rare to find this ratio in RNA extracted from mammalian tissues and a cut-off of 1.5 (Ju *et al*., 2009) and even 1.0 (Rebouissou *et al*., 2008) (quantified from agarose gel electrophoresis) has been used for extracted RNA deemed to be suitable for quantitative RT-PCR. Furthermore, a recent publication that outlines criteria for publishing RT-qPCR data suggests that a 28S:18S ratio quantified from agarose gel electrophoresis of between 1 and 2 is indicative of intact RNA (Sean Taylor, 2010).

Taken together, these data would suggest that the optimal windows for the collection of muscle and adipose tissue samples to ensure the extraction of good quality RNA to be used in gene expression studies are up to 120 and 30 minutes respectively. Conversely, in both the muscle and adipose tissue samples collected here, evaluation of myostatin expression in all animals seemed unaffected by *post-mortem* intervals of up to 6 hours. This could be interpreted to suggest that specific mRNAs might be variably robust. However, demonstration of the suitability of RNA extracted from tissues collected out with the defined optimal time-windows would

need to be demonstrated on a gene-specific basis.

Although numerous publications use abattoir-derived, *post-mortem* tissues to describe gene-expression in large animal species, data describing the suitability of RNA obtained in this manner are sparse. Two studies have suggested that skeletal muscle RNA can remain stable up to 24 hours *post-mortem* in porcine carcasses (Fontanesi *et al*., 2008) and for up to 8 days in the bovine (Bahar *et al*., 2007). In agreement with the current study, the bovine study also found that RNA extracted from subcutaneous adipose tissue was more susceptible to degradation than skeletal muscle, with 28S and 18S rRNA molecules remaining intact for 24 hours *post-mortem* (Bahar *et al*., 2007). These sampling windows greatly exceed those indicated in the current equine study and may be attributable to differences in conditions between commercial abattoir systems. The porcine and bovine studies were conducted in high throughput abattoirs where carcasses were rapidly processed and moved to cold rooms (4°C) for further sampling within 2 hours of death. This is in contrast to the low throughput system central to the current equine study and where all tissue sampling was conducted at ambient temperature (~13°C). Clearly, the impact of environmental temperatures is likely to have important implications for the long term stability and integrity of RNA and should be considered in conjunction with the *post-mortem* interval.

The second element of this study aimed to demonstrate the tissue-specific presence of myostatin, ActRIIB, follistatin and PLIN1, and is the first study to do this in equine tissues.

GeNorm and Normfinder analysis revealed that when the whole spectrum of tissues were analysed, HPRT1 expression remained stable across the spectrum of tissues in both studies. However in study two, RPL32 proved to be more stable especially across the organ tissues than beta-actin which was used for normalisation with HPRT1 in the first study (muscle and adipose tissues only). This would suggest that the same combinations of housekeeping genes are not always suitable for normalisation when a range of tissues are studied, a finding which has been demonstrated in a number of studies (Gu *et al*., 2011; Kessler *et al*., 2009;

Peters *et al.*, 2007). Hence, careful consideration must be given to ensure the stability of housekeeping genes selected for the particular tissues under consideration.

Anatomically, the expression of myostatin and its receptor (ActRIIB), both at the gene and protein transcript level would appear to be predominantly a function of skeletal muscle. Conversely, PLIN1 expression was primarily restricted to adipose tissue depots while the follistatin gene was more ubiquitously expressed across a range of diverse tissues. The small population size in this study combined with large differences in gene expression between animals may be accounting for the lack of statistically significant differences between tissues in the gene expression studies, however it is clear from the gene and protein expression data that there are differences between tissues and a greater study population may have increased the statistical significance.

Studies of myostatin in large animal species have generally focused on associations between myostatin gene mutations and carcass traits in breeds of cattle (Gill *et al.*, 2009), sheep (Hickford *et al.*, 2010), and pigs (Tu *et al.*, 2012) as an adjunct to selective breeding programs for optimal meat production. There are few studies in the horse and to date, only one report has identified myostatin precursor and mature myostatin protein expression in the skeletal muscles (*Semitendinosus, Semimembranosus, Splenius, Gluteus medius)* of Thoroughbred and Kiso-uma breeds of horses (Hosoyama *et al.*, 2002). That gene and protein expression for myostatin and its receptor were largely exclusive to skeletal muscle, suggested that as reported for other species, myostatin may have an important role in equine muscle function. Notably, the gene expression of myostatin has also been reported to differ between skeletal muscle fibre types; with increased mRNA expression recorded in muscles largely composed of type II fibres (Carlson *et al.*, 1999; Hennebry *et al.*, 2009). Although the skeletal muscles sampled in the current study were selected for their diversity of form and function and were therefore likely to vary in fibre composition, the relative extent of myostatin gene expression in the different muscles studied here did not prove significantly different and may be due

to the small number of horses studied.

Although myostatin and its receptor were not detected at the gene and protein level in adipose tissues, evidence from other species suggests that myostatin can interact with adipose tissue to inhibit adipocyte differentiation *in vitro* (Guo *et al.*, 2008; Hirai *et al.*, 2007a). Conversely myostatin-mediated adipose:muscle cross-talk has been demonstrated to up-regulate gene-associated adipogenesis in mice (Zhang *et al.*, 2012). The disparity of the conclusions of these studies is likely to be partially associated with the different experimental approaches used. It is possible that further studies are needed to evaluate the role of myostatin in cross-talking pathways with adipose tissues in *Equidae*.

The follistatin gene was liberally expressed across the range of tissues studied in these horses. Follistatin is a multi-functional protein, originally described as an inhibitor of follicle-stimulating hormone (FSH) secretion (Ueno *et al.*, 1987). It has since been well characterised as a binding protein that inhibits the actions of members of the TGF-β family of signalling molecules including activin, myostatin, and bone morphogenetic proteins (BMPs) (Cash *et al.*, 2012; Keutmann *et al.*, 2004). Expression of the follistatin gene has been demonstrated across a wide range of human tissues (Tortoriello *et al.*, 2001). Notably, this human study did not evaluate follistatin expression in adipose tissues which is one tissue in which follistatin gene was expressed in the current study. More recently, follistatin gene expression has been detected in human adipose tissues with greater expression noted in subcutaneous as opposed to visceral adipose depots (Flanagan *et al.*, 2009). These data agree with the findings of the current study where follistatin expression was notably minimal in omental and mesenteric depots, in marked contrast to the clear expression noted in the other adipose tissue depots studied. The same study also demonstrated that treatment with exogenous follistatin could promote adipogenesis in cultured human progenitor cells and could reverse adipogenic inhibition by myostatin (Flanagan *et al.*, 2009). Additionally, follistatin reversed the inhibitory effects of activin A on the differentiation of bovine pre-adipocytes (Hirai *et al.*, 2007b), whilst it was also identified that follistatin binds

myostatin to a slightly lesser extent than it binds activin A (Sidis *et al.*, 2006). Activin A has been implicated as a key player in human adipogenesis (Zaragosi *et al.*, 2010). In the current study, follistatin gene expression was greater in subcutaneous depots (crest and tailhead) relative to visceral (omental) fat. Although not measured in the current study, it could be suggested that if activin A is expressed in equine adipose tissues it may associate with follistatin to aid in the regulation of adipogenesis.

In the relatively lean horses used in the current study, PLIN1 (gene and protein), was almost exclusively expressed by adipose tissues, and was remarkably consistent between regional adipose depots. This contrasts with human work which indicated that PLIN1 gene expression was greater in subcutaneous than visceral adipose tissues (Arvidsson *et al.*, 2004; Wang *et al.*, 2003), but that the PLIN1 protein concentrations were similar between omental and subcutaneous fat depots (Arvidsson *et al.*, 2004; Wang *et al.*, 2003). This may suggest that PLIN1 expression is subject to post-transcriptional modification. PLIN1 expression has previously been shown to be modified in an obese state, with expression positively correlated to percentage body fat in human subjects (Gjelstad *et al.*, 2012; Kern *et al.*, 2004). Conversely, it was observed that whilst PLIN1 gene and protein expression in subcutaneous adipose tissue was significantly decreased in obese as opposed to lean humans, PLIN1 mass per adipocyte was constant between obese and non-obese people (Wang *et al.*, 2003). In agreement with this, a further study also found that PLIN1 protein content was relatively decreased in subcutaneous adipose tissues from obese compared to lean women (Mottagui-Tabar *et al.*, 2003). These data suggested that PLIN1 concentrations were positively associated with rates of basal lipolysis. These conflicting data may in part be associated with differences in the case-definitions for obesity between the two studies. Indisputably, PLIN1 plays a vital role in the regulation of lipolysis (Shen *et al.*, 2009; Tansey *et al.*, 2004) and it could be suggested that variations in PLIN1 expression are both the cause of the metabolic dysregulation apparent in obesity and a consequence of obesity itself. To the authors' knowledge, this is the first study to identify PLIN1 expression in the horse and future studies may aid in the resolution of the precise contribution of this protein in the fat biology of the horse.

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5.6 Conclusion

This study clearly demonstrated that RNA remains intact up to 2 hours *post-mortem* in equine *masseter* muscle and up to 30 minutes *post-mortem* in perirenal adipose tissue in all three horses studied. Furthermore, the tissue distributions for myostatin, follistatin, ActRIIB and PLIN1 in the horse have been described. More focused research into how these factors are altered in settings of energy imbalance such as observed in obesity or weight loss are required to better understand their physiological roles.

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Chapter 6

Preliminary investigation into a potential role for myostatin and its receptor (ActRIIB) in lean and obese horses and ponies

During the writing of this thesis, this chapter has been accepted for publication:

Preliminary data from this chapter were also presented as an abstract:

6.1 Abstract

Obesity is a widespread problem across the leisure population of horses and ponies in industrialised nations. Skeletal muscle is a major contributor to whole body resting energy requirements and communicates with other tissues through the secretion of myokines into the circulation. Myostatin, a myokine and negative regulator of skeletal muscle mass, has been implicated in obesity development in other species. This study evaluated gene and protein expression of myostatin and its receptor, ActRIIB in adipose tissues and skeletal muscles and serum myostatin concentrations in six lean and six obese animals to explore putative associations between these factors and obesity in horses and ponies. Myostatin mRNA expression was increased while ActRIIB mRNA was decreased in skeletal muscles of obese animals but these differences were absent at the protein level. Myostatin mRNA was increased in crest fat of obese animals but neither myostatin nor ActRIIB proteins were detected in this tissue. Mean circulating myostatin concentrations were significantly higher in obese than in lean groups; 4.98ng/ml (± 2.71) and 9.00ng/ml (± 2.04) for the lean and obese groups, respectively. In addition, there was a significant positive association between these levels and myostatin gene expression in skeletal muscles (average R^2 = 0.58; p < 0.05). Together, these results provide further basis for the speculation that myostatin and its receptor may play a role in obesity in horses and ponies.

6.2 Introduction

Epidemiological studies continue to report a high prevalence of obesity amongst the leisure population of horses and ponies in the UK (Giles *et al*., 2014; Harker *et al*., 2011). The well-documented negative impacts of obesity on health and performance have led to obesity being considered one of the major welfare issues in horses and ponies facing industrialised nations today (Owers and Chubbock, 2013). Obese animals are at an increased risk of developing insulin dysregulation and the severely painful and often life-threatening condition, laminitis, although the precise mechanisms linking these conditions are not yet fully understood.

The organ systems involved in energy homeostasis work in synergy to achieve the maintenance of whole body energy balance. As the largest metabolically active tissue in the body (comprising around 40% body mass, (Dugdale *et al*., 2011; Webb and Weaver, 1979)), skeletal muscle is a key determinant of resting energy expenditure and therefore plays a vital role in maintaining energy balance. Communication with other organs, including adipose tissue, is achieved through the secretion of molecular messengers into the circulation, termed myokines. Myostatin, a member of the transforming growth factor β (TGFβ) family of secreted growth factors, is one such myokine. The initial studies showed that mice lacking the myostatin gene were extremely hypermuscular and had minimal body fat when compared to their wild-type counterparts (McPherron *et al*., 1997). To date, myostatin has been widely characterised as a potent negative regulator of skeletal muscle mass (Joulia *et al*., 2003; Langley *et al*., 2002; Whittemore *et al*., 2003) and methods to inhibit myostatin function as a potential therapeutic treatment for increasing muscle mass in diseases such as muscular dystrophy and cancer cachexia have been explored (Benny Klimek *et al*., 2010; Wagner *et al*., 2008).

Myostatin is synthesised as an inactive precursor protein which subsequently undergoes two cleavages to produce the mature, active form of the protein. Mature myostatin is bound noncovalently to its propeptide and circulates in serum as an inactive complex (Hill *et al*., 2002). Active, mature myostatin binds selectively

to the activin type II receptor kinase, ActRIIB. Studies in rodents and humans generally report that myostatin expression levels are highest in skeletal muscle, although it has also been identified in adipose tissue (McPherron *et al*., 1997). Previous work from this laboratory supports these findings and extends them to the horse. These data confirmed that myostatin gene and precursor protein expression is greatest in skeletal muscles and that in the horse, although low levels of expression were detected in adipose tissue at the gene level, myostatin precursor protein was absent (Morrison *et al*., 2014).

Work in murine models and humans has identified that myostatin may have an important role in obesity development. Myostatin knock-out (KO) mice offered high-fat diets are resistant to gains in body fat (Dilger *et al*., 2010; Hamrick *et al*., 2006), and although this effect may be secondary to the increases in lean body mass, myostatin had direct effects on adipocyte differentiation (Guo *et al*., 2008a; Hirai *et al*., 2007). Furthermore, blocking myostatin increased the functional capacity of brown adipose tissue (BAT) (Fournier *et al*., 2012) and may even drive the browning of white adipose tissue through the up-regulation of BAT-specific genes (Shan *et al*., 2013). Myostatin gene expression was positively associated with obesity in both mouse (Allen *et al*., 2008) and human studies (Hittel *et al*., 2009), whilst blocking myostatin function in mature mice elicited positive effects on glucose and insulin dynamics (Cleasby *et al*., 2014). In comparison to human and rodent studies, there are fewer studies of myostatin in horses and ponies, and the extant reports generally focus on the identification of a number of single nucleotide polymorphisms (SNP's) in the myostatin gene. SNPs have been associated with different attributes including breeds of different morphological type (Dall'Olio *et al*., 2010), optimal race distance in Thoroughbred horses (Hill *et al*., 2010) and skeletal muscle fibre type proportions in Quarter horses (Petersen *et al*., 2013).

To date, no work has been conducted to characterise the expression of myostatin and its receptor against the setting of obesity in the horse or pony. The current study was designed to explore possible differences in myostatin and ActRIIB expression between lean and obese animals by quantifying myostatin and ActRIIB

gene and protein expression in skeletal muscle and adipose tissue, and measuring serum myostatin concentrations.

6.3 Materials and methods

Animals and tissue collection

Tissues from six lean (body condition score (BCS) /9 = 3.07 ±0.50, where 1 = emaciated and 9 = obese (Kohnke, 1992)) and six obese (BCS /9 = 7.7 ± 0.46) mature, mixed breed horses and ponies were obtained *post-mortem*. All animals were in good general health and were euthanased for reasons unrelated to this study (Table 6.1). The horses were slaughtered in a commercial abattoir (LJ Potters, Taunton, Somerset) in accordance with EU legislations EC 852/2004, 853/2004 and 854/2004 on several dates between March 2013 and January 2014. *Ante-mortem* data collection included BCS (/9, (Kohnke, 1992)), breed type, gender, estimated withers height and age. For assessment of BCS, six areas of the body (neck, withers, loin, tailhead, ribs and shoulder) are assigned a number from 1 (emaciated) to 9 (obese) based on detailed descriptors. The average of these six numbers is calculated and this number equates to the final BCS score for the animal (Kohnke, 1992).

To evaluate gene and protein expression of myostatin and ActRIIB, a total of five anatomically-discrete adipose depots and four functionally distinct skeletal muscles were sampled. Strict anatomical descriptors were used to ensure that tissue samples were collected from the same site in each animal (Table 6.2 and Appendix E). Tissue samples were obtained as rapidly as possible *post-mortem* (adipose tissues within 30 minutes; skeletal muscles within 1 hour) using sterile equipment, as recommended previously (Morrison *et al.*, 2014).

All samples were minced with scissors and snap frozen in liquid nitrogen before being stored at -80°C pending RNA and protein extraction. For the measurement of myostatin protein in serum, blood samples (~10ml) were collected into plain tubes (BD Vacutainer) at exsanguination and allowed to clot before centrifuging at 2000g for 10 minutes at 4°C. Serum was collected and stored at -20°C pending myostatin protein measurement by ELISA.

Table 6.1. Phenotypic descriptors for the animals used in this study. Body condition score (BCS), age and gender are indicated.

Horse ID		Gender	Age (years)	BCS (/9)	Breed type
Lean	1	Gelding	8	3	Welsh Pony
	2	Mare	5	3.8	Welsh Pony
	3	Gelding	15	2.5	Sport horse
	4	Gelding	6	3	Sport horse
	5	Mare	10	3.5	Sport horse
	6	Gelding	4	2.6	Sport horse
Obese	7	Mare	6	7	Cob horse
	8	Mare	13	8	Cob pony
	9	Mare	5	7.3	Sport horse
	10	Gelding	15	8.2	Cob pony
	11	Gelding	7	7.9	Cob pony
	12	Mare	15	7.8	Cob horse

RNA extraction

Total RNA was extracted from all frozen tissue samples using TRIzol reagent (Invitrogen, Paisley, UK), in accordance with the manufacturers protocol. RNA concentration and purity was quantified spectrophotometrically (Eppendorf Biophotometer, Hamburg, Germany) and all optical density A260/280 ratios were within acceptable ranges (1.7-2.0). Reverse transcription (RT) was carried out in a 10µl final reaction volume containing 0.5µg RNA using an iScript cDNA synthesis kit (Bio-Rad Hemel Hempstead, UK). The resulting cDNA was diluted at 1:4 and used as a template for real-time PCR analysis.

Table 6.2: Specific anatomical descriptors used to locate the tissue collection points for the 5 regionally discrete adipose tissue depots and 4 skeletal muscles sampled from horses used in the current study. Where relevant, tissues were collected from the left side following carcass-splitting.

Tissue		Anatomical descriptors for sample sites
Adipose tissues	Ventro-abdominal	~3cm^3, collected from the left split-carcass midline at a point equidistant between xiphisternum and pubis.
	Epicardial	~2cm^3 from the coronary groove and overlying the left coronary artery
	Omental	Variable area of omentum, sufficient to harvest ~2cm^3 of adipose tissue, from a region adjoining the greater curvature of the stomach and bearing visible adipose.
	Crest	~3cm^3 from the left split-carcass at the deepest part of the crest, midway between wither and poll extremities.
	Tailhead	~2cm3 from the subcutaneous adipose tissue overlying the gluteal muscles of the left carcass.
Skeletal muscles	*Rectus abdominis,*	~3cm^3, collected from the left split-carcass midline at a point equidistant between xiphisternum and pubis.
	Longus colli,	~3cm^3, from its severed cranial extremity in the left split-carcass.
	Pectoralis transversus	~3cm^3, collected from the exposed midline section of the muscle at a point just ~10cm caudal to the thoracic inlet.
	Pectoralis profoundus	~3cm^3, collected from the exposed midline section of the muscle, immediately deep to the collection site for *Pectoralis transversus.*

Quantitative Real-Time PCR

The expression of myostatin, ActRIIB and four housekeeping genes previously used in other studies in horses and ponies (Ahn *et al.*, 2011; Bogaert *et al.*, 2006) (GAPDH, Beta-actin, HPRT1 and RPL32) was determined in all tissues from the twelve animals. GeNorm software (GenEx, Germany) was used to assess the two 'most stably' expressed genes to be used for normalisation. Gene expression was determined by quantitative real-time PCR performed in duplicate using the Stratagene Mx3005P detection system (Agilent Technologies, California USA). Primer sequences for all four housekeeping genes were obtained from previously published data (HPRT1 and RPL32, GAPDH (Bogaert *et al.*, 2006), and beta-actin (Ahn *et al.*, 2011)) and 100% homology was confirmed by performing a basic local alignment search tool (BLAST). Primer and Taqman probe sequences for myostatin and ActRIIB, were designed using Beacon Designer (Premier Biosoft, USA). All primers were designed to be exon-spanning. All primer/probe sets were purchased from Eurogentec (Belgium) (Table 6.3). Serial dilutions of pooled cDNA were used to calculate Taqman primer efficiencies. The PCR cycling conditions (using Taqman probe and primers) for myostatin and ActRIIB were as follows: 10 minutes at 95°C, followed by 40 cycles of 30 seconds at 95°C, 1 minute at 55°C and 1 minute at 72°C. Cycling conditions for housekeeping genes (using SYBR green method) were as follows: 10 minutes at 95°C followed by 40 cycles of 15 seconds at 95°C and 30 seconds at 60°C and ending with, 1 minute at 95°C, 30 seconds at 55°C and 30 seconds at 95°C.

Relative gene expression was calculated using the comparative Ct method ($2^{-\Delta Ct}$) (Schmittgen and Livak, 2008). All gene expression data were normalised to 2 internal housekeeping genes.

Table 6.3. Nucleotide sequences of primers and probes used in the current study.

