Effect of increased adiposity on insulin sensitivity and adipokine concentrations in different equine breeds adapted to cereal-rich or fat-rich meals

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Abstract

The relationships between diet, obesity and insulin dysregulation in equids require further investigation due to their association with laminitis. This study examined the effect of dietary glycaemic load and increased adiposity on insulin sensitivity and adipokine concentrations in different equine breeds. Equal numbers of Standardbred horses, mixed-breed ponies and Andalusian horses were provided with *ad libitum* hay plus either cereal-rich (CHO; n=12), fat-rich (FAT; n=12) or control (CON; n=9) meals over 20 weeks. The isocaloric CHO and FAT diets were fed to induce obesity by gradually increasing the supplementary feeds to provide 200% of daily digestible energy requirements by Week 20. The CON group were fed a basal ration only and maintained moderate body condition.

At Week 20, the CHO and FAT groups demonstrated significantly increased body condition score, body weight, total body fat mass and plasma leptin concentrations compared with the CON group (all P<0.001). The CHO group was found to have lower insulin sensitivity (SI; P<0.001) and higher acute insulin response to glucose (P=0.002) compared with the CON group. In contrast, the FAT group was no different to the controls. Ponies and Andalusians had lower SI values compared with Standardbreds, regardless of diet group (P=0.001). Adiponectin concentrations were similar between the FAT and CON groups, but were significantly lower in the CHO group (P=0.010). The provision of cereal-rich meals appeared to be a more important determinant of insulin sensitivity than the induction of obesity *per se*. Whether hypoadiponectinaemia is a cause or consequence of insulin dysregulation warrants further investigation.

Keywords: Nutrition; Equine; Hyperinsulinaemia; Insulin resistance; Laminitis; Obesity

Introduction

Laminitis associated with insulin dysregulation is an important cause of morbidity in domestic equine populations (Harris et al., 2006; Katz and Bailey, 2012). Insulin dysregulation is an umbrella term that includes insulin resistance, fasting hyperinsulinaemia and/or exaggerated insulin responses to oral carbohydrates (Frank and Tadros, 2014). Together with obesity (generalised or regional adiposity), insulin dysregulation has been considered to be a central component of equine metabolic syndrome (EMS) – the clinical phenotype of many equids predisposed to pasture-associated laminitis (Frank et al., 2010). Pasture-associated laminitis also occurs in non-obese horses and ponies (Bailey et al., 2007; Geor, 2010); therefore, the link between obesity and insulin dysregulation requires further investigation. Other aspects of EMS that warrant additional study include alterations to adipokines (adipose-derived hormones such as leptin and adiponectin) and proinflammatory cytokines (Burns et al., 2010; Caltabilota et al., 2010; Wooldridge et al., 2012; Wray et al., 2013).

An apparent association between the induction of obesity and the development of hyperinsulinaemia and insulin resistance was demonstrated in a controlled study of Arabian geldings (Carter et al., 2009a). These changes occurred when horses were provided with multiple 'sweet feed' (cereal-rich) meals per day. The role of diet in the development of insulin dysregulation is an important consideration, because the adaptation of horses to 'sweet feed' meals can induce insulin resistance independent of obesity (Hoffman et al., 2003; Treiber et al., 2005). There is also evidence that weight gain can occur without reduced insulin sensitivity when horses and ponies are provided with relatively low-glycaemic rations (Quinn et al., 2008; Bamford et al., 2015a). Additionally, a once-daily oral glycaemic load

appeared to improve insulin sensitivity in a group of horses and ponies (Bamford et al., 2015a). Therefore, multiple daily episodes of hyperinsulinaemia may be a necessary precedent of insulin resistance through the chronic over-stimulation of insulin receptors (Kronfeld et al., 2005; Suagee et al., 2011). The breed of animals studied also needs to be considered, as differences in the innate insulin sensitivity of different breeds will influence the insulinaemic response of an individual to oral non-structural carbohydrates (Bamford et al., 2014).

The purpose of the study reported here was to examine the relative influence of a prolonged twice-daily dietary glycaemic load, compared with an isocaloric intake of vegetable fat, on insulin sensitivity and adipokine concentrations after the induction of obesity in horses and ponies. In addition, the metabolic responses of different equine breeds were compared by enrolling three groups with previously-documented differences in innate insulin sensitivity: Standardbred horses, mixed-breed ponies and Andalusian horses (Bamford et al., 2014). We hypothesised that animals gaining weight on a cereal-rich diet would demonstrate lower insulin sensitivity than animals that gained weight on a fat-rich diet.

