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Investigation of glucagon-like peptide-1 response to six oral carbohydrates in ponies

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ABSTRACT

Glucagon-like peptide-1 (GLP-1), the principal incretin in horses, may play a role in the pathophysiology of insulin dysregulation (ID). This study aimed to describe its concentration in response to three preserved forages and four dynamic tests for ID in ponies. Twelve adult ponies of mixed ID status were given a meal of hay, soaked hay or haylage, an in-feed oral glucose test (OGT), oral sugar test (OST), an oral test using a proprietary breakfast cereal (WEET) or a combined glucose-insulin tolerance test (CGIT) weekly in a randomised cross-over study. Glucose, insulin and GLP-1 concentrations were measured before and following each intervention. Ponies were designated ID or non-ID and insulin resistant (IR) or non-IR according to OGT and CGIT results, respectively.

All interventions apart from the CGIT provoked a GLP-1 response within 30 min. The OGT and WEET interventions, (containing the greatest dose of non-structural carbohydrate, 1.06 and 1 g/kg BW, respectively), resulted in a greater area under the curve (AUC) for GLP-1 compared to all other interventions ($P < 0.001$). No difference in GLP-1 response was detected according to ID or IR status, despite there being strong positive correlations (r_s [95 % CI]) between GLP-1 and insulin concentrations measured at individual time points (0.67 [0.62 – 0.71]; $P < 0.001$) and as AUC (0.66 [0.49–0.79], $P < 0.001$). These data do not support of the use of GLP-1 as an adjunctive diagnostic test for ID or IR, as defined by conventional intravenous or oral dynamic tests.

Introduction

An association between hyperinsulinaemia and laminitis has been demonstrated in numerous experimental and clinical trials (Treiber et al., 2006; Asplin et al., 2007). The gastrointestinal potentiation of pancreatic insulin secretion is termed the enteroinsular axis (EIA) (de Graaf-Roelfsema, 2014; de Laat and Fitzgerald, 2023), evidenced in both humans and horses by a greater insulinaemic response following oral glucose compared to an isoglycaemic dose administered intravenously (Hampton et al., 1986; Duehlmeier et al., 2010; de Laat et al., 2016). The EIA response is partially mediated by hormones called incretins. These include glucagon-like peptide-1 (GLP-1), which forms the major component of the EIA in horses, and glucose-dependent insulinotropic peptide (GIP). Variation in GLP-1 concentration was estimated in one study to cause 23 % of variation in insulin concentration (de Laat et al., 2016). Other effects of GLP-1 in humans include inhibition of glucagon secretion, appetite and gastric motility (Campbell and Drucker, 2013). These additional effects have not been investigated in horses; however, it has been demonstrated that the GLP-1 receptor is distributed across a wide range of equine tissues (Kheder et al., 2018).

The measurement of insulin concentration following oral carbohydrate administration, or basal insulin concentration during consumption of preserved forage or pasture is advocated for detection of an exaggerated response and the diagnosis of ID (Durham et al., 2019). Oral tests have the advantage over intravenous tests of including an assessment of the EIA, and so may be more appropriate for detecting ID (Frank and Tadros, 2014; Durham et al., 2019). There is, however, suboptimal repeatability and agreement between different oral carbohydrate and basal tests (Borgia et al., 2011; de Laat and Sillence, 2017; Knowles et al., 2017) and reference intervals or diagnostic thresholds are not well established.

Assuming the EIA, in particular GLP-1 concentration, is contributing to an exaggerated postprandial hyperinsulinaemia, measurement of GLP-1 could offer useful additional diagnostic information for the assessment of insulinaemic responses to feeding and ID. However, the relationship between GLP-1 and insulin concentrations is incompletely

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Table 1
Amount administered and non-structural carbohydrate dose (g/kg bodyweight [BW]) for the seven interventions as determined by wet chemistry nutritional analysis or published data.