Gene	Primer	Sequence	Amplification efficiency
Beta-actin	Forward	GGACCTGACGGACTACCTC	97%
	Reverse	CACGCACGATTTCCCTCTC	
HPRT1	Forward	GGCAAAACAATGCAAACCTT	94.5%
	Reverse	CAAGGGCATATCCTACGACAA	
GAPDH	Forward	CAGAACATCATCCCTGCTTC	95%
	Reverse	ATGCCTGCTTCACCACCTTC	
RPL32	Forward	AGCCATCTACTCGGCGTCA	94%
	Reverse	TCCAATGCCTCTGGGTTTC	
Myostatin	Forward	GCAGTGATGGCTCTTTGGAAG	97.9%
	Reverse	GCATTAGAAGATCAGACTCTGTAGG	
	Probe	ACCACGCGACGACGGAAACAATCAT	
ActRIIB	Forward	GCCTCGCTGTTCGGTTTGAG	92.9%
	Reverse	GGCTCCCTCAAGCACCTCAG	
	Probe	ACCGCCGTGTGCCCACCTGC	

Protein Extraction and Western Blotting

Soluble protein was extracted from frozen tissues by homogenising around 100mg of tissue in a SHE buffer (250mM sucrose, 1mM HEPES, 0.2mM EDTA) containing both phosphatase and protease inhibitor cocktails (both Sigma, Poole, Dorset, UK). Samples were centrifuged and the soluble fraction was used for determining protein concentration by the bicinchoninic acid (BCA) method (Smith *et al*., 1985).

Thirty micrograms of protein extract were separated on 10% SDS-polyacrylamide gels under reducing conditions and proteins were transferred onto nitrocellulose membranes (Hybond-C Extra, Amersham Bioscience, Buckinghamshire, UK) by electroblotting (Turbo transfer, BioRad). Membranes were stained in Ponceau S reversible stain to verify the success of protein transfer and then blocked for 1 hour in 5% BSA in Tris-buffered saline containing 0.1% Tween 20 (TBST). Commercially available primary antibodies (listed below) were used and were selected on the basis that they were listed as having 'equine cross-reactivity'. They were added at

the following concentrations: myostatin precursor (MSTN), 1:2500 [ab98337 Abcam, Cambridge, UK], myostatin receptor (ACTRIIB), 1:2000 [sc-25453 Santa Cruz, Dallas, Texas, USA] and the serine/threonine Akt (AKT), 1:3000 [#9272 Cell Signalling, Danvers, MA, USA]) in blocking buffer and incubated overnight at 4°C. The myostatin antibody detected the precursor form of the protein (~43kDa). The membranes were washed and then incubated for 1 hour with a secondary antibody (Cell Signalling) at appropriate concentrations. Signals were detected by chemiluminescence using a SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, US) and visualised and quantified on a Molecular Imager ChemiDoc XRS+ System (Bio-Rad). The results were normalised to the value of AKT. To ensure the reliability of data, western blots for myostatin and ActRIIB proteins in skeletal muscles were repeated three times and average densitometric values were calculated.

Myostatin ELISA

Mature myostatin protein concentration was measured using a commercially available ELISA kit (R&D Systems, Catalogue number: DGDF80) which has been validated for use on 'equine serum and plasma samples' by R&D systems (www.rndsystems.com/pdf/DGDF80.pdf) and employs the quantitative sandwich enzyme immunoassay technique to measure mature myostatin concentration. Prior to running the plate, samples were subjected to acid activation and neutralisation to remove the pro-peptide from myostatin. Samples were run in duplicate and the ELISA was run according to the manufacturer's protocol. Myostatin concentration (ng/ml) was calculated from a standard curve.

Statistical Analysis

Statistical analyses were performed using STATA version 12.1 (StataCorp, Texas). Non-parametric, analytical methods were employed to assess gene and protein expression data. The Kruskal Wallis test was used to assess differences in gene and protein expression, along with differences in circulating myostatin concentrations between lean and obese animals. Associations between circulating myostatin and

myostatin plus ActRIIB gene and protein expression were analysed using linear regression. Significance was set at $p < 0.05$.

6.4 Results

Animals

The animals used in this study were slaughtered in a commercial abattoir for non-research purposes. The BCS in the population fell within the commercial range and lean and obese BCS categories were selected to give clear differences in body fat content (Dugdale *et al.*, 2012).

Myostatin and ActRIIB gene expression

Myostatin gene expression across all skeletal muscles studied was significantly greater in the obese animals compared to the lean animals ($p < 0.05$) (Figure 6.1 and Table 6.4). In contrast, ActRIIB gene expression was significantly lower in obese animals in three out of the four skeletal muscles studied ($p < 0.05$) (Figure 6.1 and Table 6.4). While myostatin gene expression was considerably lower in adipose tissues in comparison to skeletal muscles, expression was significantly greater in the crest fat of obese animals compared with lean animals ($p < 0.05$) (Figure 6.2 and Table 6.5). No difference was observed between lean and obese animals for ActRIIB gene expression in adipose tissues (Figure 6.2 and Table 6.5).

Table 6.4: Median and interquartile range (IQR) for myostatin and ActRIIB gene expression (normalised data) in skeletal muscles of lean and obese horses and ponies.

Muscle	Lean		Obese		P value
Myostatin	**Median**	**IQR**	**Median**	**IQR**	
Rectus abdominis	0.0007	0.0010	0.003	0.001	0.04
Longus colli	0.0009	0.0004	0.003	0.003	0.02
Pectoralis profoundus	0.0003	0.0006	0.002	0.001	0.03
Pectoralis transversus	0.0004	0.0008	0.004	0.004	0.01
ActRIIB					
Rectus abdominis	0.003	0.002	0.001	0.001	0.15
Longus colli	0.004	0.005	0.001	0.0009	0.03
Pectoralis profoundus	0.001	0.002	0.0004	0.0006	0.04
Pectoralis transversus	0.0003	0.0005	0.0001	0.0001	0.03
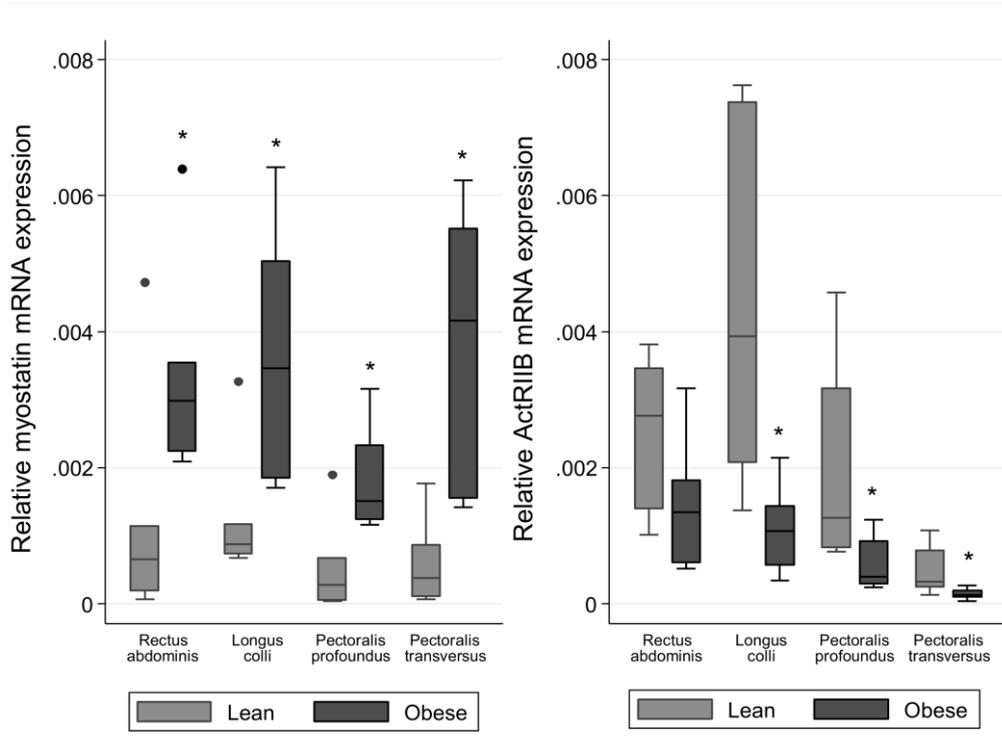
Figure 6.1: Myostatin and ActRIIB gene expression in skeletal muscles of lean and obese horses and ponies. *denotes where values differ significantly (p < 0.05) from lean group. *n* = 6/group.
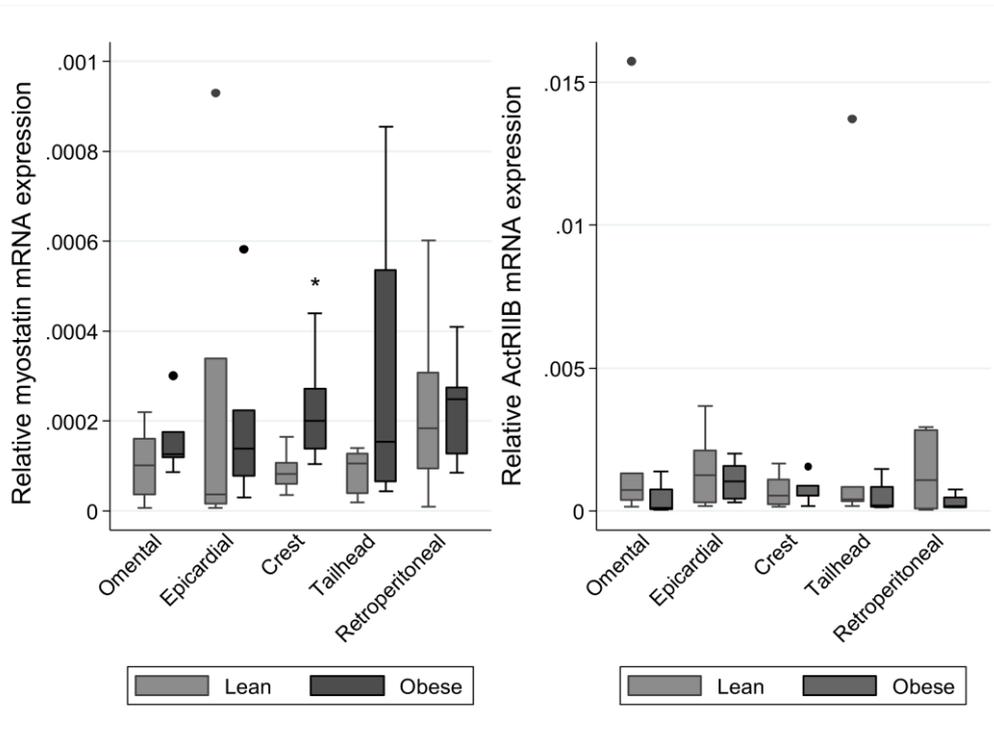
Figure 6.2: Myostatin and ActRIIB gene expression in adipose tissues of lean and obese horses and ponies. *n* = 6/group.

Table 6.5: Median and interquartile range (IQR) for myostatin and ActRIIB gene expression (normalised data) in adipose tissues of lean and obese horses and ponies.

Depot	Lean		Obese		P value
Myostatin	**Median**	**IQR**	**Median**	**IQR**	
Omental	0.0001	0.0001	0.0001	0.00006	0.42
Epicardial	0.00004	0.0003	0.0001	0.0001	0.34
Crest	0.00008	0.00005	0.0002	0.0001	0.02
Tailhead	0.0001	0.00009	0.0001	0.0004	0.33
Retroperitoneal	0.0002	0.0002	0.0002	0.0001	0.75
ActRIIB					
Omental	0.0007	0.0009	0.00009	0.0007	0.11
Epicardial	0.001	0.002	0.001	0.001	0.87
Crest	0.0005	0.0009	0.0005	0.0004	0.86
Tailhead	0.0004	0.0005	0.0002	0.0007	0.20
Retroperitoneal	0.001	0.003	0.0002	0.0003	0.52

Myostatin and ActRIIB protein expression

Myostatin precursor protein expression was quantified across the four skeletal muscles by western blotting in three separate experiments. Although the average densitometric data showed no significant differences between lean and obese animals for any skeletal muscle studied (*Pectoralis transversus*, p = 0.75; *Longus colli*, p = 0.42; *Rectus abdominis*, p = 0.26; *Pectoralis profoundus*, p = 0.08), obese animals tended to have greater myostatin protein expression compared with lean animals (Figure 6.3). Similarly ActRIIB protein expression was quantified across the four skeletal muscles by western blotting in three separate experiments. No significant differences in protein expression between lean and obese animals were observed in the skeletal muscles studied (Figure 6.4).
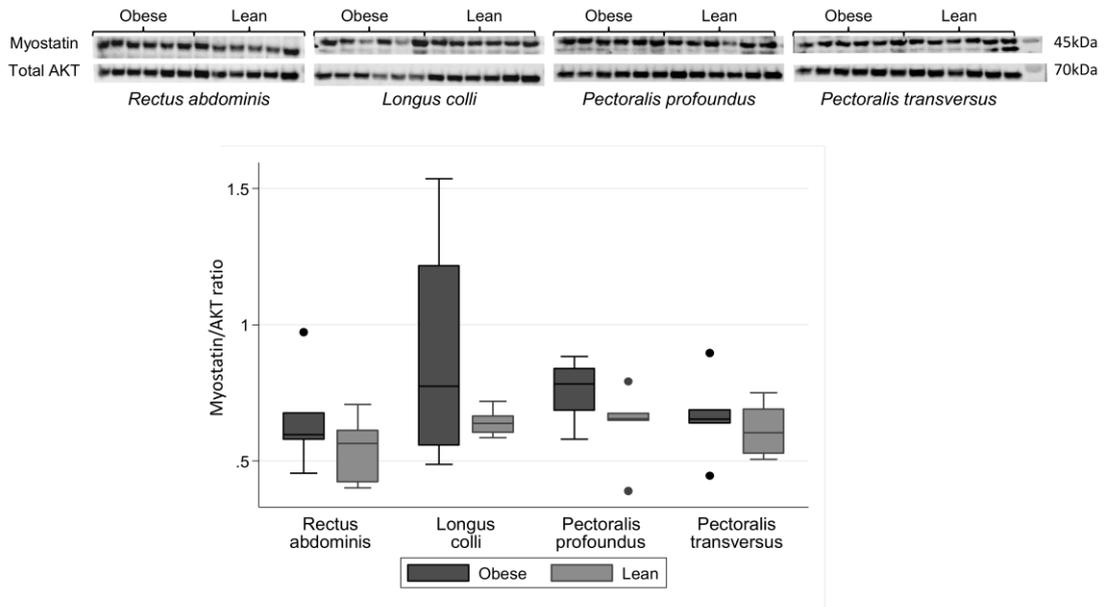
Figure 6.3: Protein expression of myostatin precursor protein in skeletal muscles of lean and obese horses and ponies. *n* = 6/group.
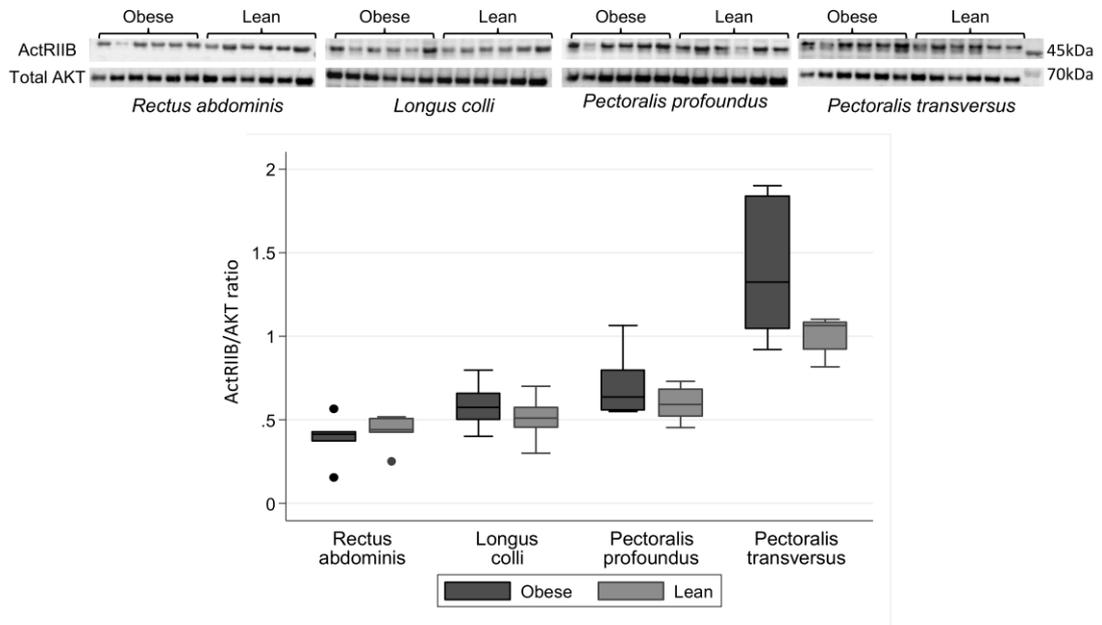
Figure 6.4: Protein expression of ActRIIB protein in skeletal muscles of lean and obese horses and ponies. *n* = 6/group.

Due to the differences observed at the gene level in crest fat for myostatin, we sought to identify whether these differences were translated into differences at the protein level for both myostatin and ActRIIB. However, Figure 6.5 clearly demonstrates there was no protein detected in either the lean or obese animals for myostatin precursor protein or ActRIIB.

Figure 6.5: Protein expression of myostatin precursor and ActRIIB proteins in crest fat of lean and obese horses and ponies.

Circulating myostatin concentration

Circulating, mature myostatin protein was detected in serum samples from all animals studied. Overall, mean serum myostatin concentration was 6.99ng/ml ($\pm$ 3.10); the range was 2.72ng/ml to 11.40ng/ml. The mean values for the lean and obese groups were 4.98ng/ml ($\pm$ 2.71) and 9.00ng/ml ($\pm$ 2.04), respectively. Kruskal Wallis test revealed significant differences between lean and obese animals ($p < 0.05$) (Figure 6.6). Univariate analysis revealed positive associations between serum myostatin concentrations and myostatin mRNA expression in skeletal muscle for all muscles studied, irrespective of whether muscles were considered independently or collectively (average $R^2 = 0.58$, $p < 0.05$) (Table 6.6). Associations between myostatin serum concentrations and the magnitude of muscle myostatin protein expression were weaker than those recorded for gene expression (average $R^2 = 0.28$). Only myostatin protein expression in *Pectoralis profoundus* had a significant association with serum myostatin concentration ($R^2 = 0.50$, $p = 0.01$) (Table 6.6). No associations were identified between myostatin serum concentration and ActRIIB gene or protein expression (Table 6.6).
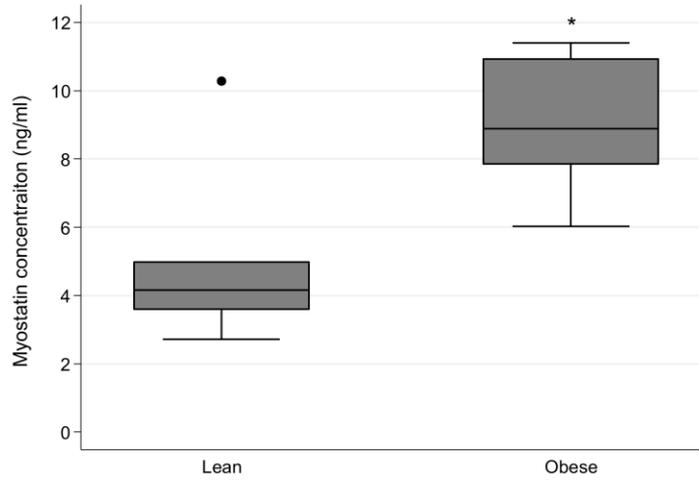
Figure 6.6: Circulating concentrations of myostatin protein in lean and obese horses and ponies. *denotes where values differ significantly (p < 0.05) from lean group. *n* = 6/group.

Table 6.6: Univariate regression analysis results for myostatin ELISA data. Myostatin concentration was the outcome variable and myostatin and ActRIIB gene and protein expression data for the individual skeletal muscles was offered as explanatory variables.

Variable		Coefficient	Adjusted R^2	95% CI	P value
Myostatin gene expression	Rectus abdominis	1287.62	0.65	622.89 to 1952.35	0.002
	Longus colli	1179.68	0.54	409.69 to 1949.68	0.007
	Pectoralis profoundus	2811.07	0.78	1761.03 to 3861.11	< 0.001
	Pectoralis transversus	836.77	0.36	57.41 to 1616.13	0.04
ActRIIB gene expression	Rectus abdominis	-201.53	0.01	-2083.14 to 1680.08	0.82
	Longus colli	-583.66	0.23	-1336.72 to 169.40	0.12
	Pectoralis profoundus	-1332.49	0.32	-2710.86 to 45.89	0.06
	Pectoralis transversus	-5812.13	0.33	-12115.38 to 491.11	0.07
Myostatin protein expression	Rectus abdominis	7.28	0.13	-6.17 to 20.74	0.26
	Longus colli	5.36	0.26	-0.95 to 11.67	0.09
	Pectoralis profoundus	16.40	0.50	4.81 to 27.99	0.01
	Pectoralis transversus	12.58	0.24	-3.39 to 28.54	0.11
ActRIIB protein expression	Rectus abdominis	-8.91	0.15	-23.98 to 6.15	0.22
	Longus colli	1.43	0.004	-13.84 to 16.71	0.84
	Pectoralis profoundus	10.35	0.25	-2.37 to 23.08	0.10
	Pectoralis transversus	6.80	0.17	-3.62 to 17,23	0.17

6.5 Discussion

This study presents preliminary data which provide the first indication of a possible association between BCS and myostatin gene expression and secretion in horses and ponies.

The increased gene expression of myostatin in skeletal muscles of obese animals is in agreement with similar data for mice where myostatin mRNA levels were significantly greater in *tibialis anterior* muscle in *ob/ob* mice compared to wild-type mice (Allen *et al*., 2008). In that study, the expression of ActRIIB was not different

between lean and obese animals for skeletal muscle, whereas in the current study, ActRIIB mRNA was significantly down-regulated in three out of the four skeletal muscles studied. This may be suggestive of some element of negative feedback regulation between myostatin and ActRIIB.