Materials and methods

Animals

Eleven Standardbred horses (9.5 ± 1.8 years, 457 ± 8 kg, BCS 5.0 ± 0.2), 11 mixed-breed ponies (9.0 ± 1.2 years, 305 ± 17 kg, BCS 5.3 ± 0.3) and 11 Andalusian-cross horses (8.3 ± 1.2 years, 475 ± 17 kg, BCS 5.5 ± 0.2) were studied. No animals demonstrated evidence of pituitary *pars intermedia* dysfunction when screened with a low-dose

dexamethasone suppression test (McFarlane, 2011), nor did they have clinical or radiographic evidence of prior laminitis. They were kept in large dry lot paddocks with *ad libitum* access to fresh water and hay for at least eight weeks prior to the study. Routine hoof trimming, dental prophylaxis and anthelmintic treatments were provided as appropriate. The use of animals in this study was approved by the University of Melbourne Animal Ethics Committee (ID 1011918).

Study design and diets

Animals were blocked by breed and randomly assigned to one of three diet groups: a cereal-rich diet (CHO), a fat-rich diet (FAT) or a control diet (CON). The CHO and FAT groups contained 12 animals (four of each breed) and received a hypercaloric ration to induce obesity. The CON group contained nine animals (three of each breed) and received only the basal ration.

Over a 20-week study period, all animals were provided with *ad libitum* access to fresh water and the same hay in dry lot paddocks. Diet groups differed in the type and amount of complementary feed provided in twice-daily meals (fed at 08:00 and 16:00) on each day of the study period (Table 1). To facilitate the individual provision of meals, animals were fed in separate yards along the perimeter of the dry lot paddocks. All meals contained a base ration of soaked soyahull pellets (Maxisoy, Energreen Nutrition) and lucerne chaff, with a balanced vitamin and mineral supplement (60 mg/kg BW; Ranvet) added to the morning meals. Animals in the CHO group received additional energy in the form of micronised maize (Micrmaize, Hygain). The amount of micronised maize added to the base ration was gradually increased over the study period to allow for gastrointestinal adaptation (Figure 1).

113 The final amount of micronised maize in the diet reached 4.55 g/kg BW (providing 3.34 g/kg
114 BW of additional non-structural carbohydrate), with the total ration providing approximately
115 200% of daily digestible energy (DE) requirements (NRC, 2007). Animals in the FAT group
116 received an isocaloric amount of supplementary vegetable fat as an equal mix (by weight) of
117 liquid oil (Energy Gold, Kohnke's Own) and granulated (Cool Calories, Buckeye Nutrition)
118 fats. Mirroring the gradual increase in micronised maize for the CHO meals, supplementary
119 vegetable fat was gradually increased in the FAT meals over the study period to allow for
120 gastrointestinal adaptation (Figure 1). To control for seasonal and environmental influences,
121 animals in the CON group also had *ad libitum* access to hay and received meals containing
122 the base ration only throughout the study.
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124 Hay consumption was accurately quantified on three separate occasions (Week 0,
125 Week 12 and Week 20) when horses and ponies were kept in individual yards for a 24-hour
126 period.
127
128 *Assessment of adiposity*
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130 Body weight was measured weekly using calibrated scales. Percentage change from
131 Week 0 (ΔBW) was calculated to account for differences in average starting body weight
132 between breeds. Body condition score was determined weekly by an experienced observer
133 using a 9-point scale (Henneke et al., 1983; Kohnke, 1992). Regional adiposity along the
134 nuchal ligament was assessed using the cresty neck score (CNS) described by Carter et al.
135 (2009b). Total body fat mass (TBFM) was accurately determined during Week 0 and Week
136 20 using deuterium oxide (D_2O) dilution (Dugdale et al., 2011). Briefly, a dose of 0.12 g/kg
137 BW D_2O (Cambridge Isotope Laboratories) was administered through a temporary catheter in

the left jugular vein. Blood samples (20 mL) were collected by venepuncture of the right jugular vein immediately before and 4 hours after D_2O infusion. Syringes were weighed to determine the exact weight of D_2O administered to each animal. Heparinised plasma samples were analysed using gas isotope ratio mass spectrometry (Iso-Analytical Ltd.). Total body fat mass was determined using previously described calculations (Dugdale et al., 2011).