Intervention	Amount administered	Total non-structural carbohydrate dose (g/kg BW)
Hay	0.25 % BW DM	0.4
Soaked Hay	0.25 % BW DM	0.27
Haylage	0.25 % BW DM	0.46
OGT	1 g/kg BW glucose powder 1 g/kg BW DM chaff 1 mL/kg BW water	1.06
OST	0.15 mL/kg BW corn syrup	0.14 (Jocelyn et al., 2018) 0.15 (Schuver at al., 2014)
WEET	1.46 g/kg BW DM cereal 1 g/kg BW water	1
CGIT	150 mg/kg BW glucose (intravenous) 0.1 IU/kg neutral insulin (intravenous)	0.15 (intravenous)

BW, body weight; DM, Dry matter content; OGT, Oral glucose test; OST, Oral sugar test; WEET, oral test using proprietary breakfast cereal; CGIT, combined glucose-insulin tolerance test. All interventions were administered orally except those marked IV (intravenous).

described. Insulin and GLP-1 concentrations were positively correlated following non-structural carbohydrate (NSC) ingestion (Bamford et al., 2015, de Laat et al., 2016) and grazing (Fitzgerald et al., 2019), whereas there was no relationship following an OST (Frank and Walsh, 2017) or high NSC feed (Meier et al., 2020). Evidence for an association between GLP-1 concentration and ID status is also mixed (Bamford et al., 2015; Chameroy et al., 2016; de Laat et al., 2016; Fitzgerald et al., 2019). Data are lacking on GLP-1 concentration following consumption of preserved forages. Further work is required to help understand the contribution of the enteroinsular axis to ID, and its potential in the assessment of ID and laminitis risk.

This study describes GLP-1 response to six different forms of oral carbohydrate and a combined glucose insulin tolerance test (CGIT) in a group of twelve ponies of varying ID status. We hypothesised that GLP-1 response would be associated with NSC content of the feed, and that it would be greater in ID-positive ponies. Further, we hypothesised there would be minimal GLP-1 response to intravenously administered glucose, and that insulin and glucose concentrations would be correlated with GLP-1 concentration following oral carbohydrate.

Materials and methods

The study was conducted under the Animals (Scientific Procedures) Act 1986 (project licence PPL 40/3715; Approval date, 6 September 2013).

Study protocol

This was part of a larger 7-way, randomised (Research Randomizer, 2023) cross over study in 12 adult ponies which was conducted in May and June in northwest England. Details of the recruitment, management and monitoring of the ponies before and during the study have been reported previously (Carslake et al., 2018, Carslake et al., 2023). Briefly, the ponies were screened before the start of the study to ensure they were free of confounding disease, and that the group included a wide range of insulin dysregulation. During a 2-week habituation period and throughout the study they were stabled individually, apart from 2 h/day of turn out on pasture, fitted with a closed grazing muzzle. Daly feed intake was 2 % body weight (BW) fresh weight hay, divided into two daily meals, and 200 g/pony/day of a proprietary feed balancer (Lite Balancer, Spillers Feeds). Weekly measurements of body weight, girth and belly circumference, body condition score (BCS, Kohnke, 1992) and cresty neck score (CNS, Carter et al. 2009) were obtained. The cross-over design resulted in each pony receiving each of seven interventions on one occasion, with the order for each pony randomised. Each week over the 7-week study period a catheter was placed in the jugular vein. That evening, hay provision was restricted (1 % BW fed at 17:00) to ensure complete consumption by midnight. The following morning, each pony had one of the following interventions: a single forage meal (0.25 % BW dry matter [DM]) of hay, soaked hay or haylage, or an oral glucose test (OGT), oral sugar test (OST), oral test using a proprietary breakfast cereal (WEET) or intravenous combined glucose-insulin tolerance test (CGIT). Time to complete all meals was recorded, and any residual feed was removed and weighed after 40 min. The catheter was removed after the last blood sample and ponies returned to their regular routine.