Increased expression of myostatin protein has been identified in the *vastus lateralis* muscle from extremely obese human subjects (BMI ≥40kg/m^2) (Hittel *et al*., 2009). Perhaps the lack of statistical significance observed in the current study may be due to absolute differences in body fat content between species. Obese horses and ponies were found to have up to 30% body fat recorded in a previous study (Argo *et al*., 2012), which is considerably lower than the body fat content of morbidly obese humans which was found to average 48.5% (Vijgen *et al*., 2011). The finding of altered myostatin and ActRIIB mRNA expression in muscle without parallel changes in protein expression has previously been shown (Baumann AP, 2003; Smith *et al*., 2010). It is known that the mRNA expression of a particular gene is not always predictive of protein expression, and the correlation between the two can vary significantly (Guo *et al*., 2008b). There are several possible explanations for the differences between the gene and protein expression including variation in protein half-lives, complex post-transcriptional mechanisms, and different sensitivities in methodologies for detecting mRNA and protein expressions (Greenbaum *et al*., 2003). Furthermore, the lack of differences between lean and obese animals observed at the protein level may be due to an increased secretion of myostatin protein from skeletal muscle, although the kinetics of myostatin secretion has yet to be explored for the horse.

This preliminary study examined both myostatin gene and protein expression in skeletal muscles. These techniques have not been performed previously in abattoir derived equine material in lean and obese animals, thus there was a total absence of prior data to inform study design. Therefore it was not possible to perform *a priori* sample size calculations since the magnitude of differences in expression on which to base such calculations were unknown. Differences in gene expression were demonstrated between lean and obese animals and *post hoc* power

calculations confirmed that in this case the study had sufficient power (82%). However in the case of protein expression, no differences were observed at the P < 0.05 level. Two hypotheses for this result may be formulated – firstly there was a true difference in protein expression but the sample size was insufficient to detect it at a P value < 0.05 or secondly no difference existed, which may be due to reasons outlined earlier.   A *post hoc* sample size calculation was performed based on the protein expression data from *pectoralis profoundus* muscle. This suggested that whilst a mean difference of 0.123 (2 tailed P = 0.114) in myostatin protein expression was observed between groups, the power of the study (based on the mean difference and P value obtained) was only 35% and a sample size of 21 animals per group would likely be required to achieve a statistical significance of P < 0.05 with 80% power. The validity and utility of *post hoc* power calculations are widely challenged by many biostatisticians e.g. (Goodman et al., 1994), therefore whilst the results of post hoc power calculations should not be taken definitively regarding sample sizes, it does indicate that a larger sample size may be required for future studies.

Circulating concentrations of myostatin were significantly higher in obese than in lean animals in the current study. This is consistent with previous observations of increased myostatin secretion from myotubes derived from muscle of extremely obese humans (Hittel *et al*., 2009). In the current study there was one clear outlier in our lean group of animals for both circulating concentrations and mRNA expression of myostatin which upon investigation was found to be the Welsh pony mare (Horse 2; Table 1). It could be speculated that this may be indicative of an increased propensity towards obesity based on a finding from a murine study in which obesity-susceptible strain of mice (C57BL/6) had increased mRNA expression of myostatin in skeletal muscle compared to an obesity-resistant strain of mice, SWR/J (Lyons *et al*., 2010).

Myostatin gene expression was generally low in adipose tissues but was significantly higher in crest fat from obese than lean animals. Increased fat deposition in this subcutaneous fat depot along the nuchal crest of the neck in

horses and ponies has been associated with laminitis risk (Carter *et al*., 2009b), hyperinsulinemia (Carter *et al*., 2009a), and has been proposed to be an important source of pro-inflammatory cytokines (Bruynsteen *et al*., 2013). Differences in myostatin gene expression between crest fat samples from lean and obese animals were not reflected in protein expression in this tissue. Data indicated that neither myostatin precursor nor ActRIIB proteins were detectable in the crest fat of either lean or obese animals by the methods used in the current study. This is in agreement with data presented in an earlier study which similarly failed to detect either myostatin precursor or ActRIIB proteins in crest and other adipose tissues from lean (BCS < 4/9) animals (Morrison *et al*., 2014).

6.6 Conclusion

These preliminary data offer some evidence that the 'myostatin system' may differ at both the gene and protein level in lean and obese horses and ponies. Further work is needed, and these findings now provide the basis for future prospective studies in horses and ponies to explore the previous speculation from human studies (Hittel *et al*., 2009) that circulating myostatin levels and/or associated factors might act as biological marker(s) for metabolic conditions including obesity.

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Chapter 7

Expression of perilipin and hormone-sensitive lipase in adipose tissues from lean and obese horses and ponies

7.1 Abstract

Adipose tissue is a highly active endocrine organ, specialised in the storage of excess energy as triglycerides in intracellular lipid droplets within adipocytes. Lipolysis is a key physiological process by which stored triglycerides are catabolised in a stepwise manner by the actions of several proteins to produce fatty acids and glycerol. Obesity in other species is associated with increases in adipocyte area and perturbations in lipolysis. In addition, the expression of key lipolytic proteins is altered in an obese state. A high prevalence of obesity continues to be reported in the UK leisure based population of horses and ponies; however, whether lipolytic proteins are altered in an obese state is not currently know. Therefore the current study was designed to characterise the expression of two key lipolytic proteins, hormone-sensitive lipase (HSL) and the lipid droplet associated protein, perilipin 1 (PLIN1) in a range of adipose depots (retroperitoneal, omental, crest and tailhead) from lean and obese horses and ponies. Adipocyte area was significantly increased in obese animals for all depots except for epicardial WAT. The ratio of lipid:extracted protein was strongly associated with adipocyte area for all depots except epicardial WAT. Furthermore, PLIN1 and HSL protein expression was significantly lower in obese animals for retroperitoneal fat in both internatant (largely cytosolic) and fat cake (lipid droplet associated) protein fractions, whilst fewer differences were noted for the other depots. In conclusion, these results indicate that obesity in horses and ponies is associated with changes in the expression of lipolytic proteins in certain depots which may indicate clear functional differences between regional adipose depots in the horse.

7.2 Introduction

The high prevalence of obesity in our leisure population of horses and ponies has potential negative health implications, including an increased risk of developing debilitating and metabolically deleterious conditions, including laminitis and insulin dysregulation (Carter *et al*., 2009). Such welfare considerations have warranted consideration of the key regulators of energy balance and how they might be altered in obesity. White adipose tissue (WAT) is a highly metabolically active tissue, specialised in the storage of excess energy as triglycerides in intracellular lipid droplets within adipocytes (Brown, 2001). These stored triglycerides are mobilised to supply the body with energy during times of negative energy balance (e.g. during fasting, exercise or when natural foods are sparse) through the delivery of fatty acids in the plasma to skeletal muscle and liver to be metabolised. An imbalance between fat deposition and mobilisation can lead to metabolic disorders associated with obesity in other species such as diabetes, fatty liver disease and dyslipidaemia (Dandona *et al*., 2005).

The catabolism of stored triglycerides into labile fatty acids, lipolysis, is a highly regulated process, promoted by catecholamines such as noradrenaline and adrenaline and inhibited by the actions of insulin. The hydrolysis of triacylglycerol (TAG) is catalysed in a step-wise manner by the action of adipose triacylglycerol lipase (ATGL), hormone-sensitive lipase (HSL) and monoglyceride lipase. Hormone-sensitive lipase activity is considered to be a major determinant of the maximal lipolytic capacity of human fat cells (Large *et al*., 1998) and the expression of HSL protein in adipocytes is reduced in the obese state (Jocken *et al*., 2007; Ray *et al*., 2009). Furthermore, a null mutation in the HSL gene has been associated with an increased risk of type 2 diabetes development in humans (Albert *et al*., 2014). These data confirm HSL as a key protein in the normal control of lipid and glucose metabolism.

The requirement for the strict regulation of lipolysis is highlighted by specific functional disorders within the pathway. Obesity is characterised by the expansion

of adipose tissue by both hypertrophy and hyperplasia of adipocytes; however continued nutrient excess beyond the adipocyte expandability limits will result in a pathogenic state (Rutkowski *et al.*, 2015). In Man, obesity is associated with increased concentrations of plasma fatty acids (Arner, 2005); an effect which may be partly mediated by correspondingly increased concentrations of the circulating inflammatory factor tumour necrosis factor alpha (TNF-α), which activates the extracellular signal-related kinase pathway, elevating intracellular cAMP levels, leading to an enhanced basal lipolytic rate (Zhang *et al.*, 2002). In addition to this, increased visceral fat deposition may also contribute to elevated circulating fatty acids as visceral fat is more lipolytically active than subcutaneous fat (Van Harmelen *et al.*, 1997). Increased circulating fatty acid concentrations are known to promote ectopic lipid deposition and contribute to the development of insulin resistance in skeletal muscle, liver and other tissues (Boden *et al.*, 2001; Boden and Shulman, 2002). Furthermore, obesity has been associated with a blunted response to catecholamine-stimulated lipolysis (Langin *et al.*, 2005), which has been suggested to be a mechanism to reduce the negative effects of insulin resistance by lowering fatty acid flow from adipose tissue (Jocken *et al.*, 2007).

For all species, lipid droplets residing in adipocytes are surrounded by a phospholipid monolayer which is coated with proteins. One of the key proteins coating the lipid droplet is perilipin (PLIN1), the founding member of the perilipin protein family (Bickel *et al.*, 2009). Under basal conditions, PLIN1 prevents lipases from accessing the enclosed triglycerides to initiate lipolysis. PLIN1 may also function in controlling lipid droplet expansion through an interaction with Fat-specific protein (FSP) 27 (Fsp27) (Sun *et al.*, 2013). Catecholamine signalling through β-adrenergic receptors increases cellular cAMP concentrations. This in turn activates Protein Kinase A (PKA) which causes phosphorylation of PLIN1 and HSL, whilst the translocation of phosphorylated HSL from the cytoplasm to the lipid droplet surface initiates lipolysis. It has been demonstrated that PKA mediated phosphorylation of PLIN1 is not essential for HSL translocation but it is essential for lipid droplet interactions between PLIN1 and HSL to allow lipolysis to proceed (Miyoshi *et al.*, 2006).

PLIN1 has been extensively studied in both man and rodents and its close functional association with HSL has established that it is a critically important protein in lipid and glucose homeostasis. PLIN1 knock-out (KO) mice are phenotypically characterised as having increased rates of basal lipolysis and attenuated catecholamine stimulated lipolytic responses, suggesting that PLIN1 is required for maximal lipolytic activity through a direct interaction with HSL at the lipid droplet surface (Zhang *et al.*, 2002). Furthermore, a resistance to diet-induced obesity has also been observed in PLIN1 KO mice; however they have been shown to develop insulin resistance with ageing (Tansey *et al.*, 2001). In humans, PLIN1 protein expression is down-regulated in obesity (Ray *et al.*, 2009) and a number of polymorphisms of the PLIN1 gene have been identified and associated with both obesity risk and weight-loss resistance (Corella *et al.*, 2005).

To date, there are few studies describing the expression of lipolysis-related proteins in horses and ponies. It has previously been demonstrated that in horses, PLIN1 is almost exclusively expressed in WAT (Morrison *et al.*, 2014). Furthermore, *ex vivo in vitro* assays using equine adipocytes demonstrated that lipolysis rates were significantly greater for ponies than horses when stimulated with adenosine deaminase and norepinephrine, although inhibition of lipolysis by insulin was comparable between horses and ponies (Breidenbach *et al.*, 1999).

The current study was therefore designed to characterise adipocyte area as well as the gene and protein expression of PLIN1 and HSL across a range of adipose depots in lean and obese horses and ponies, in order to establish whether these proteins are altered in states of positive energy balance.

7.3 Materials and methods

Animals and tissue collection

Tissues from six lean (body condition score [BCS] /9 = 3.07 ±0.50, where 1 = emaciated and 9 = obese (Kohnke, 1992) and six obese (BCS /9 = 7.7 ± 0.46) mature

(age range 5-15 years), mixed breed horses and ponies were obtained *post-mortem*. All animals were in good general health and were euthanased for reasons unrelated to this study (Table 7.1). The horses were slaughtered in a commercial abattoir (LJ Potters, Taunton, Somerset) in accordance with EU legislations EC 852/2004, 853/2004 and 854/2004 on several dates between March 2013 and January 2014. *Ante-mortem* data collection included BCS (/9, (Kohnke, 1992)), breed type, gender, estimated withers height and age. For assessment of BCS, six areas of the body (neck, withers, loin, tailhead, ribs and shoulder) are assigned a number from 1 (emaciated) to 9 (obese) based on detailed descriptors. The average of these six numbers is calculated and this number equates to the final BCS score for the animal.

Table 7.1: Phenotypic descriptors for the animals used in this study. Body condition score (BCS), age and gender are indicated. Breed types are as denoted in animal passports and/or confirmed by visual inspection.

	Horse ID	Gender	Age (years)	BCS (/9)	Breed type
Lean	1	Gelding	8	3	Welsh Pony
	2	Mare	5	3.8	Welsh Pony
	3	Gelding	15	2.5	Sport horse
	4	Gelding	6	3	Sport horse
	5	Mare	10	3.5	Sport horse
	6	Gelding	4	2.6	Sport horse
Obese	7	Mare	6	7	Cob horse
	8	Mare	13	8	Cob pony
	9	Mare	5	7.3	Sport horse
	10	Gelding	15	8.2	Cob pony
	11	Gelding	7	7.9	Cob pony
	12	Mare	15	7.8	Cob horse

To evaluate the expression of PLIN1 and HSL, a total of five anatomically-discrete adipose depots were sampled. Strict anatomical descriptors were used to ensure that tissue samples were collected from the same site in each animal (Table 7.2). Tissue samples were obtained as rapidly as possible *post-mortem* (within 30 minutes) using sterile equipment, as recommended previously (Morrison *et al.*, 2014). For the measurement of adipocyte area, a section of the adipose tissue sample ($\sim$1cm^3) was fixed by placing it in 4% paraformaldehyde prior to haematoxylin and eosin (H&E) staining. The remainder of each sample was chopped finely with scissors, packed in a tinfoil fold, snap frozen in liquid nitrogen and stored at -80°C pending RNA and protein extraction.

Table 7.2: Specific anatomical descriptors used to locate the tissue collection points for the 5 regionally discrete adipose tissue depots sampled from horses and ponies used in the current study. Approximate target sample sizes are given. Where relevant, tissues were collected from the left side following carcass-splitting.

Depot	Anatomical descriptors for sample sites
Ventro-abdominal retroperitoneal	$\sim$3cm^3, collected from the left split-carcass midline at a point equidistant between xiphisternum and pubis.
Epicardial	$\sim$2cm^3 from the coronary groove and overlying the left coronary artery
Omental	Variable area of omentum, sufficient to harvest $\sim$2cm^3 of adipose tissue, from a region adjoining the greater curvature of the stomach and bearing visible adipose.
Crest	$\sim$3cm^3 from the left split-carcass at the deepest part of the crest, midway between wither and poll extremities.
Tailhead	$\sim$2cm3 from the subcutaneous adipose tissue overlying the gluteal muscles of the left carcass.

Analysis of adipocyte area

Fixed adipose tissues were dehydrated, cleaned and embedded in paraffin wax prior to sectioning (5µm) and staining with haematoxylin and eosin. Digital photographic images were collected using a microscope (Nikon 026435, Mason

Microscopy) and digital camera (Nikon 7420364, Japan). On initial microscopic appraisal, clear differences in adipocyte size were apparent between samples collected from lean and obese animals. Therefore, for the purposes of data analysis, nine non-overlapping images were taken from each depot at 20 x magnification in the lean horses and 10 x magnifications in the obese horses. Adipocyte area (μm^2) was calculated using ImageJ software (National Institutes of Health, USA) by outlining the perimeter of each intact adipocyte in the nine fields of view per depot (average of 225 adipocytes measured per depot per horse). Areas were corrected for differences in image magnification between lean and obese animals.

RNA extraction

Total RNA was extracted from all frozen tissue samples using TRIzol reagent (Invitrogen, Paisley, UK), in accordance with the manufacturer's protocol. RNA concentration and purity was quantified spectrophotometrically (Eppendorf Biophotometer, Hamburg, Germany) and all optical density A260/280 ratios were within acceptable ranges (1.7-2.0). Reverse transcription (RT) was carried out in a 10μl final reaction volume containing 0.5μg RNA using an iScript cDNA synthesis kit (Bio-Rad Hemel Hempstead, UK). The resulting cDNA was diluted at 1:4 and used as a template for real-time PCR analysis.

Quantitative Real-Time PCR

The expression of PLIN1 and four housekeeping genes previously used in other equine studies (Morrison *et al.*, 2014) (GAPDH, Beta-actin, HPRT1 and RPL32) was determined in all tissues from the twelve animals. GeNorm software (GenEx, Germany) was used to assess the two 'most stably' expressed genes to be used for normalisation. Gene expression was determined by quantitative real-time PCR performed in duplicate using the Stratagene Mx3005P detection system (Agilent Technologies, California USA). Primer sequences for all four housekeeping genes were obtained from previously published data (HPRT1 and RPL32, GAPDH (Bogaert *et al*., 2006), and beta-actin (Ahn *et al*., 2011)) and 100% homology was confirmed by performing a basic local alignment search tool (BLAST). Primer and Taqman

probe sequences for PLIN1were designed using Beacon Designer (Premier Biosoft, USA). All primers were designed to be exon-spanning. All primer/probe sets were purchased from Eurogentec (Belgium). Full details of primer/probe sets have been published (Morrison *et al.*, 2014). Serial dilutions of pooled cDNA were used to calculate Taqman primer efficiencies. The PCR cycling conditions (using Taqman probe and primers) for PLIN1was as follows: 10 minutes at 95°C, followed by 40 cycles of 30 seconds at 95°C, 1 minute at 55°C and 1 minute at 72°C. Cycling conditions for housekeeping genes (using SYBR green method) were as follows: 10 minutes at 95°C followed by 40 cycles of 15 seconds at 95°C and 30 seconds at 60°C and ending with, 1 minute at 95°C, 30 seconds at 55°C and 30 seconds at 95°C.

Relative gene expression was calculated using the comparative Ct method ($2^{-\Delta Ct}$) (Schmittgen and Livak, 2008). All gene expression data were normalised to 2 internal housekeeping genes.

Protein Extraction and Western Blotting

Adipose tissue protein was extracted from two discrete fractions within each centrifuged tissue homogenate: internatant (largely 'fat-free' fraction) and fat cake (assumed to comprise of lipids largely originating from adipocyte lipid droplets). Briefly, frozen tissues (~200mg) were homogenised in a lysis buffer (1µl/mg frozen tissue; 250mM sucrose, 1mM HEPES, 0.2mM EDTA) containing both phosphatase and protease inhibitor cocktails (both Sigma, Poole, Dorset, UK). Centrifugation (12,000g for 15 minutes at 4°C) separated the internatant and fat cake fractions. The internatant fraction was removed and kept on ice pending protein concentration analysis. The proteins present in the fat cake fraction were extracted twice in succession by the addition of a second lysis buffer containing 125mM Tris buffer with 5% SDS, 20% glycerol and protease and phosphatase inhibitors (Ray *et al.*, 2009). Protein concentrations in both fractions (internatant and fat cake) were measured by bicinchoninic acid (BCA) assay (Pierce, Rockford, IL, US). In order to demonstrate any associations between lipid:protein ratio and adipocyte area within adipose tissue depots, data describing the individual animal ratio for end lipid weight (lipid remaining after protein extractions) to the extracted protein mass (fat

cake, internatant and total) per mg WAT were regressed on the mean adipocyte area recorded for that individual, for all study animals.

Protein samples corresponding to 30μg (internatant) or 10μg (fat cake) for comparison between lean and obese animals or 10μg of both internatant and fat cake proteins for comparison between fractions were separated on 10% SDS-polyacrylamide gels under reducing conditions and proteins were transferred onto nitrocellulose membrane (Hybond-C Extra, Amersham Bioscience, Buckinghamshire, UK) by electroblotting (Turbo transfer, BioRad). Membranes were stained in MemCode reversible stain (Pierce, Rockford, IL, US) to verify the success of protein transfer, signals were quantified on a Molecular Imager ChemiDoc XRS+ System (Bio-Rad), and then the membranes were blocked for 1 hour in 5% BSA in Tris-buffered saline containing 0.1% Tween 20 (TBST). Commercially available primary antibodies (listed below) were selected on the basis of known cross-reactivity with equine tissues. They were added at the following concentrations: PLIN1, 1:1500 [NBP1-56923 Novus Biologicals, Abingdon, UK] and total HSL, 1:1000 [NBP1-00879 Novus Biologicals, Abingdon, UK] in blocking buffer and incubated overnight at 4°C. The membranes were washed and then incubated for 1 hour with a secondary antibody (Cell Signalling) at appropriate concentrations. Signals were detected by chemiluminescence using a SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, US) then visualised and quantified on a Molecular Imager ChemiDoc XRS+ System (Bio-Rad). The results were normalised to the MemCode total protein stain.

Loading controls: Constraints and solutions

Initial studies which compared lean and obese animals within adipose tissue fractions rapidly identified that vinculin (a cytoskeletal protein routinely used as a loading control), demonstrated considerable variation in expression within the fat cake fraction between lean and obese animals. To ensure this was not due to unequal loading, 0.75μg bovine serum albumin (BSA) was loaded into each well of a gel and transferred to a membrane which was stained with MemCode total protein stain and the signal was quantified (ChemiDoc XRS+) (Figure 7.1) Hence, the data

clearly demonstrated that loading error (Coefficient of variation: 6.81%) was an unlikely cause of the variation in vinculin expression and strongly suggests that the use of a cytoskeletal protein may be an inappropriate control for lipid-associated proteins.
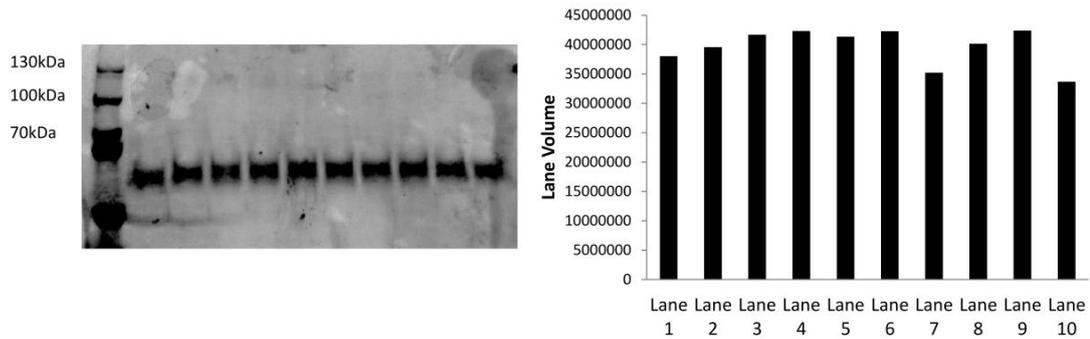
Figure 7.1: Loading variation of bovine serum albumin assessed by loading 0.75μg BSA into each lane of a gel and staining the corresponding membrane with MemCode reversible stain. The blot was imaged and densitometric signals were calculated.