Assessment of insulin sensitivity

Insulin sensitivity was assessed using a previously described insulin-modified frequently-sampled IV glucose tolerance test (FSIGT) during Week 0 and Week 20 (Hoffman et al., 2003). Briefly, horses and ponies were moved from the dry lot on the morning of testing and IV catheters were placed in the left jugular vein under local anaesthesia. Blood samples were collected 60 min, 45 min and immediately before the infusion of a glucose solution (300 mg/kg BW; 40% weight/volume) through the jugular catheter. Twenty minutes later, an insulin bolus (20 mU/kg BW; Actrapid, Novo Nordisk) was delivered by venepuncture of the right jugular vein. Blood samples (10 mL) were collected 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16, 19, 22, 23, 24, 25, 27, 30, 35, 40, 50, 60, 70, 80, 90, 100, 120, 150, 180, 210, 240, 270, 300, 330 and 360 minutes after the glucose infusion. Samples were transferred to tubes containing lithium heparin anticoagulant (Vacutainer, BD) and placed on ice until centrifugation.

Blood collection

Blood samples were collected during Week 0 and Week 20 to determine plasma concentrations of glucose, insulin, leptin, adiponectin, tumour necrosis factor-α (TNF-α) and

serum amyloid A (SAA). Samples (20 mL) were collected from the left jugular vein immediately before the morning meals and transferred to tubes containing lithium heparin (for glucose, insulin, TNF-α and SAA) or EDTA (for leptin and adiponectin) anticoagulants (Vacutainer, BD). Samples were placed on ice until centrifugation.

Laboratory analysis

Blood samples were centrifuged (1000 *g* at 4°C for 10 min), with separated plasma stored at -80°C pending analysis. In all samples, glucose concentrations were measured using an enzymatic colorimetric assay (Cayman Chemical Co.) and insulin concentrations were measured using a radioimmunoassay (Coat-A-Count, Siemens Diagnostics) previously validated for equine samples (Tinworth et al., 2011). Plasma concentrations of leptin (Coat-A-Count, Siemens Diagnostics), high-molecular weight adiponectin (Millipore), TNF-α (Thermo Fisher Scientific) and SAA (Tridelta) were measured in samples from Weeks 0 and Week 20 using previously validated assays (Buff et al., 2002; Lavoie-Lamoureux et al., 2010; Wooldridge et al., 2012).

Data analysis

Glucose and insulin curves from the FSIGT were interpreted using MinMod Millennium software (Version 6.02; University of Pennsylvania). Values of insulin sensitivity (SI), acute insulin response to glucose (AIRg), disposition index (DI) and glucose effectiveness (Sg) were obtained (Boston et al., 2003).

Statistical analyses were performed using the general linear model function in SPSS (Version 22, IBM). Each outcome variable was evaluated using the fixed effects of diet, breed and the interaction between diet and breed. Week 0 values were included as a covariate for all variables with the exception of ΔBW. Significant main effects were compared using Fisher's least significance difference test. Age and sex were not significant (P>0.20) for any of the variables and were therefore not included in the final model. Assumptions of the final model were checked using the Shapiro-Wilk test (normality of residual values) and Levene's test (homogeneity of variance). Data were reported as mean ± SEM unless stated otherwise, with significance accepted when $P<0.05$.

Results

Animals and diets

All animals remained clinically healthy throughout the study period and no episodes of laminitis were observed. The study diets were well tolerated; meal refusals were negligible and there were no signs of gastrointestinal disturbance. Hay consumption (percentage of body weight on a dry matter basis) was measured to be 2.21 ± 0.06%, 2.04 ± 0.11% and 2.39 ± 0.08% for the CHO, FAT and CON groups, respectively. Hay consumption was lower for the FAT group compared with the CON group ($P=0.027$), but was not different between other pairwise comparisons ($P=0.34$). Group hay intake over the study period was consistent with the values recorded for individual consumption.