Interventions

Details of the forage meals including nutritional analysis and the OGT, OST, WEET and CGIT protocols have been published previously (Carslake et al., 2018, Carslake et al., 2023) and are summarised in Table 1. All hay and soaked hay used for interventions were random grab samples from the same large bale of local, mixed-species hay, and haylage was from individually wrapped, small bales from a single batch (West Lancs Haylage). Forage interventions were fed as a single meal of 0.25 % BW DM. Standard, previously described protocols were used for the OGT (Borer et al., 2012), OST (Schuver et al., 2014) and CGIT (Eiler et al., 2005). Briefly, the OGT consisted of 1 g/kg BWT glucose powder mixed with 1 g/kg BWT chaff-based feed (Happy Hoof, Spillers) and 1 mL/kg bwt water. The OST consisted of 0.15 mL/kg bwt corn syrup (Karo Light Syrup, ACH Food Companies) syringed by mouth. For the WEET, animals were given 1.46 g/kg bwt of Weetabix (Weetabix Food Company), equivalent to 1 g/kg bwt NSC, mixed with 2.2 mL/kg bwt water. The CGIT consisted of glucose (150 mg/kg) rapidly administered (< 1 min) as a 50 % w/v solution, followed immediately by a bolus of neutral insulin (Humulin S, Eli Lilly) (0.1 IU/kg), both administered intravenously.

The dose of NSC provided in each intervention was calculated as the sum of starch and water-soluble carbohydrate from wet chemistry nutritional analysis for forages (Carslake et al., 2018), manufacturer-supplied nutritional analysis for Happy Hoof used in OGT and the propriety cereal used in WEET, and previously published analysis of the corn syrup (Schuver et al., 2014; Jocelyn et al., 2018) used in OST (Table 1).

A baseline blood sample (t=0) was obtained before all interventions, and then 1, 5, 15, 25, 35, 45, 60, 75, 90, 105, 120, 135, 150 and 180 min after glucose and insulin administration for the CGIT and 30, 60, 90, 120, 150, 180, 240 and 300 min following the other interventions.

Blood processing and analysis

For all time points, blood was collected in fluoride oxalate tubes,

placed in ice and then whole blood glucose concentration measured in duplicate using a glucose oxidase enzymatic method (YSI 2300 STAT) within 8 h. Blood obtained 0, 25, 45, 60, 75, 90, 120, 150 and 180 min after CGIT and at all time points following the other interventions was also analysed for active GLP-1 and insulin concentrations. For GLP-1, 2 mL of blood was immediately placed into EDTA tubes to which 40 μL of a proprietary mixture of dipeptidyl peptidase-4 (DPP-4) inhibitors (DPP-IV inhibitor, Millipore) had already been added, and stored immediately in ice. Within 5 h tubes were centrifuged at 2000 *g* for 10 min at 4 °C, and the plasma harvested and stored at −80 °C for 4–16 months until analysis. GLP-1 concentration was measured in duplicate using a high-sensitivity human active GLP-1 enzyme-linked immunosorbent assay (ELISA, Millipore), validated for use in horses (Chameroy et al., 2016; de Laat et al., 2016). Mean intra- and interassay coefficients of variation (CVs) were 8.0 % and 15.6 %, respectively. An interruption in the supply of DPP-4 inhibitor meant that 66 out of 108 GLP-1 samples in the second week were missing. Blood for insulin analysis was placed in plain tubes and left at room temperature to clot for 1–2 h, after which it was centrifuged at 2000 *g* for 10 min and the serum separated and stored at 5 °C until analysis within 8 h of collection. Insulin concentration was measured using a previously validated chemiluminescent assay (Immulite 2000, Siemens) with intra- and inter-assay CVs of 1.8–2.4 % and 3.0–7.1 %, respectively (Carslake et al., 2017).

Data analysis

Basal (fasting) GLP-1 concentration was taken as the t=0 min sample for each intervention. Time of maximal GLP-1 ($Tmax_{GLP\text{-}1}$), insulin ($Tmax_{insulin}$) and glucose ($Tmax_{glucose}$) concentrations were the time point after feeding with the greatest concentration. Obesity was defined as a BCS ≥ 7/9 and ID and IR status according to previously published cut-offs for the OGT (insulin concentration at 120 min >85 μIU/mL, (Durham et al., 2019)) and CGIT (insulin concentration at 45 min >82 μIU/mL or positive phase of glucose >45 min (Eiler et al., 2005) with a conversion factor for the insulin assay (Carslake et al., 2017)), respectively.