In an attempt to normalise data and allow comparison between lean and obese animals within adipose tissue homogenate fractions, protein signals were normalised to the MemCode protein stain. The limitations of using established loading controls under these conditions was also encountered during early attempts to compare PLIN1 and HSL protein expression within lean and obese animals between the internatant and fat cake fractions. Vinculin protein expression again demonstrated considerable variation between the adipose tissue fractions. Irrespective of equality in protein loading, the MemCode protein stain demonstrated a greater presence of protein in the internatant fraction (Figure 7.2). In a final attempt to quantify protein signals for PLIN1 and HSL between the adipose tissue fractions, samples were spiked with BSA and membranes stained with MemCode. However, the molecular weight of BSA (~66kDa) was similar to a prominent protein present in the internatant fraction and masked the signal of interest, negating the usefulness of this approach. It was therefore concluded that as outlined above, loading variation was unlikely to explain differences between fractions.
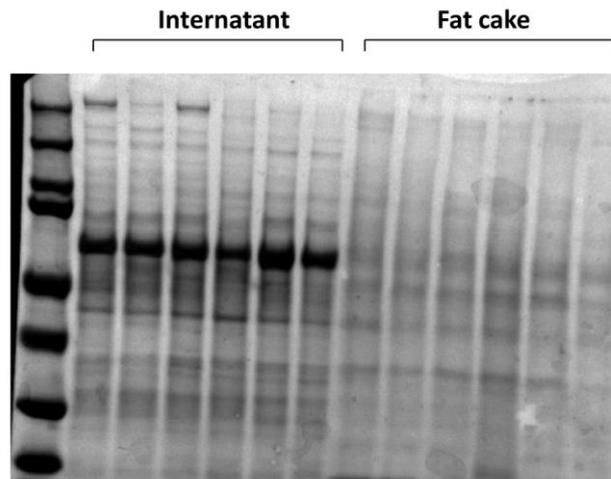
Figure 7.2: Representative MemCode reversible protein stain of internatant and fat cake protein fractions. Equal amounts of internatant and fat cake proteins (10μg) were loaded into the gel and MemCode protein stain was applied to the membrane.

Statistical analysis

Statistical analyses were performed using STATA version 12.1 (StataCorp, Texas). Non-parametric, analytical methods were employed to assess gene and protein expression data. The Kruskal Wallis test was used to assess differences in gene and protein expression, along with adipose tissue histology data between lean and obese animals. Significance was set at p <0.05.

7.4 Results

Adipocyte area

Adipocyte area was significantly greater for obese horses in 4/5 adipose depots studied (all p < 0.05, Figure 7.3, Table 7.3). For one obese animal, histological examination of the epicardial tissue sample revealed it to be largely comprised of fibroblasts. On the basis of this observation, this sample was removed from all analyses, including gene and protein expression. For retroperitoneal, crest, tailhead and omental WATs, adipocyte area was significantly greater for obese as opposed

to lean animals (Figure 7.3). Whilst obese animals did tend to have larger adipocyte areas in epicardial fat, this did not reach statistical significance (p = 0.10; Figure 7.3). Although no differences in adipocyte area between depots were noted within obese animals, median adipocyte areas were greatest in WAT derived from the retroperitoneal depot (15086μm^2; Table 7.3), whilst they were smallest for epicardial WAT (5121.60μm^2). Similarly no significant differences in adipocyte area were noted within lean animals, between depots. However, retroperitoneal WAT recovered from lean animals recorded the smallest adipocyte areas (median, 2230.65μm^2), while adipocyte area was greatest for epicardial WAT in the lean animals (3301.28μm^2).

Figure 7.4 demonstrates the distribution of adipocyte areas recorded for lean and obese animals. For the purposes of assessing differences between lean and obese animals, adipocyte areas greater than 50,000μm^2 were removed: Retroperitoneal WAT: lean animals: n = 8/2646 (0.30%) adipocyte area > 50,000μm^2 (range = 93294.88μm^2 -499879.80μm^2); obese animals: n = 22/1037 (2.12%; range = 50,710.68μm^2 -81,089.45μm^2). Tailhead WAT: lean animals: n = 5/1639 (0.31%) > 50,000μm^2 (range = 50,728μm^2 -81,435μm^2); obese animals = 51/951 (5.36%; range = 50,044μm^2 -147,707μm^2). Epicardial WAT showed a similar distribution in adipocyte area between lean and obese animals, whilst retroperitoneal fat showed the most marked difference in terms of distribution between lean and obese animals.
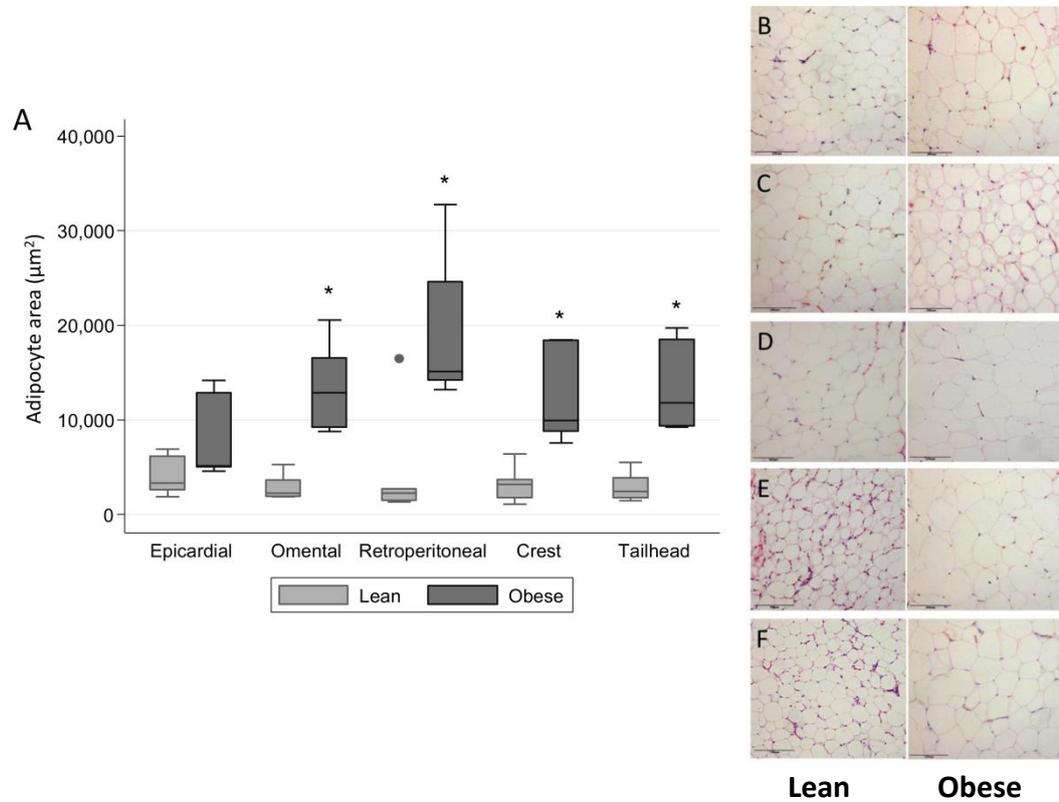
Figure 7.3. Adipocyte area in adipose tissues of lean and obese horses and ponies. The routine haematoxylin and eosin stain was applied to adipose tissue sections and adipocyte area was measured across five adipose depots in 12 animals (n = 6/group) **(A)**. Representative images are shown for all depots (Omental **(B)**, Epicardial **(C)**, Crest **(D)**, Tailhead **(E)** and Retroperitoneal **(F)**) * denotes where area is significantly different ($p < 0.05$) from corresponding lean group. Scale bar = 200μm.

Table 7.3: Median and inter-quartile range values (IQR) for adipocyte area (μm^2) and PLIN1 gene expression (normalised value) data.

Depot	Lean		Obese		P value
Adipocyte area	**Median**	**IQR**	**Median**	**IQR**	
Omental	2258.24	1724	12869.85	7383.29	< 0.001
Epicardial	3301.28	3523.99	5121.60	7908.60	0.10
Crest	3192.10	1901.31	9917.39	9607.37	< 0.001
Tailhead	2432.43	2129.40	11786.60	9174.70	< 0.001
Retroperitoneal	2230.65	1293.19	15086.00	10429.23	0.03
PLIN1 Gene expression					
Omental	0.02	0.02	0.02	0.03	0.87
Epicardial	0.03	0.07	0.02	0.01	0.20
Crest	0.07	0.04	0.04	0.04	0.05
Tailhead	0.02	0.01	0.03	0.08	1.00
Retroperitoneal	0.02	0.03	0.03	0.05	0.34
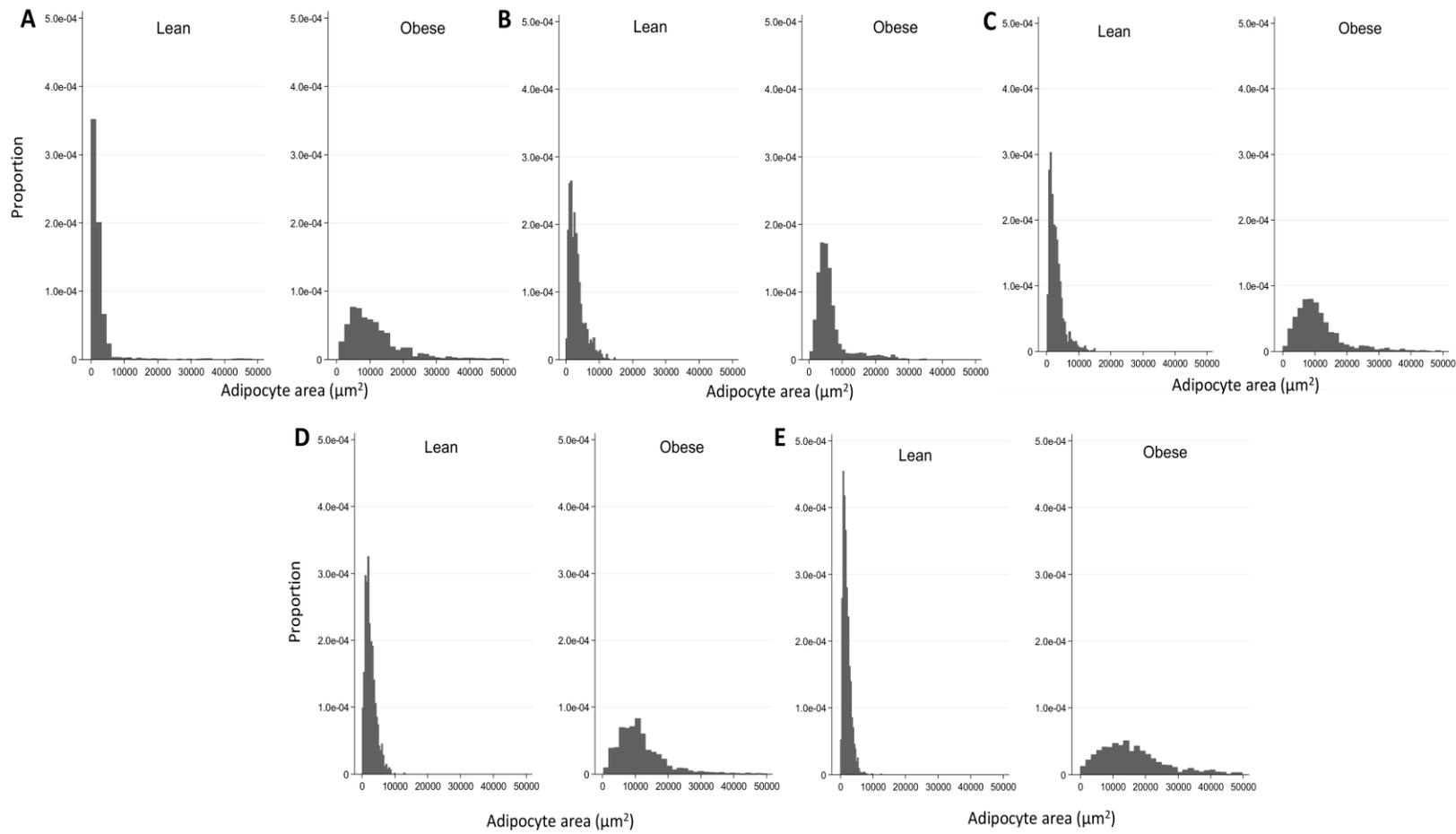
Figure 7.4: The distribution of adipocyte areas measured in lean and obese horses and ponies is shown for: **(A)** Omental, **(B)** Epicardial, **(C)** Crest, **(D)** Tailhead and **(E)** Retroperitoneal WAT depots. Distribution of adipocyte area is shown as a proportion (height of bars scaled so the sum of their height equals 1).

Protein extraction from internatant and fat cake fractions

For all WAT depots studied, significantly more protein was extracted from internatant as opposed to fat cake fractions (p < 0.01, Table 7.4). Furthermore, for all WAT depots, significantly more protein was extracted from internatant fractions derived from lean relative to obese animals (Table 7.4). By contrast, similar quantities of protein were extracted from the fat cake fractions of lean and obese animals for all WAT depots with the exception of tailhead WAT. Tailhead depots yielded significantly more protein/mg WAT from fat cake fractions derived from lean as compared to obese animals (p = 0.04).

Strong curvilinear associations were described between the ratios of lipid: total extracted protein and lipid:fat cake as well as internatant protein with adipocyte area for omental, crest and tailhead WAT, with weaker associations identified for retroperitoneal WAT and no associations for epicardial WAT (Figure 7.5).

Table 7.4: Protein extracted from internatant and fat cake adipose tissue fractions across 5 WAT depots from lean and obese horses and ponies. P value denotes difference between lean and obese animals.

Internatant protein (mg/mg WAT)	Lean		Obese		P value
Depot	Median	IQR	Median	IQR	
Epicardial	0.011	0.0006	0.005	0.0002	0.006
Omental	0.014	0.010	0.006	0.0007	0.004
Retroperitoneal	0.012	0.003	0.004	0.002	0.004
Crest	0.010	0.002	0.005	0.0006	0.004
Tailhead	0.009	0.002	0.004	0.0008	0.004
Fat cake protein (mg/mg WAT)					
Epicardial	0.002	0.0008	0.002	0.0002	0.12
Omental	0.003	0.0006	0.003	0.0005	0.13
Retroperitoneal	0.003	0.0006	0.002	0.0003	0.26
Crest	0.003	0.0006	0.003	0.0007	0.15
Tailhead	0.003	0.001	0.002	0.0003	0.04
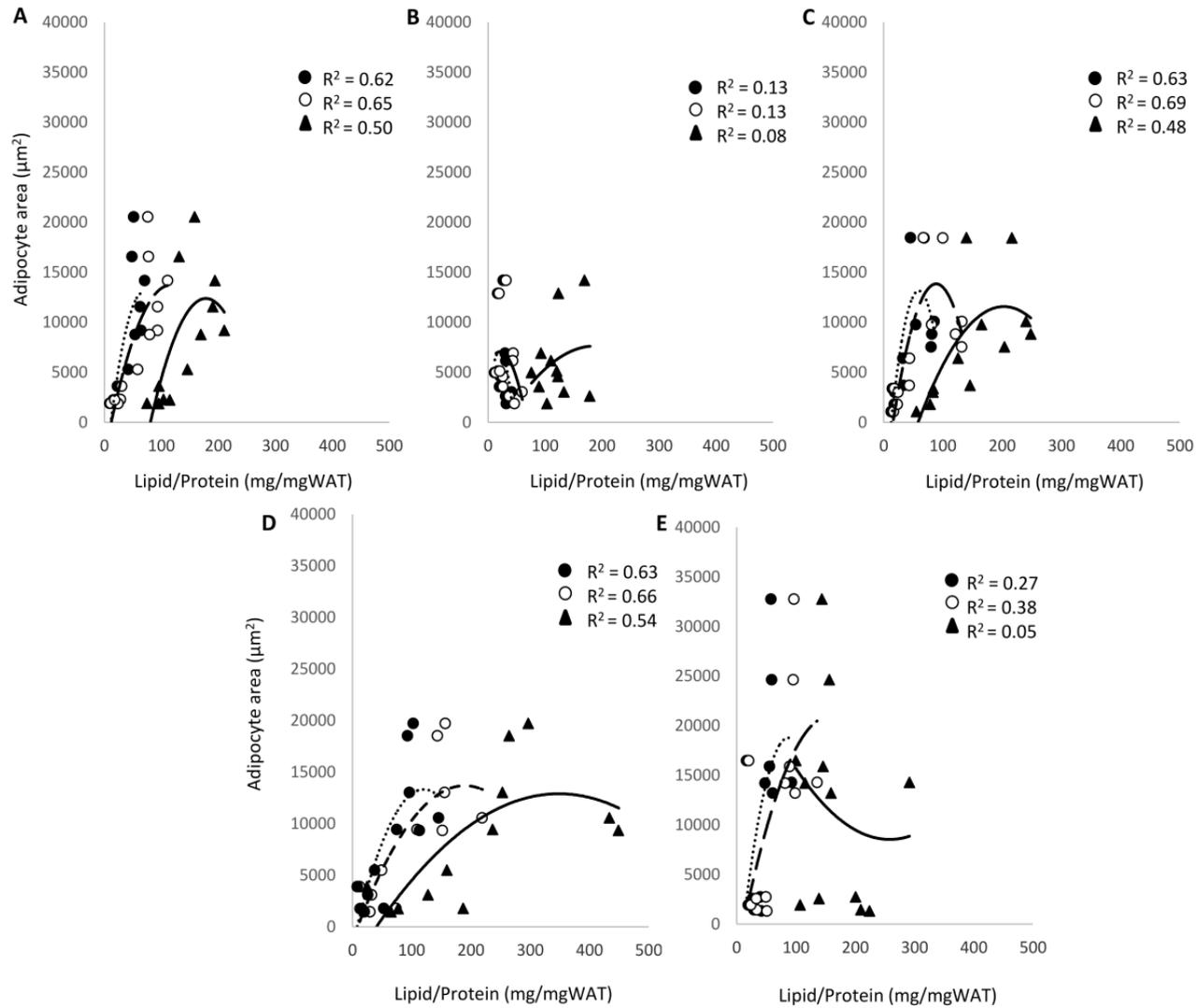
Figure 7.5: Associations between adipocyte area and lipid:protein ratio in lean and obese animals. A ratio of lipid weight (lipid remaining after protein extractions) to extracted protein concentrations in the internatant (●), fat cake (▲) and combined fractions (◯) was calculated for each animal and plotted against the corresponding adipocyte area for the individual animal. Data are shown for **(A)** Omental, **(B)** Epicardial, **(C)** Crest, **(D)** Tailhead and **(E)** Retroperitoneal WAT depots.

PLIN1 gene expression

There was a considerable range in PLIN1 gene expression for all animals. For crest WAT, there was a significantly greater expression of PLIN1 in lean animals compared to obese animals (p = 0.05; Figure 7.6 and Table 7.3), whilst no differences were observed between lean and obese animals for the other four depots studied.
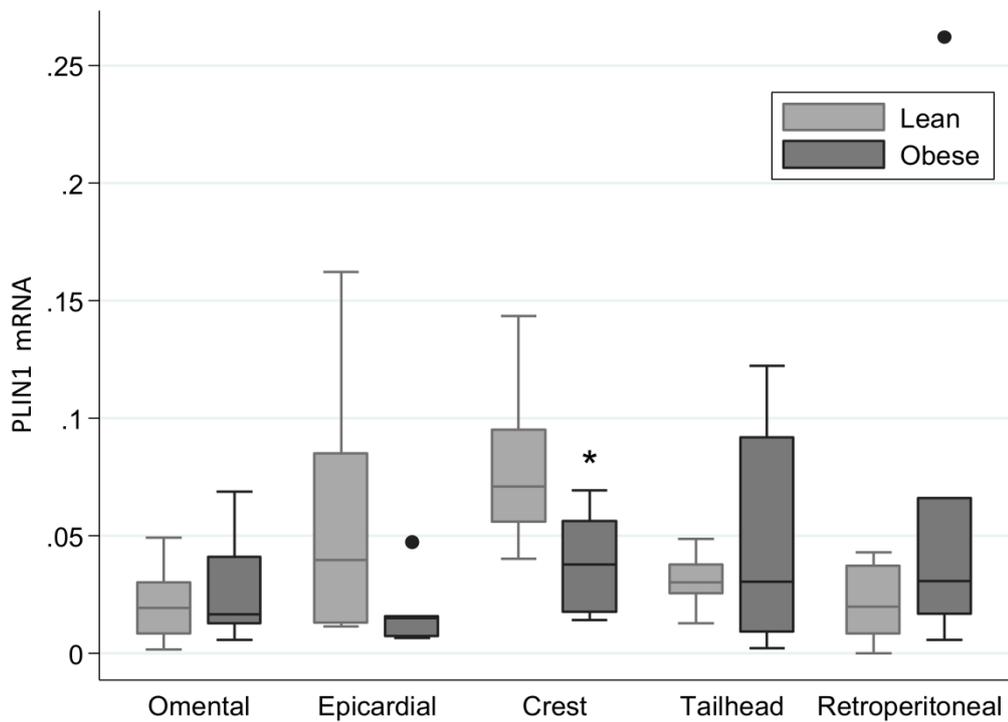
Figure 7.6: Gene expression of PLIN1 in adipose tissues of lean and obese horses and ponies analysed by real-time PCR. Box plots are shown for each depot in lean and obese animals, *n* = 6/group. *denotes where values differ significantly (p <0.05) from lean group.