Adiposity

212 Body condition score, TBFM, CNS and ΔBW were significantly increased (all
213 $P<0.001$) at Week 20 in the CHO and FAT groups compared with the CON group (Table 2;
214 Figure 2). Animals in the CHO and FAT groups were considered "obese" (BCS ≥7), whereas
215 the CON group were in "moderate" body condition (BCS ≤6). Median (range) values for
216 CNS were 3.5 (3.0 – 4.5) for the CHO group, 3.0 (2.5 – 4.5) for the FAT group and 2.0 (1.5 –
217 4.0) for the CON group. No effect of breed was detected for any of the methods used to
218 assess adiposity.
219
220 *Insulin sensitivity*
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222 Insulin sensitivity was decreased in the CHO group relative to the FAT and CON
223 groups (Table 3; $P<0.001$). There was no significant effect of the high-fat diet compared with
224 the control diet. A significant effect of breed was observed, with ponies and Andalusians
225 demonstrating lower values for SI compared with Standardbreds (Figure 3; $P=0.001$). Values
226 for AIRg were higher in the CHO group compared with the FAT and CON groups ($P=0.002$).
227 Glucose effectiveness was not different between diet groups, but there was a significant effect
228 of breed, with Standardbreds demonstrating lower Sg values than ponies and Andalusians
229 ($P=0.013$).
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231
232 *Plasma measurements*
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234 Basal glucose and insulin concentrations were not different between diet groups
235 (Table 4). Increased adiposity resulted in higher leptin concentrations in both the CHO and
236 FAT groups compared with the CON group ($P<0.001$). When breeds were compared, leptin

concentrations in the Andalusians compared with the Standardbreds and ponies resulted in a *P* value of 0.084. Adiponectin concentrations were found to be lower in the CHO group compared with the FAT and CON groups (*P*=0.010). Serum amyloid A concentrations were higher in the CHO group when compared with the FAT and CON groups (*P*=0.009), with no differences in TNF-α detected between groups.

Discussion

In the present study, the induction of obesity was associated with reduced insulin sensitivity in horses and ponies that consumed a cereal-rich ration. In contrast, animals that consumed an isocaloric fat-rich (low-glycaemic) ration did not exhibit a change in insulin sensitivity despite reaching levels of adiposity that did not differ significantly from the CHO group. There was a significant effect of breed across all diet groups, with ponies and Andalusians demonstrating lower insulin sensitivity compared with Standardbreds. Plasma adiponectin concentrations were reduced in the CHO group, supporting an association between hypoadiponectinaemia and insulin dysregulation in equids. These data enable a distinction to be made between the effects of dietary glycaemic load and short-term obesity on certain metabolic changes in equids. Furthermore, this study highlights the influence of breed when investigating these responses.

Insulin sensitivity was assessed using a FSIGT, which is considered to be one of the most accurate quantitative methods used by equine researchers (Firshman and Valberg, 2007). The SI parameter of the minimal model quantifies the ability of insulin to promote glucose uptake from the bloodstream. Significantly lower SI values were recorded in the CHO group compared with the FAT and CON groups. An effect of breed was also present,

with ponies and Andalusians having lower SI values compared with Standardbreds. A compensatory increase in insulin secretion following the IV glucose infusion was observed as higher AIRg values in the CHO group. The disposition index (multiplication product of SI and AIRg) is used to determine the adequacy of the insulin response to a given level of insulin sensitivity, which was not detectably different between diet groups. Glucose effectiveness (Sg) quantifies the ability of glucose to promote its own removal from the bloodstream. Although not different between diet groups, Sg values were found to be lower in Standardbreds compared with ponies and Andalusians. There is some evidence that insulin-independent glucose disposal may be upregulated in animals predisposed to obesity (Hoffman et al., 2003).

The finding of reduced insulin sensitivity in the CHO group is consistent with that of Carter and colleagues (2009a), who induced obesity in a cohort of Arabian geldings using 'sweet feed' (cereal-rich) meals. The mean SI value reported in the present study of 1.49 x 10^{-4}/(mU·min) is relatively modest when compared with that of the Arabians studied by Carter et al. of 0.62 x 10^{-4}/(mU·min). This is due in part to the influence of Standardbreds within each diet group; if Standardbreds are not considered, mean SI in the present study was 0.97 x 10^{-4}/(mU·min). Differences in the level of adiposity may also have influenced results from the FSIGT. The Arabian horses demonstrated slightly higher mean (± SD) values for BCS (8.0 ± 0.7) than horses and ponies in the present study (7.8 ± 0.4). Total body fat mass was also higher in the Arabian horses, but a direct comparison of TBFM values is difficult due to differences in methodology between studies (ultrasonographic fat depth vs. D_2O dilution). Carter and colleagues fed approximately 200% DE requirements for 16 consecutive weeks. In contrast, we gradually increased the amount of micronized in each meal over 20 weeks, reaching 200% DE requirements for the last 2 weeks of the study. Animal ethics

approval for the present study determined the cautious increase in grain over time due to the use of breeds potentially at risk of developing laminitis.