Analysis of differences in bodyweight, girth and belly circumferences, BCS and CNS at the times of different interventions was performed using repeated measures analysis of variance or Friedman test, according to distribution of data (Shapiro Wilk test). Change in mean bodyweight over the duration of the study was assessed using a T-test. Differences in basal GLP-1 concentration, and morphometrics according to ID and IR status were evaluated using a Mann Whitney-U test, because data did not follow a normal distribution (Shapiro Wilk test). Relationships between GLP-1 and glucose and insulin concentrations for all sampling points following oral carbohydrate interventions (hay, soaked hay, haylage, OGT, OST and WEET) was evaluated using Spearman's rank correlation coefficient (r_s), with interpretation of strength of association as follows: 0–0.19 very weak, 0.2–0.39 as weak, 0.4–0.59 as moderate, 0.6–0.79 as strong and 0.8–1 as very strong (Campbell and Swinscow, 2009). A comparison of $Tmax_{GLP\text{-}1}$ with $Tmax_{glucose}$ and $Tmax_{insulin}$ for individual oral interventions, and all oral interventions combined was performed using a Wilcoxon signed rank test.

Area under the curve (AUC) was calculated for the oral interventions (hay, soaked hay, haylage, OGT, OST, WEET) using the trapezoid method with zero as the baseline. Data from the CGIT were excluded from $AUC_{GLP\text{-}1}$ analysis as this was measured over 180 min rather than 300 min and it was not considered a comparable test. Relationships between AUC for GLP-1($AUC_{GLP\text{-}1}$) and $AUC_{glucose}$ and $AUC_{insulin}$ were evaluated using Spearman's correlation coefficient.

A 2-level linear regression model (test, horse) was established with $Log_{10}AUC_{GLP1}$ as the outcome variable, and within-horse effects due to cross over design accounted for by incorporation of horse as a random intercept term. The effect of intervention, obesity and ID and IR status were assessed by entering variables in the linear regression model. Interaction terms of intervention*ID and *IR status were assessed to determine any differing effect of the intervention in ID and IR positive or negative ponies. For each intervention, the mean (± standard error [SE]) Log_{10} $AUC_{GLP\text{-}1}$ was estimated after adjusting for clustering at the horse level by reporting the intercept. Differences in predicted mean $Log_{10}AUC_{GLP\text{-}1}$ between pairs of interventions were tested using Chi squared statistic. Model diagnostics included evaluation of the normality of the residuals using normal probability plots.

Statistical analysis was performed using commercial software (SPSS 27 (IBM) and MLwIN 3.05 (Centre for multilevel modelling, University of Bristol). Significance was defined as $P < 0.05$.

Results

Animals

All ponies were mixed, native British breeds. There were 11 mares and one gelding, aged (mean ± SD) 9.1 ± 3.4 and range of 4–15 years and with a bodyweight of 280 ± 49 kg. The median (IQR) BCS was 7.2 (6 – 7.7) out of 9, with a range of 4.2–8.5, and median (IQR) CNS was 3 (3−4). There was no significant difference in mean bodyweight, girth or belly circumference, CNS or BCS at the time of the different interventions, or between ID or IR positive and negative groups. Over the 6–week study period, there was an increase (mean ± SD) in bodyweight of 5.8 ± 4.2 kg ($P < 0.001$).

The OGT meal was entirely consumed by 9/12 ponies, taking (mean ± SD) 23.4 ± 10.7 min. Three ponies consumed between 85 % and 94 % of the OGT meal which was considered an insignificant reduction. The WEET meal was entirely consumed by 5 ponies, taking 21.8 ± 13.3 min. Six ponies consumed between 56 % and 73 % of the meal and were included in data analysis. One pony consumed only 33% of the WEET and was excluded from further analysis of WEET data. The entire hay, soaked hay and haylage meals were consumed by all ponies. All interventions were well tolerated with no adverse effects seen.