PLIN1 and HSL protein expression

Within fraction

The relative abundance of PLIN1 and HSL proteins were compared in adipose tissue fractions (internatant and fat cake) between lean and obese animals. There was no

difference between lean and obese animals for PLIN1 or HSL in the internatant or fat cake fractions for epicardial and tailhead WAT (Figure 7.7 and Table 7.5). For omental WAT, HSL expression in the fat cake fraction was greater (p = 0.02) in lean animals compared to obese animals (Figure 7.7 and Table 7.5). For crest WAT, HSL expression was greater (p = 0.05) for lean animals compared to obese animals in the internatant fraction, whilst PLIN1 protein expression was significantly greater for lean animals compared to obese animals in the fat cake fraction (p = 0.004). For both proteins and both fractions, lean animals had a significantly greater protein expression compared to lean animals for retroperitoneal WAT. A summary of the results for adipocyte area, gene and protein expression can be found in Table 7.6.
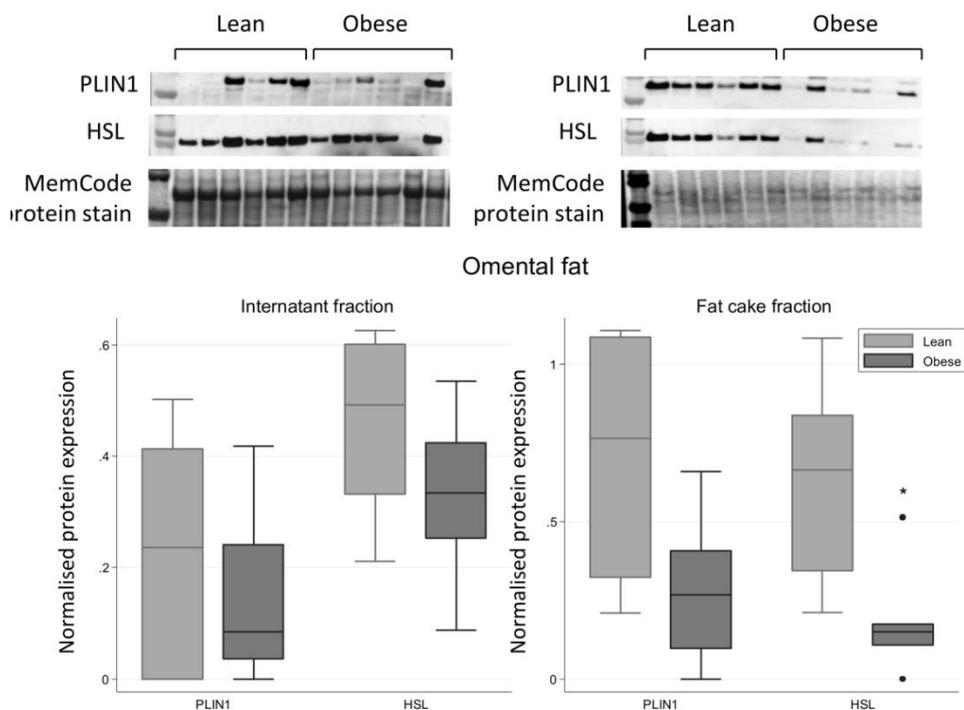
Figure 7.7: (A) Protein expression of PLIN1 and HSL in lean and obese horses and ponies between internatant and fat cake fractions of omental WAT. *denotes where values differ significantly from lean group (p < 0.05).
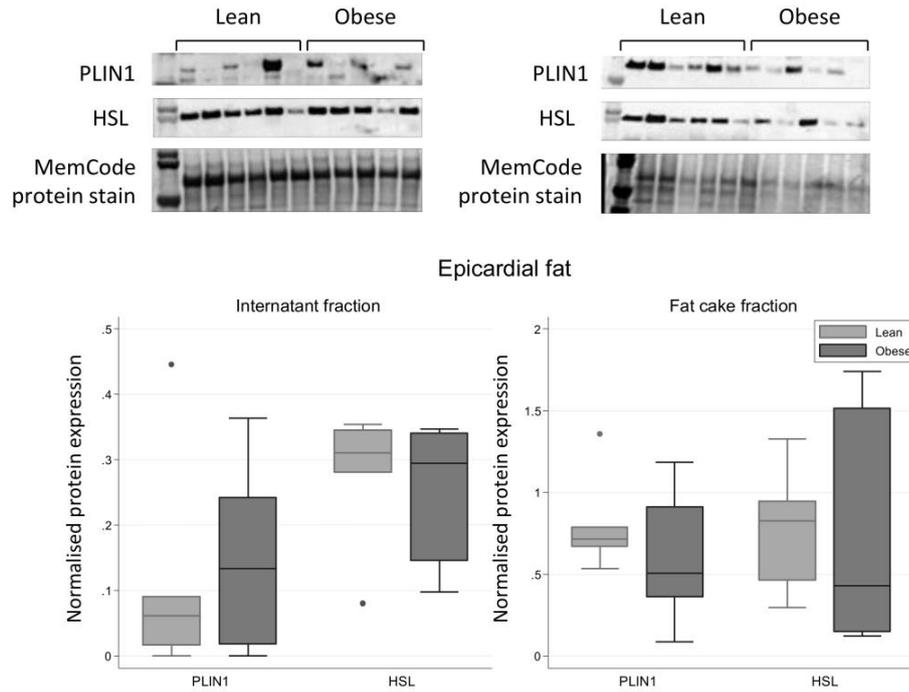
Figure 7.7: (B) Protein expression of PLIN1 and HSL in lean and obese horses and ponies between internatant and fat cake fractions of epicardial WAT. *denotes where values differ significantly from lean group (p < 0.05).
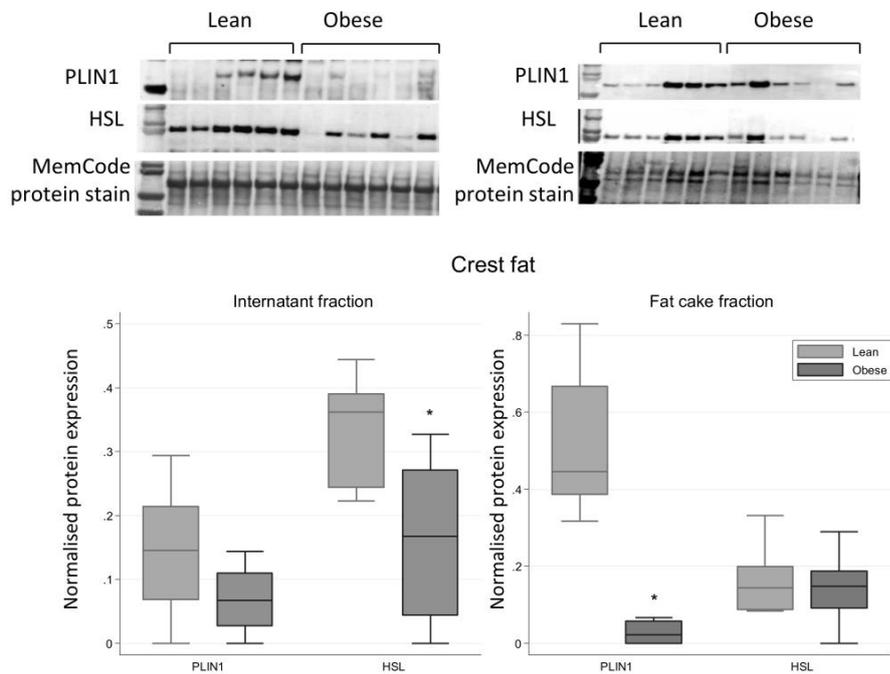
Figure 7.7: (C) Protein expression of PLIN1 and HSL in lean and obese horses and ponies between internatant and fat cake fractions of crest WAT. *denotes where values differ significantly from lean group (p < 0.05).
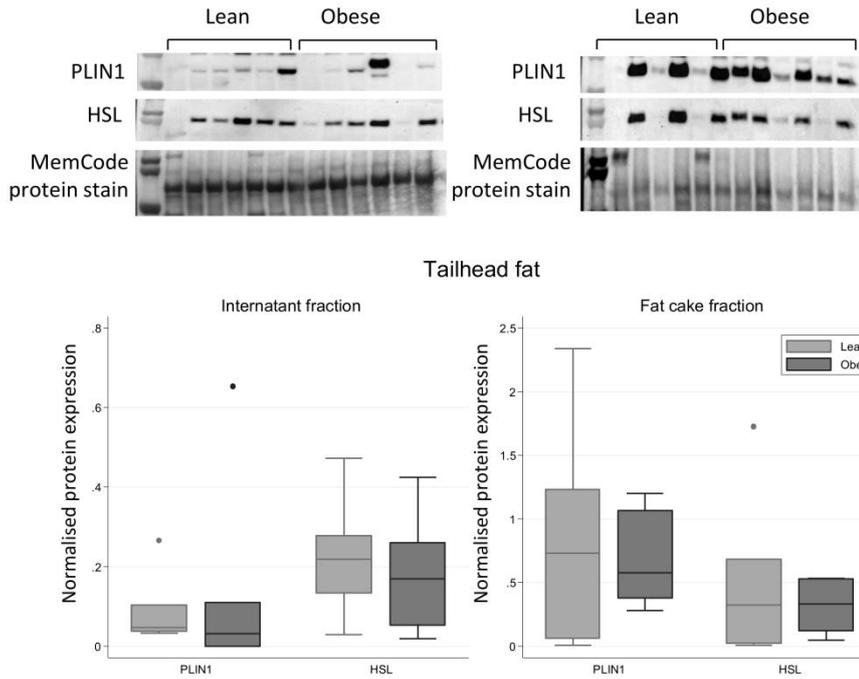
Figure 7.7: (D) Protein expression of PLIN1 and HSL in lean and obese horses and ponies between internatant and fat cake fractions of tailhead WAT. *denotes where values differ significantly from lean group (p < 0.05).
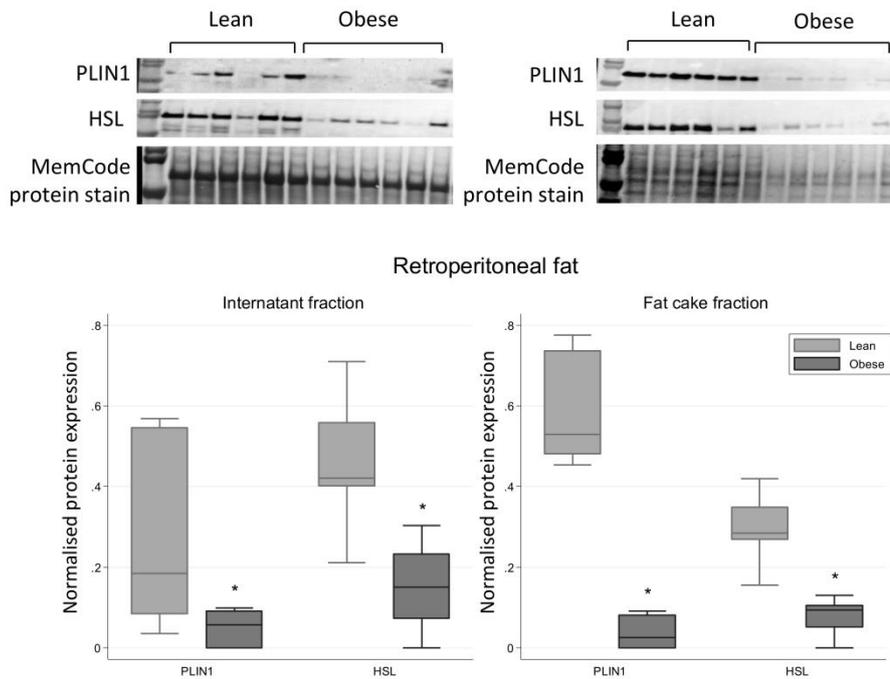
Figure 7.7: (E) Protein expression of PLIN1 and HSL in lean and obese horses and ponies between internatant and fat cake fractions of retroperitoneal WAT. *denotes where values differ significantly from lean group (p < 0.05).

Table 7.5: Protein expression data (median and IQR) for PLIN1 and HSL in adipose tissue fractions (internatant and fat cake) from lean and obese horses and ponies.

Depot		Lean		Obese		
Internatant Fraction	Protein	Median	IQR	Median	IQR	P value
Omental	PLIN1	0.24	0.41	0.09	0.20	0.75
	HSL	0.49	0.27	0.33	0.17	0.20
Epicardial	PLIN1	0.06	0.07	0.13	0.22	0.47
	HSL	0.31	0.06	0.30	0.19	0.75
Crest	PLIN1	0.14	0.15	0.07	0.08	0.17
	HSL	0.36	0.15	0.17	0.23	0.05
Tailhead	PLIN1	0.05	0.07	0.03	0.11	0.42
	HSL	0.22	0.15	0.17	0.21	0.52
Retroperitoneal	PLIN1	0.18	0.46	0.06	0.09	0.05
	HSL	0.42	0.16	0.15	0.16	0.01
Fat cake Fraction	Protein	Median	IQR	Medial	IQR	P value
Omental	PLIN1	0.76	0.76	0.27	0.31	0.08
	HSL	0.66	0.49	0.15	0.07	0.02
Epicardial	PLIN1	0.72	0.12	0.50	0.55	0.26
	HSL	0.83	0.49	0.43	1.37	0.52
Crest	PLIN1	0.45	0.28	0.02	0.06	0.004
	HSL	0.14	0.11	0.15	0.10	0.75
Tailhead	PLIN1	0.73	0.26	0.58	0.69	1.00
	HSL	0.31	0.66	0.33	0.41	1.00
Retroperitoneal	PLIN1	0.53	0.26	0.03	0.08	0.004
	HSL	0.28	0.08	0.09	0.05	0.004

Table 7.6: Summary of adipocyte area, gene and protein expression data from lean and obese horses and ponies across five adipose depots. ND, no difference.

Depot	Adipocyte area	Perilipin gene	Perilipin internatant protein	HSL internatant protein	Perilipin fat cake protein	HSL fat cake protein
Epicardial	ND	ND	ND	ND	ND	ND
Omental	Lean ↓ Ob	ND	ND	ND	ND	Lean ↑Ob
Retroperitoneal	Lean ↓Ob	ND	Lean ↑ Ob	Lean ↑Ob	Lean ↑Ob	Lean ↑Ob
Crest	Lean ↓ Ob	Lean ↑Ob	ND	Lean ↑ Ob	Lean ↑Ob	ND
Tailhead	Lean ↓ Ob	ND	ND	ND	ND	ND

Between fractions

Differences in the expression of PLIN1 and HSL between fractions were also assessed, however as outlined in the methods, between fraction comparisons must be interpreted with caution due to a lack of suitable loading control. However, it is worthy of note that visual appraisal of the western blots would strongly suggest that PLIN1 is almost exclusively present in the fat cake fraction of all depots studied, irrespective of whether animals were lean or obese (Figure 7.8). Conversely, HSL demonstrated a more ubiquitous expression between the fractions assessed for all depots (Figure 7.8).
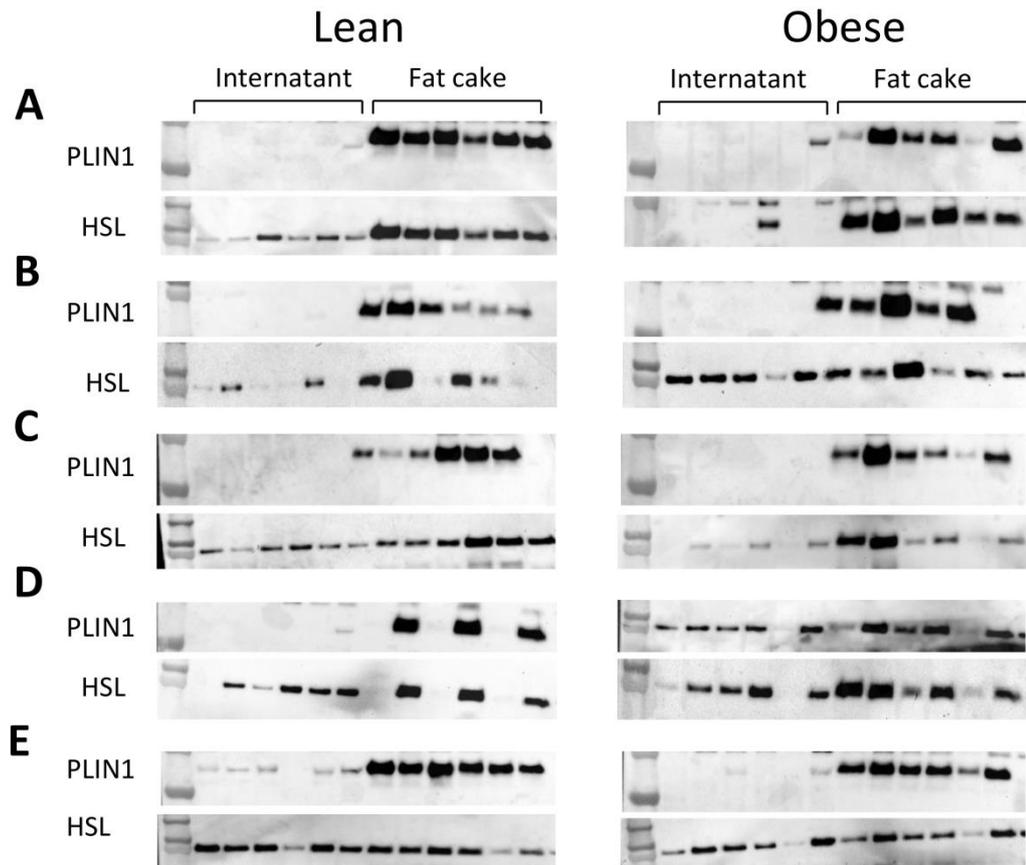
Figure 7.8: Protein expression of PLIN1 and HSL in internatant and fat cake fractions between lean and obese horses and ponies. Equal amounts of protein (10µg) from the fat cake fraction and internatant fraction were loaded into each gel. Blots are shown for lean and obese animals for each of the five adipose depots studied: **(A)** Omental, **(B)** Epicardial, **(C)** Crest, **(D)** Tailhead and **(E)** Retroperitoneal.

7.5 Discussion

This is the first study to assess adipocyte area and gene expression of PLIN1 as well as protein expression of PLIN1 and HSL across a range of adipose tissue depots in lean and obese horses plus ponies.

At the gene level, there was considerable variation in PLIN1 mRNA expression between lean and obese horses and ponies; however, for crest WAT there was a significantly greater expression of PLIN1 in lean animals compared to obese animals. These data agree with the findings of a comparable study of human

subjects where it was found that PLIN1 mRNA expression in abdominal subcutaneous adipose tissue was significantly increased in lean compared to obese people (Wang *et al.*, 2003). However, other studies have reported either no difference in PLIN1 mRNA expression for omental fat in obesity (Ray *et al.*, 2009), or an increase in PLIN1 mRNA expression in obesity for abdominal subcutaneous adipose tissue (Kern *et al.*, 2004). Differences in methodology and the criteria for obesity (in terms of BMI) may account for the discrepancies between studies.

Whilst the evaluation of gene expression is important, the assessment of protein expression is crucial in furthering our understanding into potential alterations in PLIN1 and HSL expression in obesity. Importantly, although PLIN1 and HSL function as lipid-droplet associated proteins, PLIN1 moves between the endoplasmic reticulum and lipid droplet during lipid synthesis (Skinner *et al.*, 2013). Similarly, the translocation of HSL from the cytosol to the lipid droplet in response to lipolytic stimulation has also been well characterised (Clifford *et al.*, 2000). On the basis that cellular location was likely to be indicative of protein functionality, we considered it important to characterise the cellular distribution of PLIN1 and HSL proteins in the horse. The concentration of protein extracted from the fat-free internatant (likely cytosolic) and lipid-droplet fat cake (lipid droplet associated) fractions demonstrated significant differences between lean and obese animals. Protein concentrations were significantly greater in the internatant fractions of all WAT homogenates from lean as compared to obese animals. This finding was not unexpected as a relatively greater proportion of adipocyte volume is likely to be occupied by the lipid droplet in obese animals, reducing the proportion of cytosol associated with each cell and thereby the total cytosol volume per mg WAT. By contrast, the concentrations of proteins extracted from fat cake fractions were generally similar across the lean and obese groups, an observation which is in agreement with another study in human subjects (Wang *et al.*, 2003). Interestingly, in that study, it was calculated that due to the lower number of fat cells per gram of tissue in the obese subjects, adipose tissue protein content per adipocyte was correspondingly higher in the obese group. Although the total number of fat cells in specific WAT depots could not be quantified in the current study, it is likely, given

the increased adipocyte area recorded for obese equines for the majority of depots appraised, that protein content per adipocyte would also be relatively greater for horses and ponies in the obese state.