The present study was designed similarly to a previous report by our group that described the metabolic responses of horses and ponies fed either a high-fat diet or an isocaloric diet containing a once-daily glycaemic stimulus (Bamford et al., 2015a). Yielding comparable results to the present study, a decrease in SI was not detected in the high-fat group after the induction of obesity. However, there was a significant increase in SI values for the group provided with a once-daily glycaemic stimulus (as 1.5 g/kg BW dextrose) after the induction of obesity. Based on this finding, it was hypothesised that high insulin concentrations were not sustained for long enough to cause insulin receptor down-regulation, and that chronic stimulation of the pancreas by more than one cereal-rich meal per day might be required to cause a decrease in insulin sensitivity (Williams et al., 2001; Kronfeld et al., 2005; Suagee et al., 2011).

The glycaemic and insulinaemic properties of the CHO and FAT meals used in this study have been previously reported (Bamford et al., 2015a; Bamford et al., 2015b). Maize was chosen as the supplementary cereal because of its high starch content. The micronised form ensured that starch underwent as much precaecal digestion as possible, reducing the risk of hindgut disturbances that can lead to laminitis (Kronfeld and Harris, 2003; Vervuert et al., 2004). Although the CHO meals have been shown to induce robust insulinaemic responses in a previous report, an important observation was the discrepancy in responses between different breeds (Bamford et al., 2015b).

311 Ponies and Andalusians experience a more profound postprandial hyperinsulinaemia
312 than Standardbreds, which is associated with differences in innate insulin sensitivity between
313 these breeds (Bamford et al., 2014). The hyperinsulinaemia experienced by ponies and
314 Andalusians in the CHO group of the present study may have contributed to the lower SI
315 values compared with the Standardbreds. However, despite relatively modest postprandial
316 insulin responses to the CHO meal, Standardbreds exhibited lower SI values at Week 20
317 compared with Week 0. It is not clear whether the decrease in SI values was solely due to the
318 effects of twice-daily postprandial hyperinsulinaemia, or whether there may be other effects
319 of feeding cereals that are involved. Hyperinsulinaemia has been hypothesised to represent
320 one aspect of a genetic predisposition to laminitis in horses and ponies (Harris et al., 2006;
321 Treiber et al., 2006).
322
323 No signs of gastrointestinal upset were observed for any of the diet groups, indicating
324 that the rate of supplementary feed increase was sufficiently cautious. Supplementary
325 vegetable fat was well tolerated whilst providing up to 25% of daily DE in the total ration,
326 supporting a previous finding that supplementary vegetable fat is well tolerated in the horse
327 (Harris et al., 1999; Kronfeld et al., 2004). Hay consumption in the FAT group appeared
328 slightly lower than the CHO group, although the difference between means was not
329 statistically significant. The CON group was included to verify that observations in the CHO
330 and FAT groups were due to the effects of diet and adiposity, and not related to
331 environmental or management factors. Percentage change in body weight was increased in
332 control animals at Week 20 (relative to Week 0) despite equivocal TBFM values. The
333 increase in body weight without increase in adiposity in the controls may be a limitation of
334 the study, although this finding could potentially be due to increased gut fill or more likely an
335 increase in lean body mass in these animals. This is supported by the fact that the control diet

included good quality protein from the soybean hulls and chaff; it has been previously observed that adult horses may increase rates of muscle protein synthesis in response to feeding increased protein (Urschel et al, 2011).

Leptin is an adipokine that is constitutively secreted by mature adipocytes, functioning to signal the existing state of energy balance and aid in the regulation of body weight (Jéquier, 2002). Whether a state of leptin resistance contributes to the exacerbation of obesity in horses with insulin dysregulation has not been determined. Certainly, there is a strong correlation between leptin concentrations and fat mass in horses (Buff et al., 2002; Kearns et al., 2006). In the present study, leptin mirrored adiposity; higher concentrations were present in the CHO and FAT groups compared with the CON group. Leptin was similar between the CHO and FAT groups despite differences in SI and AIRg, suggesting that leptin was reflective of fat mass and not of insulin sensitivity.