Glucose and insulin concentrations

Full results and comparisons of glucose and insulin concentrations following the forage meals (Carslake et al., 2017) and diagnostic tests (Carslake et al., 2023), have been published previously. Six out of twelve ponies were ID-positive and 4/12 were IR-positive. All IR-positive ponies were also ID-positive.

GLP-1 concentration

Due to an interruption in the supply of DPP-IV inhibitor in the 2nd week, over the whole study period 690 GLP-1 samples were included out of a possible 756.

Plasma GLP-1, blood glucose and serum insulin concentrations (mean ± SEM) following the different interventions are shown in Fig. 1. In comparison to the other interventions, OGT and WEET resulted in greater mean GLP-1 concentrations (Table 3) which remained increased compared to basal concentration throughout the 300 min testing period. The other oral carbohydrate interventions resulted in a lower, earlier peak in mean GLP-1 concentration which returned to close to basal concentration before 300 min (Figs. 1 and 2). For all oral interventions there was an early rise in GLP-1 concentration from 0 to 30 min, and then, apart from OST, a second peak after 90–180 min (Fig. 1). There was no discernible increase in mean GLP-1 concentration in response to the CGIT. Comparing all oral interventions together, median $Tmax_{GLP\text{-}1}$ was earlier than $Tmax_{glucose}$ ($P = 0.01$) and $Tmax_{insulin}$ ($P < 0.001$, Fig. 2, Table 2). For individual tests, median $Tmax_{GLP\text{-}1}$ was earlier than $Tmax_{insulin}$ for haylage ($P = 0.03$) and OGT ($P = 0.03$), and earlier than $Tmax_{glucose}$ for soaked hay ($P = 0.02$, Fig. 2, Table 2).

In relation to IR and ID status, there was no difference in median (IQR) basal GLP-1 between IR-positive (0.91 pmol/L [0.03–1.80]) and