The present finding, that adipocyte area was increased in obese animals across almost all depots is consistent with findings in other species including dogs (Grant *et al*., 2011) and cats (Van de Velde *et al*., 2013). For the horse, one study identified differences in adipocyte area between 5 anatomically-discrete WAT depots (Bruynsteen *et al*., 2013). In that study, retroperitoneal peri-renal adipose tissue had the greatest adipocyte area; a depot not evaluated in the current study. However, the mean adipocyte area for recorded for abdominal retroperitoneal WAT by that group was akin to the median value of the lean animals reported in the present data set and considerably lower than the median value of obese animals in the current study. Methodological differences clearly distinguish these reports. The study of Bruynsteen *et al*. (2014) was designed to compare the 5 WAT depots studied and body adiposity was crudely characterised across the 12 animals sampled; the standard BCS approach was not used. The study (Bruynsteen *et al*., 2014) indicated that only 1/12 animals was obese, with 5/12 animal being classed as overweight to obese. The methods used (Bruynsteen *et al*., 2014) to estimate adipocyte area also differed. The method for measuring adipocyte area in the current study is a widely used technique (Ronkainen *et al*., 2015). However, as with any measurement of sectioned material, it should be noted that it is possible that a portion of the measurements taken do not represent the maximum cross-sectional area due to the nature and structure of adipose tissue. In the current study, in excess of 200 cells were individually evaluated in histological sections. By contrast, in the earlier study (Bruynsteen *et al*., 2014), adipocyte area was reported as the area of field of view divided by the number of adipocytes identified in that field; an approach that will encounter the same issues as the current study, however this will be further compounded by the likely over-estimation of individual cell size due to the inclusion of interstitial areas. Nevertheless, it is of interest that the measurements given by Bruynsteen *et al*. (2014) are broadly consistent with the values obtained in the present study for lean animals, raising the question of the

previous study's (Bruynsteen *et al*., 2014) method of classification of body adiposity.

It was of note that the distribution of adipocyte areas in epicardial WAT was altered least of all the WAT depots studied between the lean and obese states. Interestingly, and consistent with our finding, it has been identified that epicardial adipocyte area was not significantly associated with BMI in human subjects (Eiras *et al*., 2010). In support of these data we have previously reported that the EQUIFAT score for epicardial WAT (a *post-mortem* fat score associated with depot-specific, 'visually apparent,' gross adiposity) was also not associated with BCS in horses and ponies (2014). Due to its anatomical location, this adipose depot may have a more direct function in supplying local nutrition and thermal support for the heart. This is supported by data from a human study in which the presence of the brown-fat specific gene, UCP-1 was identified in epicardial fat, suggestive of a functional role in protecting the myocardium against hypothermia (Sacks *et al*., 2009).

Although no statistically significant differences were observed for adipocyte area between depots within lean or obese animals in the current study, it has been demonstrated previously in the rat (Palou *et al*., 2009) that retroperitoneal adipocyte size is greater than mesenteric and subcutaneous (inguinal) adipocytes, which may indicate that retroperitoneal WAT has a greater capacity for lipid droplet expansion. The wide range in adipocyte area for retroperitoneal WAT between lean and obese equines and the extensive range in area noted within obese animals compared to other depots in the current study may support this contention. Furthermore, the extensive range in adipocyte area noted within obese animals for retroperitoneal WAT might further indicate that this depot demonstrates considerable plasticity and has the greatest potential to modify lipid droplet size in response to changing energy balance. In support of the hypothesis that abdominal WAT may constitute a long-term energy reserve, the measurement of belly girth (an indirect measure of abdominal WAT content in animals on a steady plane of nutrition) in a group of obese ponies was decreased progressively with dietary restriction over 14 weeks, while BCS, a subjective measure of externally palpable

adipose reserves (including crest and tailhead) decreased minimally in the same period (Dugdale *et al.*, 2010).

To establish the association between relative proportion of lipid and protein change and adipocyte area, the relationship between lipid:protein ratio and adipocyte area was evaluated. A curvilinear relationship was shown for omental, tailhead and crest WAT's. The nature of this association confirmed that as the relative lipid content increased, adipocyte area also increased; however, this association is not constant and the data suggest that once a certain ratio is attained, the adipocyte area is reduced. One explanation for this trend may be that this is a result of the addition of new smaller adipocytes (hyperplasia), which would, given their relatively increased cytosolic content, lead to an overall increase in protein concentrations per mg WAT. Mathematical modelling has demonstrated that the addition of new adipocytes occurs in mice during epididymal fat pad mass expansion in a strain- and diet-dependent manner (Jo *et al.*, 2009), whilst a greater expression of adipogenic genes, reflective of hyperplasia was evident in subcutaneous WAT compared to omental WAT in women (Drolet *et al.*, 2007). Further studies would be required to establish whether hyperplasia occurs during adipose tissue expansion in the horse. For retroperitoneal fat, a weaker association was identified for lipid to internatant protein ratio with adipocyte area, and no association was found for lipid to fat cake protein ratio with adipocyte area. In combination with the data describing the distribution of adipocyte area, this might provide further evidence that this depot demonstrates the greatest plasticity and would therefore show the greatest range in adipocyte areas at any one time.

The relative abundance of PLIN1 protein in the internatant fraction was found to be significantly lower in obese animals compared to lean animals in retroperitoneal fat only.   Furthermore, in the fat cake fractions, PLIN1 expressions were also significantly reduced in both retroperitoneal and crest fats in obese compared to lean animals. This finding is consistent with studies in humans that demonstrate a decreased expression of PLIN1 protein in obesity (Ray *et al.*, 2009). Interestingly, another study found that although the calculated mass of PLIN1 per fat cell was

constant between lean and obese humans, the relative increase in fat cell size in obesity was not accompanied with an increase in PLIN1 concentration (Wang *et al.*, 2003). Fat cell size has previously been positively correlated with basal lipolysis (Laurencikiene *et al.*, 2011), with a complete loss of PLIN1 accounting for elevated basal lipolysis observed in PLIN1 KO mice (Tansey *et al.*, 2001). Due to the nature of the current study, it was not possible to measure basal lipolysis in these animals, but it could be speculated that it may have been altered in the obese state, as it has been demonstrated previously that in humans, PLIN1 protein expression in omental and subcutaneous fats was negatively correlated with basal lipolysis (Ray *et al.*, 2009).

This study examined PLIN1 gene and protein expression in adipose tissue depots. These techniques have not been performed previously in abattoir derived equine material in lean and obese animals, thus there was a total absence of prior data to inform study design. Therefore it was not possible to perform *a priori* sample size calculations since the magnitude of differences in expression on which to base such calculations were unknown. However, clear differences in PLIN1 protein expression between lean and obese animals were demonstrated in some but not all adipose tissue depots. Two hypotheses for the lack of "statistically significant" differences observed in e.g. omental WAT depot may be formulated – firstly there was a true difference in protein expression but the sample size was insufficient to detect it at a P value < 0.05 or secondly no difference existed. For the retroperitoneal WAT depot, significant differences between lean and obese animals were demonstrated for PLIN1 protein expression, and *post hoc* power calculations confirmed that in this case, the study had sufficient power (50%). Applying *post hoc* sample size calculation to the omental WAT depot suggested that whilst a mean difference of 0.075 (2 tailed P = 0.449) in PLIN1 protein expression was observed, the power of the study was only 11% and a sample size of 40 animals per group would likely be required to achieve a statistical significance of P < 0.05 with 80% power. As outlined in Chapter 6, whilst the results of the post hoc power calculations should not be taken definitively, they do indicate that a larger sample size may be required for future studies.

The relative abundance of HSL was significantly reduced in the internatant fraction of crest and retroperitoneal WAT in obese animals, whilst it was reduced in the fat cake fraction of retroperitoneal and omental fats in obese animals. Hormone-sensitive lipase protein expression has been previously identified as a determining factor of maximal lipolytic capacity of human fat cells (Large *et al*., 1998), and its expression is reduced in an obese state (Ray *et al*., 2009). Interaction between HSL and PLIN1 and the lipid droplet is required for maximal stimulated lipolysis (Shen *et al*., 2009), therefore a reduced abundance of these proteins in the fat cake fraction of retroperitoneal and omental WAT's may suggest that for obese animals, these depots would be less sensitive to catecholamine stimulated lipolysis. In agreement with this, maximal stimulated lipolysis in response to noradrenaline was lower for omental adipocytes compared with subcutaneous adipocytes (Ray *et al*., 2009). Although the major objective of the current study was not to quantify differences in the relative abundance of PLIN1 and HSL between regional adipose depots, the finding that PLIN1 and HSL proteins are not consistently altered between depots in obesity is suggestive of some functional differences between these regional depots. No differences were observed in the relative abundance of PLIN1 or HSL in epicardial fat. This adipose depot has been associated with factors encompassing the metabolic syndrome (Iacobellis *et al*., 2003), and indicators of insulin resistance and glucose intolerance in humans (Iacobellis and Leonetti, 2005). A role for epicardial fat in insulin dysregulation in horses and ponies is yet to be established and may be an area for further research.

The sub-cellular distribution of PLIN1 and HSL proteins between adipose tissue fractions was evaluated in the current study. However, as outlined in the methods, innate differences in the population of proteins located in each fraction precluded our ability to normalise data to a standard loading control. Interestingly, this finding would appear to have been encountered by other researchers and a lack of a valid loading control for normalising between fractions has led to variation between studies in their normalisation strategies or some choosing to continue using loading controls which show no expression in the fat cake fraction, such as beta-actin (Okumura *et al*., 2014). Therefore, whilst the current results must be

interpreted with caution, our data would demonstrate that regardless of whether animals are lean or obese, PLIN1 predominantly resides in the fat cake fraction, a finding consistent with other publications (Ray *et al.*, 2009; Yang *et al.*, 2011). The distribution of HSL appears to be more similar between the internatant and fat cake fraction which may reflect the greater capacity for translocation between the lipid droplet and the cytosol fractions during catecholamine-stimulated lipolysis.

There was a degree of inter-animal variability in both gene and protein expression for some depots in the current study which may be due in part to the mixed breed and genders used in the current study. In addition to this, despite these animals being categorised as lean or obese at the time of sample collection, detailed nutritional history was unavailable for these animals so it was unknown whether they were actively gaining or losing weight which may have attributed these variations observed. It is acknowledged that this study did not assess the portion of phosphorylated PLIN1 and HSL proteins. The phosphorylation sites for these proteins have not yet been established for the horse. Therefore, for the purposes of the current study it was considered important to firstly assess the relative abundance of the total proteins before more detailed investigations are undertaken to assess the relative proportion of phosphorylated proteins in animals where a more detailed nutritional history is available.

7.6 Conclusion

The current study has established clear differences in adipocyte area and distribution between regional adipose depots in lean and obese horses and ponies. Furthermore, it has characterised the expression of the lipolytic proteins PLIN1 and HSL in adipose tissues of lean and obese horses and ponies, providing evidence that these proteins may be altered in obesity in certain adipose tissue fractions and depots, indicating important functional lipolytic differences between adipose depots in the horse. These data form the basis for further work to investigate alterations in lipolysis in equine obesity.

7.7 References

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Chapter 8

General Discussion

8.1 Overview

The use of horses and ponies in the UK has changed considerably over the past 100 years. During this time the role of the horse has shifted from primarily working purposes in agriculture, mining, transport and warfare in the 19th and early 20th centuries, to being kept almost entirely for recreational purposes as we see in today's society (Wyse *et al.*, 2008). While this shift in purpose has had many positive impacts on animal welfare, some aspects of modern management could be considered deleterious. Reduced workloads, in combination with increased availability of high-quality feedstuffs and modern husbandry techniques all of which promote positive energy balance, have likely contributed to the high prevalence of obesity recorded in leisure populations of horses and ponies in the UK (Giles *et al.*, 2014). Across species, obesity prevention and treatment is largely dependent on dietary management.

8.2 Equine obesity – can we recognise it?

The ability to recognise obesity and an appreciation of the associated health risks are essential prerequisites for the initiation of weight-loss measures at least in humans, where   individuals who perceived themselves to be 'overweight' as opposed to being 'about right' were more likely to initiate weight loss attempts (Lemon *et al.*, 2009). Furthermore, it has been shown that if individuals perceived their body weight to be a risk to their health, they were more likely to try and   lose weight (Gregory *et al.*, 2008). Whether these factors also influence willingness to initiate body weight management strategies in horses is not known and is likely to be complicated by the fact that not all obese horses and ponies will develop laminitis and/or insulin dysregulation. The exact mechanisms linking obesity with insulin dysregulation and laminitis remain to be fully elucidated. However, regardless of whether obesity is identified alone or in conjunction with laminitis and/or insulin dysregulation, controlled weight loss management remains the primary corrective therapy. For those animals who are at a high risk of developing laminitis in an obese state, a failure by owners to recognise obesity and take action

to reverse it prior to the onset of laminitis has potential ethical implications. Under these circumstances, the omission of body weight management could be deemed to cause unnecessary pain and suffering to the animal. Furthermore, implementing a weight-loss program once laminitis is established will be more difficult as increasing energy expenditure through exercise may be contraindicated. Improving our understanding of owner-perceptions of obesity in horses and ponies is the first step in establishing the reasons behind the excessive rates of obesity reported in our leisure population of animals.

Knowledge on how horse owners and enthusiasts perceive optimal body weights and obesity in their animals is lacking. To the author's knowledge, two previous studies identified an owner-underestimation of their horse or pony's body condition score (BCS) (Ireland *et al*., 2012; Wyse *et al*., 2008). Questionnaire data gathered in **Chapter 3** improved our understanding of the perceptions of obesity (Tier 1) and expanded our knowledge of the current management practices employed by horse owners (Tier 2). Tier 1 clearly demonstrated a varied ability of horse owners and enthusiasts to identify overweight animals from lateral photographic images. It was hypothesised that equine professionals would have a superior ability to identify overweight animals; however, we found limited evidence to support this. In human studies, it has been demonstrated that mothers with less education were more likely to misperceive their child's weight (Baughcum *et al*., 2000). An emerging concept in human epidemiological studies, suggests that our increased exposure to obesity in the population we see today, has upwardly altered what we perceive to be normal in terms of body weight (Burke *et al*., 2010). It is tempting to speculate that, with the current high prevalence of obesity in horses and ponies, this upward shift in our perception of normality, may be equally relevant for the horses in our care.

The large range in conformational shapes and sizes between and within different breeds of horses and ponies adds a degree of complexity to understanding obesity perceptions. Evidence from **Chapter 3** suggests that, despite the two images of cob breed horses being of an ideal body condition, they were considered to be

overweight by the majority of respondents. The classic heavy neck and loin of cob conformation is clearly at variance with that of other breeds and these data support findings in Andalusian horses that some breeds may warrant separate consideration (Martin Gimenez *et al*., 2014). It is known that breed is a risk factor for an animal's propensity to become obese (Giles *et al*., 2014). In combination, these data would suggest that further advice regarding body condition of animals of varying breeds is required in order that horse owners better understand the difference between the conformational aspects of the breed and relative adiposity.

 Tier 1 data also provided evidence to support the anecdotal wisdom that, a respondent's perception of body weight and / or body condition was clearly influenced by the equine sporting discipline or leisure role in which a specific horse or pony was intended to participate. Consistently, each animal evaluated by photographic image (sport horse, cob horse and pony) was considered to require more weight/condition for competing in affiliated (professional) showing classes than was deemed appropriate for participation in other disciplines. As discussed, this finding was in agreement with anecdotal observations and would indicate that further advice is required to encourage the showing fraternity to promote education and discourage the presentation of overweight animals within the show horse sector. A recent publication identified that 1 in 4 show dogs which competed at the premier UK dog show (Crufts) were overweight (Such and German, 2015). The concern over heightened media exposure and the accessible nature of images of show-winning dogs online was considered to have been an important contributor to the 'normalisation' of canine obesity in the public eye. This could be applied to the equine showing industry, whereby highlighting the issue and prevalence of obesity at a national showing competition such as the Horse of the Year Show might bring about a positive change in reducing obesity prevalence in these animals. Tier 2 reported on basic care and management practices of horses and ponies and adds to the current epidemiological literature for horses and ponies.

 As with any questionnaire, there are some limitations. For the first tier, assessing the weight status of animals based on a single photographic image is more

challenging than assessing animals *in vivo,* although as discussed, attempts were introduced to limit bias. For example, different breeds and colours of animals were used and they had been photographed in the summer to limit the impact of a longer winter coat length on visual perception of weight status. Future studies assessing obesity perceptions could employ the use of multiple photographic images of individual animals taken from different angles. For both aspects of the questionnaire, there is likely to be a degree of respondent bias whereby those with a genuine interest in the subject are more likely to respond. The low proportion of owner-reported obesity in Tier 2 could be due to an owner underestimation of their horse or pony's body condition or it could also be speculated that those owners who took part had a greater knowledge with respect to nutrition and a level of interest in the subject that made them less likely to own an obese animal. In addition, it is tempting to speculate that horse owners may be less able to correctly identify obesity in their own animal but are more critical and better able to recognise obesity in animals belonging to others.

Evidence from human studies indicates a discrepancy between self-reported and actual dietary intakes and physical activity levels (Elliott *et al*., 2014; Lichtman *et al*., 1992). It would be expected that this will be the case for leisure horse owners who are likely to be over-feeding and under-exercising their horse or pony, culminating in the development of an obese state. During the initial development of the studies described in **Chapter 3**, a third tier was designed to evaluate perception versus reality in terms of feeding and exercise of horses and ponies. A home visit-based, owner-interview study design was constructed - and subsequently rejected on consideration of time constraints and an inability to control confounding factors.

Whilst it is acknowledged that current husbandry methods for leisure horses and ponies are likely to persist, evidence presented in this chapter indicates that further education is required for horse owners in the correct identification of obesity that needs to become a standard part of normal husbandry. This could be delivered through various avenues including the potential inclusion of a body condition scoring tool on feed labels with advice on the risks of obesity and management. The

addition of weight management advice from veterinarians to those owners with animals deemed to be overweight or obese during routine veterinary visits may also provide a useful tool for education. Furthermore, health checks at regional and national showing competitions could be implemented to enforce the disqualification of obese animals on the grounds of animal welfare.

Future directions

It is clear from the outcomes of the above discussion that there is a need to:

- *Evaluate any associations between owner's educational status and their perception of their animal's body condition.*

- *Investigate whether, as for humans, there has been a recent upward shift in our perception of normality in terms of the ideal body condition of horses and ponies.*

- *Consider the use of focus groups for the evaluation of horse-owner perception versus reality of body condition score and feeding requirements.*

- *Characterise different ownership styles and how they might be associated with the likelihood of owning an obese horse or pony. This need was highlighted by a recent article which discussed the concept of associating parenting styles to ownership styles for pet dogs and cats and how these might predispose to obesity in these pets (German, 2015). Differences in relationships between owners of normal weight and overweight cats/obese dogs have also been assessed (Kienzle and Bergler, 2006; Kienzle et al., 1998).*

8.3 How important is regional adipose tissue distribution in *Equidae?*

It is clear from human studies that whilst obesity itself poses a major health threat, it is in fact the regional distribution of body fat that has stronger overall links with metabolic disease risk. Increased visceral fat deposition is associated with the metabolic syndrome in humans (Després, 2006). Quantifying regional body fat

distribution in living horses is problematic. Their large body size precludes the use of modern imaging modalities such as CT and MRI. Therefore our knowledge about regional adipose tissue distribution is lacking for the horse. Body condition scoring remains a useful scoring system, however it does not account for internal adipose tissue deposition. Until alternative imaging modalities allowing quantification of internal adipose depots in large animals are developed, we have an urgent need to capitalise on available *post-mortem* or surgical material in order to draw any conclusions between health and internal adiposity in horses. **Chapter 4** identified a strong, positive association between retroperitoneal EQUIFAT score and BCS. There was a weaker association between omental EQUIFAT score, and no association for mesenteric and epicardial EQUIFAT scores with BCS.

A strong association for retroperitoneal EQUIFAT score with BCS is suggestive that this depot may function as a long-term and labile reserve for excess dietary energy. In agreement with this, the complete dissection of a group of 7 Welsh mountain pony mares across a range of BCS revealed that whilst dissected WAT was equally distributed between 'internal' (body wall/organ-associated) and 'external' (intermuscular/subcutaneous) depots, the retroperitoneal depot weight revealed the greatest range between animals and the highest calorific content compared to other WAT depots (Dugdale *et al.*, 2011). Together with the finding from the current study, this would suggest that the retroperitoneal WAT depot has the greatest capacity for long-term fat deposition.

The fact that mesenteric EQUIFAT scores were not associated with BCS would suggest that this depot, in combination with omental WAT may be the most labile reserves that can be readily accessed during times of negative energy balance. The visceral depot is known to be highly metabolically active compared to subcutaneous depots, with higher rates of lipolysis noted in omental compared to subcutaneous depots in human subjects (Arner, 1995). In humans, a preferential loss of visceral adipose tissue is observed during moderate weight loss; however this preferential loss is diminished with further weight loss (Chaston and Dixon, 2008). A loss of visceral fat would be considered beneficial in terms of reducing metabolic

abnormalities associated with this depot, and improvements in insulin signalling and glucose homeostasis in a murine model of diet-induced obesity have been observed following the complete removal of visceral (epididymal and perinephric) fat pads (Pitombo *et al.*, 2006). However for human subjects, removal of the greater omentum (omentectomy) from obese subjects failed to elicit the predicted improvement in insulin sensitivity (Andersson *et al.*, 2014; Fabbrini *et al.*, 2010). Recently, it has been demonstrated in dogs that increases in visceral WAT content, affected through the sympathetic denervation of the omental adipose depot, did not result in alterations in insulin sensitivity (Castro *et al.*, 2015). Taken together, these data would suggest that whilst reductions in omental WAT may not illicit the expected positive metabolic outcomes in humans; there appears to be some differences between species in terms of the contribution of visceral depots to metabolic abnormalities associated with obesity.

A precise role for visceral WAT for the horse has yet to be established. Studies have shown that the expression of inflammatory genes in visceral WAT is not altered by an insulin resistant state in light-breed horses (Burns *et al.*, 2010), whilst the difference in expression of inflammatory genes was not markedly different between mesenteric fat and other regional adipose depots in a group of mixed-breed horses and ponies (Bruynsteen *et al.*, 2013). However, significant differences in insulin responses have been established between breeds of horses and ponies (Bamford *et al.*, 2014).