In contrast to leptin, adiponectin is often inversely proportional to adiposity (Maury and Brichard, 2010). Hypoadiponectinaemia has been postulated to play a role in the pathogenesis of several comorbidities in humans with metabolic syndrome, due to a reduction in the anti-inflammatory, anti-arthrogenic and insulin-sensitising actions of adiponectin (Fisman and Tenenbaum, 2014). Previous studies of horses have found adiponectin to be negatively correlated with basal insulin concentrations and inversely proportional to fat mass (Kearns et al., 2006; Wooldridge et al., 2012). When laminitis status was considered, previously-laminitic ponies had lower adiponectin concentrations than control ponies (Wray et al., 2013). The present study found adiponectin concentrations to be similar between the FAT and CON groups despite differences in TBFM. However, adiponectin concentrations were significantly lower in the CHO group even though leptin concentrations and TBFM

were similar to the FAT group. This finding suggests that relative hypoadiponectinaemia occurred in animals with lower insulin sensitivity, without concurrent differences in leptin concentrations or TBFM. Further work is required to determine the role of adiponectin in the pathogenesis of equine insulin dysregulation.

There is conflicting information about whether obesity represents a pro-inflammatory state in the horse (Frank and Tadros, 2014). We did not detect differences in plasma TNF-α concentration between groups, supporting a previous finding that cytokine-mediated inflammation was not associated with obesity or insulin dysregulation in horses (Holbrook et al., 2012). Recent work has indicated that SAA might be a better marker of obesity-associated inflammation in horses (Suagee et al., 2013). In the present study, higher plasma concentrations of SAA were detected in the CHO group compared with the FAT and CON groups. Adiposity was similar between the CHO and FAT groups; therefore, a possible explanation for this increase could be a reduction in anti-inflammatory activity due to the relative hypoadiponectinaemia in this group. It is important to note that absolute concentrations of SAA in the CHO group were within the reference interval for horses without infectious or inflammatory conditions (Belgrave et al., 2013).

Specific recommendations for the use of high-energy providing complementary feeds in horses predisposed to laminitis cannot be made on the basis of this study. It is clear, however, that the use of this particular form of supplementary vegetable fat did not result in decreased insulin sensitivity in a population of horses and ponies that became obese. This finding may support the rationale for the use of low-glycaemic meals as an appropriate energy source in breeds predisposed to EMS that require more calories than low non-structural carbohydrate forage can provide. A reduction in dietary glycaemic load has also

been shown to reduce basal insulin concentrations and improve insulin sensitivity in clinical cases of EMS (Morgan et al., 2015). There seems to be a threshold level of dietary non-structural carbohydrates such as starch which lead to a significant glycaemic response and sufficiently high insulin levels to lead to insulin resistance. In the present study, the feed consumed by the CON group contained relatively more starch (14.2 g per 100kg BW per meal) than the FAT group (1.6 g); however, this was still a lot less than the amount of starch consumed by the CHO group (162.3 g per 100kg BW per meal). Pilot studies showed that both the control feed and the fat-rich feed produced minimal glycaemic and insulinaemic effects, and the control feed caused no greater response than the fat-rich feed (data not shown). This probably explains why the CON group did not become more insulin resistant than the FAT group.

A limitation of the present study is that animals were only obese for a short period of time. It cannot be discounted that chronic obesity represents a different metabolic state; additional metabolic derangements may occur with long-standing obesity that predispose certain animals to endocrinopathic laminitis. Further investigation of the chronically obese equine phenotype is required.

Conclusions

The glycaemic load of the diets used in this 20-week study appeared to be a more important influence on insulin sensitivity than the induction of obesity *per se*. Differences in the glucose and insulin dynamics of horse and pony breeds persisted regardless of the diet consumed, with ponies and Andalusian horses less insulin sensitive than Standardbred horses. Adiponectin may play a role in equine insulin dysregulation and warrants further

411 investigation. These data suggest that the development of obesity and insulin dysregulation
412 may be functionally uncoupled, which is an important premise in the further study of obesity-
413 associated disorders in equids.
414
415 **Conflict of interest statement**
416
417 P.A. Harris is both a collaborating author and an employee of WALTHAM, who part-
418 funded this work. None of the authors has a financial or personal relationship with other
419 people or organisations that could inappropriately influence or bias the content of the paper.
420
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Tables

Table 1. Proximate analysis and ingredient composition of the study diets at Week 20.