Epicardial EQUIFAT score had no association with BCS in the current study. In recent years, this adipose depot has been implicated in the pathogenesis of coronary artery disease and type 2 diabetes (Okada *et al.*, 2014; Wang *et al.*, 2009). This lack of association between epicardial WAT and BCS also emphasises its distinctive functional role. The primary role of epicardial WAT may be to support local myocardial nutrition and to buffer the heart against hypothermia. This hypothesis is supported by the observation that expression of the brown-fat specific gene UCP-1 is relatively increased in epicardial WAT depots in other species in comparison to other depots (Sacks *et al.*, 2009). This raises interesting

considerations for the diverse horse breeds, where in evolutionary terms; exposure to severe low temperature environments would have been highly variable.

Until imaging technologies can be developed to assess regional adiposity *in vivo* for *Equidae*, we remain unable to assess changes in internal, visceral depots during weight gain and weight loss. The distribution of the EQUIFAT scoring system in 'user-friendly' score cards to clinicians and researchers are now vital in directing a co-ordinated research effort into regional adiposity and disease risk.

Future directions

On the basis of the above discussion, the following questions remain unanswered:

- *Could EQUIFAT scoring system bring us closer to defining associations between regional adiposity and disease risk (e.g. pedunculated lipomas)?*

- *Does the expression of inflammatory factors differ between: a) breeds of horses and ponies of common BCS and b) between regional adipose depots of differing EQUIFAT scores, independent of BCS?*

- *Is the role of epicardial adipose tissue in myocardial support and insulin sensitivity related to the evolutionary adaptations of diverse equine breeds?*

8.4 Prerequisites to molecular biology investigations

Given difficulties exploring regional depots *in vivo*, there is a need to find another route to explain likely differences and implications of fat deposition/mobilisation in various reserves on health. It was therefore considered important to conduct more in-depth molecular biology studies, focused on the effect of obesity on key regulators of energy balance. The primary targets for more in-depth investigations were key proteins that had established roles in other species including humans, in the regulation of energy balance and whole-body metabolism, Myostatin and Perilipin 1 (PLIN1). Despite the widespread use of *post-mortem* / abattoir material for molecular biology studies in the horse, the has been a conspicuous lack of evidence to underpin the time limits between death and tissue collection within

which samples intended for RNA extraction can usefully be taken. The conduct of studies in the absence of this fundamental information would question the validity of a considerable proportion of published data (Brown *et al*., 2012; Echigoya *et al*., 2011; Manso Filho *et al*., 2007). It was therefore considered 'good practice' in the current thesis to evaluate the time limits for RNA extraction and demonstrate the tissue-specific expression of the proteins of interest prior to undertaking more detailed molecular studies.

In agreement with the current findings (**Chapter 5**), RNA extracted from bovine adipose tissue appeared less stable than that extracted from skeletal muscle in bovine tissues (Bahar *et al*., 2007). In the current study, RNA integrity was assessed through agarose gel electrophoresis and resulting bands were quantified by densitometry. Although modern technologies employing microfluidics, capillary electrophoresis and fluorescence now exist for assessing RNA integrity, this type of system was not available in our laboratory. Despite this, our results allowed us to recommend time-limits for the extraction of quality RNA from adipose tissue and skeletal muscle. A time limit of 30 and 120 minutes *post-mortem* was deemed an appropriate window to ensure the extraction of good quality RNA from adipose tissue and skeletal muscle, respectively.

In addition to establishing time limits for RNA recovery, **Chapter 5** confirmed findings in other species regarding the anatomical distribution of myostatin and PLIN1. Myostatin gene and protein expression was largely restricted to the skeletal muscles, whilst the expression of PLIN1 was predominant in adipose tissues. As outlined, **Chapter 5** formed the foundation for subsequent studies: 1) assessing the expression of myostatin and its receptor (ActRIIB) in skeletal muscles of lean and obese horses and ponies (**Chapter 6**), and 2) characterising the expression of PLIN1 and HSL across a range of adipose tissue depots of lean and obese horses and ponies (**Chapter 7**).

Lessons learned:

- *Prior to undertaking molecular biology studies using post-mortem material, it is crucial to ascertain the time-frame for obtaining tissues suitable for subsequent analysis.*

8.5 A role for the myostatin system in equine obesity

Chapter 6 provided evidence that myostatin gene expression was up-regulated in skeletal muscles in obese horses and ponies, whilst ActRIIB gene expression was down-regulated in the obese state for 3 out of the 4 skeletal muscles studied. These findings were partly in agreement with similar studies in mice (Allen *et al.*, 2008) and humans (Hittel *et al.*, 2009) and indicate some form of negative feedback between myostatin and ActRIIB. It was important to note that these differences were not translated into differences at the protein level, a finding which has also been confirmed in other studies (Baumann AP, 2003; Smith *et al.*, 2010), and highlights the dangers of using gene expression data alone to elucidate physiological principles. It must be noted that the precursor form of the myostatin protein was studied in this thesis. At the time at which the studies were performed, no antibody was available against the mature form of the protein.

Of interest, it was found that gene expression of myostatin in the crest WAT was up-regulated in obese animals, although the precursor form of the protein was not detected in this tissue. It is worthy of note that in the horse, crest adipose tissue is histologically distinct from other adipose tissue depots and is considered to be comprised of a greater amount of connective tissue compared to other depots. It is likely that the elevated myostatin gene expression may be a function of the different cell types present in crest fat. Differences in the heterogeneity of cell types within the discrete adipose tissues should always be considered as a source of variation in molecular expression studies. The ELISA used in the current study assessed the concentration of the circulating mature form of myostatin which was found to be significantly elevated in obese animals, a finding which has been

recently supported in humans (Zhu *et al.*, 2014). The reduction in the relative proportion of skeletal muscle to adipose tissue in obesity may partly explain the increased circulating myostatin observed in obese horses and ponies. Furthermore the cause and effect is not clear. The up-regulation of myostatin concentrations may be a direct result of obesity. Conversely, these animals may have become obese partly due to their elevated myostatin concentrations. However, this preliminary study provides some evidence that the myostatin system might be altered in equine obesity. As myostatin inhibition has such profound effects on skeletal muscle mass, it is perhaps unsurprising that myostatin has also been implicated in glucose uptake and insulin sensitivity. Inhibition of myostatin signalling is associated with improvements in whole body insulin sensitivity in mice (Akpan *et al.*, 2009; Guo *et al.*, 2009), whilst more recently, elevated myostatin expression in mice following a high fat diet has been demonstrated to induce insulin resistance in mice through the up-regulation of Casitas B-lineage lymphoma b (Cblb) which led to a subsequent degradation of the insulin receptor IRS-1 in C2C12 myotubes and HepG2 cells (Bonala *et al.*, 2014). Due to the abattoir environment in which samples for the current study were collected, it was inappropriate to assess insulin dynamics or even basal plasma insulin concentrations. The long-term nutritional history of the animals was unknown and all animals had been fasted immediately prior to slaughter. Variation in individual animal responses to fasting and the inevitable stressors of the novel environment, both of which are known to impact on insulin concentrations, were considered to negate the usefulness of measured insulin concentrations as markers of animal health.

Myostatin expression is known to be fibre-type specific, with a greater expression of myostatin noted in fast-twitch fibres (Allen and Unterman, 2007; Kawada *et al.*, 2001). Fibre type proportions were not studied in this chapter; however it is known that weight-loss sensitivity in humans is associated with a higher proportion of type 1 fibres in the *vastus lateralis* muscle (Gerrits *et al.*, 2010). A large genome-wide analysis in horses revealed that a promoter variant and intronic SNP in the myostatin gene was associated with greater proportions of Type 2B fibre types in

Quarter horse breeds, known for their sprinting ability over 1/4 mile (Petersen *et al.*, 2013). Further genetic-based studies may be warranted to investigate whether fibre type proportions are associated with obesity/obesity-predisposition and whether any established polymorphisms in the myostatin gene are associated with obesity/obesity-predisposition in the horse.

The control of myostatin expression has been considered to be regulated by the transcription factor MyoD (Spiller *et al.*, 2002), although it has also been shown to auto-regulate its own expression through Smad-7 (Forbes *et al.*, 2006). Interestingly, myostatin function may also be regulated by post-transcriptional mechanisms through the action of microRNAs (miRNAs) (Allen, 2010; McFarlane *et al.*, 2014; Miretti *et al.*, 2013). MicroRNAs are short, non-coding RNAs that regulate the expression of many genes by binding to target mRNAs to repress translation or induce mRNA degradation. *In vitro* studies have identified several miRNA's that are implicated in physiological processes such as adipogenesis (Xie *et al.*, 2009) and lipid metabolism (Iliopoulos *et al.*, 2010). The discovery of circulating miRNA's has led to the consideration that specific miRNA's may provide a therapeutic treatment for obesity (Peng *et al.*, 2014). Whether specific miRNA's are altered in obesity in the horse remains to be identified, however recent work indicates a differential expression pattern of circulating miRNA's between insulin sensitive and insulin resistant horses (da Costa Santos *et al.*, 2015). Further investigations would be required to establish any differences between lean and obese animals.

Future directions

- *Consolidate current findings by evaluating the expression of the mature form of myostatin protein in equine skeletal muscles.*

- *Conduct prospective weight gain / weight loss studies to evaluate whether changes in serum myostatin concentration or polymorphisms within the myostatin gene could be quantitative markers for obesity predisposition and / or weight loss resistance.*

- *Establish a role for myostatin in insulin dysregulation in the horse.*

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8.6 Lipolytic proteins in equine obesity

Chapter 7 demonstrated clear increases in adipocyte area for obese animals compared to lean animals for 4/5 adipose depots studied. In agreement with findings from **Chapter 4**, whereby epicardial EQUIFAT score was not associated with BCS, no difference in adipocyte area for the epicardial depot was noted between lean and obese animals in **Chapter 7**. Unsurprisingly, no association was found between lipid:protein ratio with adipocyte area for epicardial fat, whilst no difference in the relative abundance of PLIN1 and HSL was identified between lean and obese animals for either internatant or fat cake fractions. Taken together, this would indicate that epicardial fat has a relatively low capacity for fat deposition and likely has alternative functions. As described earlier, epicardial fat has increased gene expression of the brown fat marker, UCP-1 compared to other adipose depots. The presence of brown fat has not yet been established for the adult horse or pony. It could be speculated that the deposition of epicardial fat would differ between breeds of horses and native ponies that are adapted to survive outdoors during harsh winters due its insulator properties. Epicardial fat can be visualised and measured using standard two-dimensional echocardiography in humans and this could also be a method to evaluate epicardial fat thickness in the horse.

Despite the fact that adipocyte area was found to be significantly greater in obese animals in tailhead adipose tissue, and strong associations were noted between lipid:protein ratio and adipocyte area; there were no differences between lean and obese animals for PLIN1 or HSL protein abundance in either the internatant (largely cytosolic) or fat cake (largely lipid droplet) fractions of tissue homogenates. Additionally, there was considerable variation in terms of relative protein abundance between animals, especially in the fat cake fractions for the tailhead depot. This may suggest that those obese animals that had a greater abundance of PLIN1, might have been losing weight, resulting in smaller lipid droplets and therefore a greater relative abundance of PLIN1. As a detailed nutritional history was not available for these animals, interpreting these results proved difficult.

The greatest range in adipocyte area between lean and obese animals was

observed in retroperitoneal adipose tissues. In lean animals, this retroperitoneal depot had a significantly greater abundance of both PLIN1 and HSL in both the internatant and fat cake fractions. The large range in cell size noted in the obese animals may be indicative of hyperplasia. Protein signals were more consistent between animals for this depot. It is likely that retroperitoneal fat has a greater capacity for lipid storage and lipid mobilisation compared to other depots and is therefore the dominant long-term storage depot for excess energy. This would agree with data from **Chapter 4** whereby retroperitoneal EQUIFAT score was strongly associated with BCS. As discussed in **Chapter 7,** it could be speculated that the quantity of PLIN1 and HSL proteins per fat cell remains unchanged between lean and obese animals and the apparent reduction in the relative abundance of PLIN1 and HSL may be consequence of the increased size of adipocytes in the obese animals. The expansion of lipid droplets in obesity would therefore not be accompanied by a relative increase in PLIN1 protein concentration which may lead to lipid droplet instability and elevated basal lipolysis rates. It is acknowledged that lipid droplet size was not quantified in this study, however it is generally accepted that increased adipocyte size observed in obesity is primarily due to the expansion of the lipid droplet within the adipocyte.

Fewer alterations in the abundance of PLIN1 and HSL proteins were noted between lean and obese animals for crest and omental WAT depots. If the above speculation regarding cell size and relative protein abundance can be confirmed, the lower range of adipocyte areas between lean and obese animals for crest and omental depots may account for the less pronounced differences in the relative abundance of PLIN1 and HSL between lean and obese animals.

In addition, an interesting concept being addressed in the human literature surrounds the notion of adipose tissue expandability limits. This theory suggest that it may not be the absolute amount of fat deposited that causes metabolic disease but rather that exceeding the functional capacity of adipose tissue to expand that results in a pathogenic state (Virtue and Vidal-Puig, 2010). In a human study, it was found that the gene expression of lipid-droplet associated molecules, PLIN1, the

Cide domain-containing protein, Cidea and Cidec/FSP27 were significantly elevated in omental and subcutaneous adipose tissue of obese, insulin-resistant compared to obese, insulin-sensitive individuals (Puri *et al.*, 2008). Whilst protein expression was not assessed by these authors, this finding may support the hypothesis that lipid-droplet associated proteins play an important role in expandability limits of adipose tissues. Although the relative abundance of PLIN1 protein was consistently lower in all obese animals compared with lean animals for retroperitoneal adipose tissue (a depot which appeared to have the greatest capacity for expandability), the variation between animals for other depots eluded to earlier, may have been associated with unmeasured differences in insulin dynamics between individual obese animals and this in turn could be related to differences in adipose tissue expandability limits. Furthermore, the lack of consensus regarding associations between inflammatory factors and obesity in horses and ponies and the fact that not all obese horses and ponies will develop insulin dysregulation or laminitis may be linked to the individual's ability to expand adipose tissue appropriately.

In the context of comparison of protein abundances between lean and obese animals within adipose tissue fractions, the assessment of PLIN1 and HSL protein expression between the internatant and fat cake fractions was also described in **Chapter 7**. However due to methodological limitations discussed, it was not appropriate to quantify these differences due to a lack of a suitable loading control to compare between fractions. However, identifying a protein that is expressed to the same degree in both fractions may not be possible due to the inherent differences between fractions. The visible differences when the MemCode protein stain was applied between fractions was stark, despite the same amount of protein loaded. This may be due to a relatively low abundance of numerous proteins in the fat cake fraction compared with high abundance of proteins at around 65kDa in the internatant fraction. The MemCode stain is a highly sensitive protein stain with a lower limit of detection of 25-50ng protein per band. In **Chapter 7**, spiking samples with BSA was attempted, however it was found that the band displayed was at a similar molecular weight to an already prominent band in the internatant fraction. This method could prove successful for comparing between fractions if a protein

with either high or low molecular weight was used.

Further analysis of adipose tissues employing methods such as Oil-red O to assess lipid droplet size, would be beneficial and add to our understanding of lipid droplet dynamics in obesity. Estimating the contribution of hyperplasia to the growth of adipose depots would be less straightforward. Knowledge of the total weight of individual depots would be required to gain an accurate indication of adipocyte numbers. However, the Coulter counting method described by Hirsch and Gallian (Hirsch and Gallian, 1968) could be employed to assess the number of adipocytes present per unit weight of tissue.

Obtaining adipose tissue samples from a rigorously phenotyped group of animals with known nutritional history and assessing the relative contributions of the phosphorylated forms of PLIN1 and HSL would aid in our understanding of the regulation of lipolysis in obesity for the horse. It was not intended to establish differences in the relative abundance of PLIN1 and HSL between depots in the current study and further studies would be required to establish depot-differences in these proteins which may reflect regional differences in lipolysis rates as observed in humans.

One limitation of Chapters 6 and 7 is the relatively small sample size used and the mixed-breed nature of these animals. In an ideal scenario, we had hoped to obtain tissues from lean and obese animals from the same breed type; however it became apparent that the population of animals observed at the abattoir was not representative of the general leisure horse population in the UK. Very few truly obese animals were presented at the abattoir. The dichotomy in phenotype (gender, breed, BCS) between the abattoir population and those included in quantitative surveys evaluating obesity prevalence in the wider UK equine population was noteworthy and intimates limited overlap between sectors. For example, whilst racehorses will account for only a relatively low proportion of the population of horses and ponies owned in the UK, animals from this industry appear to be over-represented in the abattoir population. Given time constraints within the project, it was not possible to wait for obese animals of the same breed

to be presented. In addition, no nutritional history was available for the animals used, therefore it is unknown whether the obese animals had been in that body condition for a considerable length of time and likewise for the lean animals it is unknown whether these animals were actively losing or gaining weight at the time of sampling. Given the relative infrequency of encountering lean or obese slaughter animals, it was not possible to account for seasonal changes. Samples were collected over a period of about 18 months in total, with lean animal samples collected in March (x 3), May, June and December and obese animals samples collected in March, September (x 2), October, November and December.

At the time that the ideas behind this thesis were conceived, both myostatin and PLIN1 were emerging as highly topical areas of research in the fields of obesity and associated metabolic disorders. Whilst blocking of myostatin function was considered to be an exciting therapeutic target for obesity treatment, the discovery that the loss of myostatin function resulted in an upregulation of genes involved in brown adipose tissue formation (Zhang *et al.*, 2012) generated considerable interest. The secretion of a novel myokine, irisin from skeletal muscle of myostatin-null mice was clearly implicated in driving the 'browning' of white adipose tissue (Shan *et al.*, 2013), and since then, a wealth of studies have emerged describing associations between irisin and obesity and associated metabolic derangements observed in obesity. The ability to increase energy expenditure through the conversion of white adipose tissue to brown adipose tissue poses as an effective therapeutic target to treat obesity. More recently, irisin infusion in obese mice has been proposed to improve insulin sensitivity and enhance lipolysis through increased HSL expression and phosphorylation in combination with a reduced expression of PLIN1 (Xiong *et al.*, 2015). Recently, fibronectin type III domain-containing protein 5 (FNDC5), the gene encoding irisin was found to be up-regulated by exercise training in Thoroughbred horses (McGivney *et al.*, 2014). Whilst it remains to be known whether adult horses possess any brown fat and secrete irisin, this could certainly be an area for further investigations.

Future directions

- *Evaluate any associations between PLIN1 protein concentration and basal lipolysis rates in horses.*

- *Determine whether specific breed and / or adipose tissue depot differences are implicated in the capability of individual adipose tissues to expand. This may improve our understanding the metabolically healthy / unhealthy phenotypes.*

- *There is a requirement to establish a suitable loading control for the quantification of lipid droplet proteins.*

- *Assessing lipid droplet size by a staining technique such as Oil-red O or electron microscopy would enable the quantification of lipid droplet size and changes in obesity.*

- *Conduct in-depth studies using samples obtained from rigorously-phenotyped horses and ponies to evaluate the precise contribution of phosphorylated PLIN1 and HSL to lipolysis and establish adipose depot-differences in lipolysis regulation.*

- *Establish whether functional brown fat is present in adult horses and ponies and evaluate whether the myokine irisin is present and plays a role in 'browning' fat.*

8.7 Conclusions

Evidence presented in this thesis encompasses a range of aspects in equine obesity and adds to our current knowledge of obesity perceptions, differing functional aspects of the adipose depots in the horse and provides novel information concerning how the myostatin system and elements of the lipolytic system are altered in equine obesity. The primary conclusions are as follows:

- Horse owners and enthusiasts have a limited ability to recognise overweight horses and ponies from photographic images and it was deemed more acceptable for horses and ponies to carry more weight/condition for competing in affiliated showing classes (**Chapter 3**).

- Retroperitoneal EQUIAT fat score has a strong association with BCS whilst omental EQUIFAT score had a weaker association and mesenteric as well as epicardial scores had no association with BCS, indicating different functions of the regional adipose depots in the horse (**Chapter 4**).

- RNA remains intact up to 30 minutes and 120 minutes *post-mortem* in adipose tissue and skeletal muscle, respectively. The expression of myostatin is generally restricted to skeletal muscles, whilst PLIN1 expression is predominant in adipose tissues in the horse (**Chapter 5**).

- Circulating myostatin concentration in blood serum and myostatin gene expression in skeletal muscles is elevated in obese horses and ponies (**Chapter 6**).

- Adipocyte area was increased in obese animals for all depots except epicardial fat. PLIN1 and HSL relative protein abundance was reduced in obese animals in retroperitoneal fat for both fat cake and internatant fractions. Fewer differences were identified between lean and obese animals for the other depots studied (**Chapter 7**).