		Supplementary feed		
	Hay	CHO	FAT	CON
Energy				
DE (MJ/kg feed, DM basis)	7.1	12.4	16.4	9.4
DE (as fed; MJ/100 kg BW)		13.1	13.1	3.8
Nutrient (%)				
CP	7.7	15.6	14.7	11.9
ADF	46.0	22.1	27.3	37.9
NDF	75.8	33.1	38.7	58.6
NSC	9.2	35.9	5.9	18.4
WSC	7.3	5.3	5.5	11.4
Starch	1.8	30.6	0.4	7.0
Fat	1.8	4.0	27.8	3.8
Ash	5.5	5.0	5.9	5.7
Ingredient (g/100 kg BW)				
Soyahull pellets		300	300	200
Chaff		300	300	200
Micronized maize		455	0	0
Fat supplement		0	200	0
Vitamin/mineral supplement		6	6	6

Proximate analysis performed at Equi-Analytical Laboratories. Hay was sourced from a single batch for the duration of the study. Animals were fed either cereal-rich (CHO), fat-rich (FAT) or control (CON) supplementary feeds divided into 2 daily meals. DM, dry matter; DE, digestible energy; CP, crude protein; ADF, acid detergent fibre; NDF, neutral detergent fibre; NSC, non-structural carbohydrate; WSC, water soluble carbohydrate.

608 **Table 2.** Morphometric measurements (mean ± SEM) of horses and ponies fed a cereal-rich
609 (CHO; n = 12), fat-rich (FAT; n = 12) or control (CON; n = 9) diet. Each diet group consisted
610 of an equal number of Standardbred horses, mixed-breed ponies and Andalusian horses.

		Diet Group			*P* value		
Variable	Week	CHO	FAT	CON	Diet	Breed	Diet x Breed
BCS (1-9 scale)	0	5.5 ± 0.2	4.9 ± 0.2	5.3 ± 0.3			
	20	7.8 ± 0.1[a]	7.4 ± 0.1[a]	5.6 ± 0.2[b]	<0.001	0.80	0.25
TBFM (%)	0	8.1 ± 0.9	8.9 ± 0.9	7.7 ± 1.9			
	20	16.6 ± 0.7[a]	16.0 ± 1.0[a]	7.8 ± 1.4[b]	<0.001	0.83	0.99
CNS (1-5 scale)	0	2.1 ± 0.1	2.0 ± 0.2	2.0 ± 0.3			
	20	3.6 ± 0.1[a]	3.2 ± 0.2[a]	2.2 ± 0.3[b]	<0.001	0.59	0.52
ΔBW (%)	0	0	0	0			
	20	16.0 ± 0.7[a]	14.6 ± 0.7[a]	8.1 ± 1.7[b]	<0.001	0.95	0.91

611 BCS, body condition score (Henneke et al., 1983; Kohnke, 1992); TBFM, total body fat mass
612 (determined by deuterium oxide dilution); CNS, cresty neck score (Carter et al., 2009b);
613 ΔBW, percentage change in body weight from Week 0. *P* values represent the effects on
614 Week 20 values. [a,b]Different superscript letters indicate significant difference between diet
615 groups at Week 20 ($P<0.05$).

Table 3. Minimal model analysis of an insulin-modified frequently-sampled IV glucose tolerance test (FSIGT; mean ± SEM) in horses and ponies fed a cereal-rich (CHO; n = 12), fat-rich (FAT; n = 12) or control (CON; n = 9) diet. Each diet group consisted of an equal number of Standardbred horses, mixed-breed ponies and Andalusian horses.

		Diet Group			*P* value		
Variable	Week	CHO	FAT	CON	Diet	Breed*	Diet x Breed
SI ($x10^{-4}$/[mU·min])	0	3.66 ± 0.61	2.48 ± 0.32	2.85 ± 0.68			
	20	1.49 ± 0.23[a]	2.65 ± 0.46[b]	2.66 ± 0.94[b]	<0.001	0.001	0.61
AIRg ([mU·min]/L)	0	280 ± 58	289 ± 61	193 ± 36			
	20	502 ± 76[a]	281 ± 43[b]	229 ± 40[b]	0.002	0.38	0.40
DI ($x10^{-2}$)	0	9.14 ± 2.02	5.88 ± 1.03	4.93 ± 1.50			
	20	6.58 ± 0.94	6.53 ± 1.54	5.72 ± 0.92	0.75	0.31	0.93
Sg ($x10^{-2}$/min)	0	1.64 ± 0.22	1.80 ± 0.35	1.18 ± 0.34			
	20	2.22 ± 0.23	1.92 ± 0.20	1.73 ± 0.37	0.37	0.013	0.15

SI, insulin sensitivity; AIRg, acute insulin response to glucose; DI, disposition index; Sg, glucose effectiveness. *P* values represent the effects on Week 20 values. [a,b]Different superscript letters indicate significant difference between diet groups at Week 20 ($P<0.05$). *Significant effect of breed indicative of lower SI values in ponies and Andalusians compared with Standardbreds ($P<0.05$) and lower Sg values in Standardbreds compared with ponies and Andalusians ($P<0.05$).

626 **Table 4.** Plasma concentrations (mean ± SEM) in horses and ponies fed a cereal-rich (CHO;
627 n = 12), fat-rich (FAT; n = 12) or control (CON; n = 9) diet. Each diet group consisted of an
628 equal number of Standardbred horses, mixed-breed ponies and Andalusian horses.

		Diet Group			*P* value		
Variable	Week	CHO	FAT	CON	Diet	Breed	Diet x Breed
Glucose (mmol/L)	0	5.0 ± 0.1	4.9 ± 0.1	4.9 ± 0.2			
	20	5.0 ± 0.1	5.1 ± 0.1	5.1 ± 0.1	0.38	0.19	0.68
Insulin (mU/L)	0	6.7 ± 1.3	4.5 ± 0.8	4.1 ± 0.6			
	20	7.1 ± 0.6	6.6 ± 0.8	4.4 ± 0.6	0.088	0.99	0.75
Leptin (ng/mL)	0	0.80 ± 0.18	1.33 ± 0.27	1.62 ± 0.36			
	20	7.07 ± 0.56[a]	7.29 ± 0.59[a]	1.97 ± 0.29[b]	<0.001	0.084	0.33
Adiponectin (µg/mL)	0	4.88 ± 0.78	3.99 ± 0.62	3.15 ± 0.73			
	20	2.15 ± 0.27[a]	4.14 ± 0.66[b]	3.89 ± 0.48[b]	0.010	0.18	0.72
TNF-α (ng/mL)	0	0.58 ± 0.26	0.86 ± 0.56	1.58 ± 1.06			
	20	0.58 ± 0.25	0.63 ± 0.27	1.50 ± 1.01	0.44	0.99	0.36
SAA (µg/mL)	0	1.69 ± 0.28	0.93 ± 0.14	1.76 ± 0.49			
	20	6.34 ± 1.12[a]	2.46 ± 0.60[b]	2.72 ± 1.13[b]	0.009	0.58	0.55

629 TNF-α, tumour necrosis factor-α; SAA, serum amyloid A. *P* values represent the effects on
630 Week 20 values. [a,b]Different superscript letters indicate significant difference between diet
631 groups at Week 20 ($P<0.05$).

632 **Figure legends**

633

634 **Figure 1.** Amount of micronized maize (left y axis) or fat supplement (right y axis) added to
635 the base ration in the cereal-rich (CHO) and fat-rich (FAT) meals over the study period. The
636 fat supplement consisted of an equal mixture (by weight) of liquid oil and granulated
637 vegetable fats. The total amount of each supplement was divided into twice-daily meals.

638

639 **Figure 2.** Weekly measurements (mean ± SEM) of body condition score (BCS; A) and
640 percentage change in body weight (ΔBW; B) in the cereal-rich (CHO; n = 12), fat-rich (FAT;
641 n = 12) and control (CON; n = 9) diet groups. Each diet group consisted of an equal number
642 of Standardbred horses, mixed-breed ponies and Andalusian horses.

643

644 **Figure 3.** Insulin sensitivity (SI; A), acute insulin response to glucose (AIRg; B) and glucose
645 effectiveness (Sg; C) determined by a FSIGT. Equal numbers of Standardbred horses (white
646 bars), mixed-breed ponies (stippled bars) and Andalusian horses (grey bars) were fed either
647 cereal-rich (CHO; n = 12), fat-rich (FAT; n = 12) or control (CON; n = 9) meals over 20
648 weeks. Data are expressed as mean ± SEM. *Indicates significant difference between diet
649 groups (P<0.05). The model indicated a significant effect of breed for SI, with lower values
650 for ponies and Andalusians compared with Standardbreds (P=0.001). The model indicated a
651 significant effect of breed for Sg, with lower values for Standardbreds compared with ponies
652 and Andalusians (P=0.013).

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