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Appendix A

Appendix A: Kohnke body condition scoring system

Condition Score	General Condition	Neck	Withers	Loin	Tailhead	Ribs	Shoulder
1	Very Poor	Individual bone structure visible	Bones easily visible. No fat	Spine bones visible. Ends feel pointed	Tailhead & hip bones very visible	Ribs v. visible & skin furrows between ribs	Bone structure v. visible
		Animal extremely emaciated; no fatty tissue can be felt					
2	Very Thin	Bones just visible. Animal emaciated	Withers obvious, very minimal fat covering	Slight fat covering over vertical & flat spine projections. Ends feel rounded	Tailhead, hip bones obvious	Ribs prominent, slight depression between ribs	Bone structure can be outlined
3	Thin	Thin, flat muscle covering	Withers accentuated with some fat cover	Fat build-up halfway on vertical spines, but easily discernible. Flat spinal bones not felt	Tailhead prominent. Hip bones appear rounded but visible. Pin bones covered	Slight fat cover over ribs. Rib outline obvious	Shoulder accentuated, some fat
4	Moderately Thin	Neck some fat, not obviously thin	Withers not obviously thin, smooth edges	Slight ridge along back	Fat can be felt	Faint outline visible	Shoulder not obviously thin
5	Moderate	Neck blends smoothly into body	Withers rounded over top	Back level	Fat around tailhead beginning to feel spongy	Ribs can't be seen but can be easily felt	Shoulder blends smoothly into body
6	Moderately Fleshy	Fat can be felt	Fat can be felt	May have slight inward crease	Fat around tailhead feels soft	Fat over ribs feels spongy	Fat layer can be felt
7	Fleshy	Visible fat deposits along neck	Fat covering withers is firm	May have slight inward crease down back	Fat around tailhead is soft & rounded off	Individual ribs can still be felt	Fat build up behind shoulder
8	Fat	Noticeable thickening of neck	Area along withers filled with fat	Crease down back evident	Tailhead fat v. Soft & flabby	Difficult to feel ribs	Area behind shoulder filled in flush with body
		Fat deposited along inner buttocks					
9	Extremely Fat	Bulging fat	Bulging fat	Obvious deep crease down back	Bulging fat around tailhead	Patchy fat over ribs	Bulging fat
		Fat along inner buttocks may rub together. Flank filled in flush					
Score (1-9)							
Overall BCS = Total/6							

Adapted from: Kohnke, J. 1992. Feeding and nutrition: The making of a champion. Birubi Pacific, Australia. pp.163-166.

Appendix B

Appendix B: Tier 1

1. Which region of the United Kingdom do you live in?

a. Northern Scotland   b. North East Scotland   c. North West Scotland
d. Eastern Scotland   e. Western Scotland   f. Southern Scotland
g. South East Scotland   h. South West Scotland   i. Wales
j. Northern Ireland   k. Midlands   l. North East England
m. North West England   n. South East England   o. South West England
p. Southern England   q. Eastern England

2. What age category do you fall into?

a. Over 60   b. 41-60   c. 26-40   d. 18-25   e. Under 18

3. Are you:

a. Male   b. Female

4. Do you currently:

a. Own your own horse/pony
b. Loan a horse/pony
c. Don't currently own/loan but have done previously
d. Ride at a local riding school
e. Have never owned/loaned a horse/pony but have a keen interest
f. Other (please specify)

5. Overall, how long have you had an active interest in horses and/or riding/driving horses?

a. More than 40 years   b. 20-40 years   c. 6-19 years
d. 1-5 years   e. Less than 1 year

6. Which of the following disciplines are you interested in/compete in?

a. Dressage   b. Eventing   c. Showjumping
d. Showing   e. Pony Club/Riding Club f. Driving
g. None of the above – leisure rider/happy hacker   h. Other (please specify)

7. Which of the following best describes your involvement/interest in horses and ponies?

a. They form part of or my entire job (I make money from my interest/involvement with horses/ponies)
b. My interest/involvement is purely for fun/enjoyment
c. Other (please specify)

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8. Based on the pictures below, which of the following horses/ponies do you consider to be overweight? You can choose as many as you wish.

9. Based on the picture above, and based only on the weight/condition of the horse/pony, select whether the horse/pony is the appropriate weight for the activity stated below:

	Very Underweight	Slightly Underweight	About Right	Slightly Overweight	Very Overweight
Mainly staying in the field with the occasional weekend hack					
Competing at affiliated one day events					
Competing in affiliated showing classes					
Competing at affiliated dressage					

10. Based on the picture below, and based only on the weight/condition of the horse/pony, select whether the horse/pony is the appropriate weight for the activity stated below:

	Very Underweight	Slightly Underweight	About Right	Slightly Overweight	Very Overweight
Mainly staying in the field with the occasional weekend hack					
A busy summer involving Pony Club camp and one day events					
Competing in affiliated showing classes					

11. Based on the picture above, and based only on the weight/condition of the horse/pony, select whether the horse/pony is the appropriate weight for the activity stated below:

	Very Underweight	Slightly Underweight	About Right	Slightly Overweight	Very Overweight
Mainly staying in the field with the occasional weekend hack					
Competing at affiliated one day events					
Competing in affiliated showing classes					
Competing at affiliated dressage					

Based on the above pictures, match each picture to the appropriate scenarios in the following questions:

12. This horse/pony is obese and needs to go on a strict diet ASAP

         A   B   C   D

13. This horse/pony could do with gaining a little bit of weight/condition before a busy summer competing

         A   B   C   D

14. This horse/pony has just finished the hunting season and is ready for a busy summer competing in one day events

         A   B   C   D

15. This horse/pony could do with losing some weight before attending Pony Club camp

         A   B   C   D

16. Based on the pictures above, rank these horses/ponies in order of increasing body weight/condition (1 = very thin to 5 = very fat)

	1	2	3	4	5
A					
B					
C					
D					
E					

Appendix C

Appendix C: Tier 2

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SECTION 1: About You   (Please circle appropriate answers)

1. Which region of the United Kingdom do you live in?

a. Northern Scotland   b. North East Scotland   c. North West Scotland

d. Eastern Scotland   e. Western Scotland   f. Southern Scotland

g. South East Scotland   h. South West Scotland   i. Wales

j. Northern Ireland   k. Midlands   l. North East England

m. North West England   n. South East England   o. South West England

p. Southern England   q. Eastern England

2. What age category do you fall into?

a. Over 60   b. 41-60   c. 26-40   d. 18-25   e. Under 18

3. Are you:

a. Male   b. Female

4. How many horses or ponies do you own/care for? ....

(If more than one horse or pony owned pleased select only one to complete the rest of the questionnaire)

5. Name of horse/pony chosen to complete questionnaire: ....

6. Where do you keep your horse/pony?

a. At home   b. At a friend's   c. Full livery   d. Part livery

e. DIY livery   f. Full livery + riding and/or competing for you

7. Which of the following best describes you as a horse/pony keeper?

a. Pet keeper (keeping for pleasure, don't ride)

b. Professional yard manager

c. Professional rider/instructor/competitor (this is your full-time job)

d. Hobbyist/leisure rider/driver

e. Amateur competitor (affiliated and unaffiliated riding/driving competitions)

f. Keeping for someone else

g. Pleasure rider/competing locally at unaffiliated riding/driving competitions

8. Which of the following societies/associations are you a current member of?

a. British Horse Society   b. Endurance GB

c. Riding Club   d. British Eventing

e. British Showjumping   f. British Dressage

g. Association of British Riding Schools   h. British Carriage driving

i. The Pony Club   j. None

k. Other, please specify: ....

9. Overall, how long have you had an active interest in horses and/or riding/driving horses?

a. More than 40 years   b. 1-5 years   c. 6-19 years

d. 20-40 years   e. Less than 1 year

SECTION 2: About your horse/pony

10. How old is your horse/pony?

a. Less than 4 years old   b. 4-10 years old   c. 11-16 years old

d. 17-21 years old   e. More than 21 years old

11. How many years have you owned/cared for your horse/pony?

a. 11 years or more   b. 6-10 years   c. 1-5 years

d. Less than 1 year

12. Which of the following height categories does your horse/pony fit into?

a. Under 12hh   b. 12.1-14.2hh   c. 14.3-16hh   d. Over 16hh

13. Gender:

a. Mare/Filly   b. Gelding   c. Stallion/Colt

14. Colour:

a. Chestnut   b. Black   c. Bay   d. Grey

e. Coloured   f. Roan   g. Palomino   h. Dun

i. Other, please specify: ....

15. Which of the following breed categories does your horse/pony best fit into?

a. UK native breed of horse/pony   b. Thoroughbred/Thoroughbred type

c. Warmblood/Warmblood type   d. Cob

e. Draught horse   f. Arab/Arab type

g. Irish Draught/Irish Draught type   h. Cross-breed pony

i. Sport horse/pony   j. Other, please specify:

SECTION 3: Health and Wellbeing of your horse/pony

16. In the past 3 months, has a vet attended your horse/pony? *IF YES - PLEASE ANSWER Q17*

a. Yes   b. No

17. For what reason did a vet attend your horse/pony?

a. Lameness   b. Laminitis   c. Colic

d. Dental   e. Respiratory infection   f. Wound

g. Routine vaccination   h. Microchipping   i. Castration

j. Skin condition   k. Other, please specify: ....

18. To the best of your knowledge, has your horse/pony ever had laminitis?

IF YES – PLEASE ANSWER Q19 & 20

a. Yes   b. No

19. Was it diagnosed by a vet?

a. Yes   b. No

20. Has your horse/pony had more than one laminitic episode?

a. Yes   b. No

21. Has your horse/pony ever been diagnosed with Equine Metabolic Syndrome (EMS)?
IF YES - PLEASE ANSWER Q22

a. Yes   b. No

22. Was it diagnosed by a vet?   *IF YES - PLEASE ANSWER Q23*

a. Yes   b. No

23. Was it diagnosed with a blood test?   *IF YES - PLEASE ANSWER Q24*

a. Yes   b. No

24. Please provide any further information including the type of blood test performed if known: ……….

25. Has your horse/pony ever been diagnosed with Cushings disease (Pars pituitary intermedia dysfunction, PPID)?

a. Yes   b. No

26. How often do you worm your horse/pony?

a. Never   b. Every 4 weeks   c. Every 6 weeks

d. Every 8 weeks   e. Every 12 weeks   f. If indicated to do so from

worm egg counts   g. Other, please specify: ………………………………

27. Do you measure your horse/pony's body weight with a weigh tape or scales?

a. Yes   b. No

28. What do you think your horse/pony weighs to the nearest 50kg? ....

29. Which one of the following descriptors best describes the weight/condition of your horse/pony during the different seasons? (Please tick the appropriate boxes)

	Very Overweight	Slightly Overweight	About Right	Slightly Underweight	Very Underweight
Spring					
Summer					
Autumn					
Winter					

30. Compared to this time last year, do you think that your horse/pony has:

a. Put on weight/condition   b. Lost weight/condition

c. Is at a similar weight/condition

31. In your opinion, rank the following in order of importance, with 1 being the most important and 6 the least important:

a. Maintaining your horse/pony at a healthy weight/condition: ……………

b. Having your horse/pony's feet regularly trimmed/shod: …………………..

c. Having your horse/pony's teeth regularly checked: …………………….

d. Picking your horse/pony's feet out on a daily basis: …………………….

e. Having your horse/pony's back regularly checked: …………………….

f. Grooming your horse or pony on a daily basis: …………………………..

32. On a scale from 1 (very poor) to 9 (extremely fat) what number would you assign your horse/pony in its' current condition?

………………………………………

SECTION 4: Use and Exercise of your horse or pony

33. Which of the following best describes the main reason for which your horse/pony is kept?

a. Pet/companion

b. Hacking/schooling/hunting (non-competitive riding)

c. Breeding

d. Riding club/Pony club/Driving (unaffiliated competitions)

e. Affiliated competitions (Dressage/SJ/Eventing/Driving etc.)

34. Is your horse/pony current exercised?

a. No – not currently in any work   b. Yes – primarily ridden work

c. Yes – primarily driven work   d. Yes – primarily in-hand/lunging work

35. Which one of the following best describes the current workload of your horse/pony?

a. Light work   b. Medium work

c. Hard work   d. Not in work

36. On average, how many days do you ride per week in the following months:

a. December-February: ....   b. March-May: ....

c. June – August: ....   d. September – November: ....

37. On average, how many hours do you ride per week in:

a. December-February: ....   b. March-May: ....

c. June – August: ....   d. September – November: ....

38. Which one of the following descriptors best describes the current fitness of you horse/pony?

a. Unfit   b. Moderately fit   c. Very fit   d. Extremely fit

SECTION 5: Nutrition and management of your horse/pony in the summer months (May to September)

39. In the summer months (May-September), do you ROUTINELY feed any of the following?

a. Straights (oats, bran etc.)   b. Combination of feeds

c. Conditioning mix/cubes   d. Stud mix/cubes

e. Competition mix/cubes   f. Pasture/leisure/pony mix/cubes

g. Fibre blend   h. Balancer

i. None   j. Other, please specify: ....

40. In the summer months (May-September), do you EVER OCCASIONALLY feed any of the following?

a. Straights (oats, bran etc.)   b. Combination of feeds

c. Conditioning mix/cubes   d. Stud mix/cubes

e. Competition mix/cubes   f. Pasture/leisure/pony mix/cubes

g. Fibre blend   h. Balancer

i. None   j. Other, please specify: ....

41. In the summer months (May-September), do you provide your horse/pony with supplements (excluding vitamins and minerals)?

a. No   b. Yes – Joint   c. Yes – Hoof   d. Yes – Digestion

e. Yes – Behaviour   f. Yes - Immunity   g. Yes – Lifestyle   h. Yes – Wellbeing

i. Yes – Electrolytes   j. Yes – Weight management

k. Other, please specify: ....

42. Which of the following best describes your horse/pony's daily routine in the summer months (May – September)?

a. Lives out at grass – may only come into a stable to be tacked up or groomed

b. Is turned out either at night or during the day (~8 hours at grass)

c. Out at grass for part of the night or day (between 1 and 8 hours at grass)

d. Stabled at all times (except when ridden/driven)

e. Other, please specify: ....

43. Does your horse/pony have access to hay/haylage/soaked hay on a typical summer day (May-September)?

a. Yes – receives ad libitum (as much as it can eat) of hay/haylage/soaked hay when stabled

b. Yes – receives restricted amounts of hay/haylage/soaked hay when stabled

c. Yes – receives ad libitum hay/haylage/soaked hay in the field

d. Yes – receives restricted amounts of hay/haylage/soaked hay in the field

e. No

f. Other, please specify: ....

44. If stabled in the summer months (May-September), what type of bedding is used?

a. Shredded paper/cardboard   b. Shavings/sawdust

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c. Straw   d. Rubber matting only

e. N/A   f. Other, please specify: ....

45. If stabled in the summer months (May-September), is your horse/pony:

a. N/A

b. Able to see other horses/ponies (i.e. Over door, through grill etc.)

c. Isolated from seeing other horses/ponies

46. Is your horse/pony usually clipped during the summer months (May-September)?

a. Yes   b. No

47. Which of the following best describes the type of grazing your horse/pony has access to during the summer months (May-September)?

a. Unrestricted grazing

b. Restricted grazing – strip grazing

c. Restricted grazing – starvation paddock

d. Restricted grazing – limited by time availability

e. No access to grazing

48. If out at grass during the summer months (May-September), is your horse/pony:

a. On its own

b. Out with others (including livestock)

c. No access to grazing

49. If your horse/pony has access to grazing during the summer months (May-September), which of the following best describes the pasture type?

a. Unknown   b. Specific for horses

c. Originally planted for cattle/sheep   d. No access to grazing

e. Hill grazing   f. Permanent pasture

g. Unimproved grass   h. Other, please specify: ....

50. Is the pasture used for grazing fertilised?

a. Yes   b. No   c. Unknown   d. No access to grazing

51. Does your horse/pony typically wear a grazing muzzle when out at grass during the summer months (May-September)?   *IF YES, PLEASE ANSWER Q52*

a. Yes   b. No

52. On average, how many hours during the summer months (May-September) does your horse/pony wear a grazing muzzle?

a. Less than 3 hours   b. Between 3 and 8 hours   c. More than 8 hours

SECTION 6: Nutrition and management of your horse/pony in the winter months (November-March)

53. In the winter months (November-March), do you ROUTINELY feed any of the following?

a. Straights (oats, bran etc.)   b. Combination of feeds

c. Conditioning mix/cubes   d. Stud mix/cubes

e. Competition mix/cubes   f. Pasture/leisure/pony mix/cubes

g. Fibre blend   h. Balancer

i. None   j. Other, please specify: ....

54. In the winter months (November-March), do you EVER OCCASIONALLY feed any of the following?

a. Straights (oats, bran etc.)   b. Combination of feeds

c. Conditioning mix/cubes   d. Stud mix/cubes

e. Competition mix/cubes   f. Pasture/leisure/pony mix/cubes

g. Fibre blend   h. Balancer

i. None   j. Other, please specify: ....

55. In the winter months (November-March), do you provide your horse/pony with supplements   (excluding vitamins and minerals)?

a. No   b. Yes – Joint   c. Yes – Hoof

d. Yes – Digestion   e. Yes – Behaviour   f. Yes - Immunity

g. Yes – Lifestyle   h. Yes – Wellbeing   i. Yes – Electrolytes

j. Yes – Weight management   k. Other, please specify: ....

56. Which of the following best describes your horse/pony's daily routine in the winter months (November-March)?

a. Lives out at grass – may only come into a stable to be tacked up or groomed

b. Is turned out either at night or during the day (~8 hours at grass)

c. Out at grass for part of the night or day (between 1 and 8 hours at grass)

d. Stabled at all times (except when ridden/driven)

e. Other, please specify: ....

57. Does your horse/pony have access to hay/haylage/soaked hay on a typical winter day (November-March)?

a. Yes – receives ad libitum (as much as it can eat) of hay/haylage/soaked hay when stabled

b. Yes – receives restricted amounts of hay/haylage/soaked hay when stabled

c. Yes – receives ad libitum hay/haylage/soaked hay in the field

d. Yes – receives restricted amounts of hay/haylage/soaked hay in the field

e. No

f. Other, please specify: ....

58. If stabled in the winter months (November-March), what type of bedding is used?

a. Shredded paper/cardboard   b. Shavings/sawdust

c. Straw   d. Rubber matting only

e. N/A   f. Other, please specify: ....

59. If stabled in the winter months (November-March), is your horse/pony:

a. N/A

b. Able to see other horses/ponies (i.e. Over door, through grill etc.)

c. Isolated from seeing other horses/ponies

60. Is your horse/pony usually clipped during the winter months (November-March)?

a. Yes   b. No

61. Which of the following best describes the type of grazing your horse/pony has access to during the winter months (November-March)?

a. Unrestricted grazing

b. Restricted grazing – strip grazing

c. Restricted grazing – starvation paddock

d. Restricted grazing – limited by time availability

e. No access to grazing

62. If out at grass during the winter months (November-March), is your horse/pony:

a. On its own

b. Out with others (including livestock)

c. No access to grazing

63. If your horse/pony has access to grazing during the winter months (November-March), which of the following best describes the pasture type?

a. Unknown   b. Specific for horses

c. Originally planted for cattle/sheep   d. No access to grazing

e. Hill grazing   f. Permanent pasture

g. Unimproved grass   h. Other, please specify: ....

64. Does your horse/pony typically wear a grazing muzzle when out at grass during the winter months (November-March)?   *IF YES, PLEASE ANSWER Q65*

a. Yes   b. No

65. On average, how many hours during the winter months (November-March) does your horse/pony wear a grazing muzzle?

a. Less than 3 hours   b. Between 3 and 8 hours   c. More than 8 hours

Appendix D

EQUIFAT: Regional adipose tissue scoring system

	OMENTAL FAT	
	Evaluate ~30cm of omentum extending distally from the midpoint of the greater curvature of the stomach	
Score	**Descriptor**	**Exemplar**
1	No or minimal fat visible	
2	Fat in immediate vicinity of the gastroepiploic vessels (GEVs) but vessels still clearly visible.	
3	Distinct fat deposits around and beginning to fill the spaces between GEVs. GEVs partially obscured by fat.	
4	Extensive accumulations of fat largely obscuring and filling the spaces between most GEVs	
5	Omental peritoneum and GEVs completely obscured by fat.	

	MESENTERIC FAT	
	Evaluate ~30cm of mesentery extending distally from the serosal margin of a ~0.5 m loop of proximal jejunum.	
Score	**Descriptor**	**Exemplar**
1	No or minimal fat visible.	
2	Fat in the immediate vicinity of the superior mesenteric vessels (SMVs) but arterial arcades still clearly visible.	
3	Distinct fat deposits around and beginning to fill the spaces between SMVs. SMVs partially obscured by fat.	
4	Extensive accumulations of fat largely obscuring and filling spaces between most arcades of the SMVs.	
5	Mesenteric peritoneum, SMVs completely obscured by fat.	

EPICARDIAL FAT

Evaluate left heart

Score	Descriptor	Exemplar
1	No or minimal fat visible	
2	Fat limited to immediate vicinity of coronary groove (CG) and paracornial interventricular branch of left coronary artery (PIBLCA). Entire PIBLCA visible. Fat 'level' with adjacent myocardium.	
3	Fat 'tendrils' extend from vicinity of PIBLCA across myocardium. Proximal limits of PIBLCA partially obscured by fat. Fat minimally protruding over myocardial surface.	
4	Lobular fat emanating from CG & PIBLCA only distal PIBLCA visible. Fat protruding above myocardium but ≥50% of ventricular myocardium visible.	
5	CG & PIBLCA completely obscured by lobular fat. Fat in folded bulges with < 50% ventricular myocardium visible.	

NUCHAL CREST FAT:	Depth at craniocaudal midpoint				
Score	1	2	3	4	5
Depth (cm)	0 – 2.9	3 – 5.9	6 – 8.9	9 – 11.9	≥12

RUMP FAT

Evaluate dorsal rump from point of tailhead over loin

Score	Descriptor	Exemplar
1	No / minimal fat cover. Flesh clearly visible. (**<10% fat cover**)	
2	Visible fat on tailhead, flesh remains visible lower down rump (**10 – 25% fat cover**).	
3	Small patches of flesh may remain visible. (**25 – 50% fat cover**).	
4	Flesh not clearly visible, fat may have bulging appearance. (**50 – 75% fat cover**).	
5	Fat appears thick and more protruding. (**>75% fat cover**)	

ABDOMINAL RETROPERITONEAL FAT: Cr/Cau midpoint					
Score	1	2	3	4	5
Depth (cm)	0 – 1.9	2 – 3.9	4 – 5.9	6 – 7.9	≥8

Appendix E

Appendix E: Anatomical location of skeletal muscles sampled

Adductor

(Chapter 5)

Rectus abdominis

(Chapter 5 & 6)

Pectoralis profoundus
(Chapter 6)

Pectoralis transversus

(Chapter 5 & 6)

Longus colli

(Chapter 5 & 6)

Appendix F