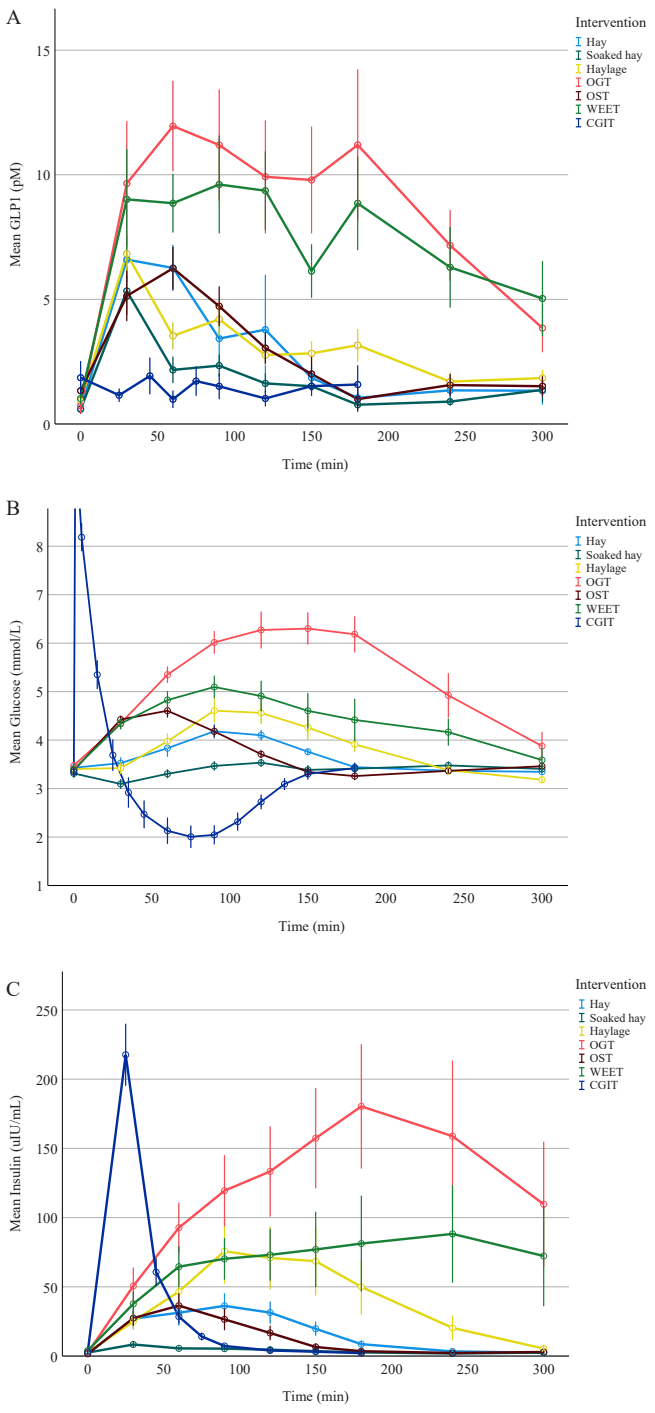

Fig. 1. Plasma concentration (mean ± SEM) of glucagon-like peptide-1 (GLP – 1) following hay ($n = 11$), soaked hay ($n = 9$), haylage ($n = 11$), oral glucose test (OGT. $n = 11$), oral sugar test (OST, $n = 10$), Weetabix test (WEET, $n = 9$) and combined glucose-insulin tolerance test (CGIT, $n = 11$) (A); mean ± SEM glucose (B) and insulin (C) concentrations following the same seven interventions (all $n = 12$).

IR-negative (0.69 pmol/L [0–1.45], $P = 0.60$), or between ID-positive (0.72 pmol/L [0.14–1.48]) and ID-negative (0.75 pmol/L [0–1.55], $P = 0.73$) animals.

Following oral carbohydrate interventions there was a strong correlation (r_s [95 % CI]) between GLP-1 and insulin (0.67 [0.62–0.71]; $P < 0.001$) concentrations and a moderate correlation between GLP-1 and glucose (0.52 [0.46–0.58]; $P < 0.001$) concentrations from the same time points.

GLP-1 area under the curve

The missing GLP-1 concentration data resulted in an inability to calculate $AUC_{GLP\text{-}1}$ for 11/72 oral interventions: 1 x hay, 3 x soaked hay, 1 x haylage, 1 x OGT, 2 x OST, 3 x WEET (including the pony excluded for inadequate consumption) and 1 x CGIT (CGIT data not included in analyses).

Following oral carbohydrate interventions there was a strong positive correlation (r_s [95 % CI]) between $AUC_{GLP\text{-}1}$ and $AUC_{insulin}$ (0.66 [0.49–0.79], $P < 0.001$) and a moderate correlation between $AUC_{GLP\text{-}1}$ and $AUC_{glucose}$ (0.58 [0.39 – 0.73], $P < 0.001$).

The multilevel linear regression model showed there was a significant effect of intervention on $Log_{10}AUC_{GLP\text{-}1}$ ($P < 0.001$). There was no significant effect of obesity, ID or IR status (Fig. 3) or of the interaction terms tested. The predicted mean $Log_{10}AUC_{GLP\text{-}1}$ from the regression model, adjusted for within-horse clustering is shown for each intervention in Table 3. $Log_{10}AUC_{GLP\text{-}1}$ was significantly greater after OGT and WEET compared to all other interventions ($P < 0.001$). Haylage, hay and OST caused a greater response than soaked hay ($P = 0.02$, 0.02 and 0.046, respectively). Within-horse variance (SE) was 0.018 (0.010). A scatterplot and normal probability plot of standardised residuals showed homoscedasticity and that the assumption of normality was reasonable, respectively.

Discussion

This study provides further evidence of a functional enteroinsular axis in ponies and a detectable GLP-1 response to a variety of feeds, including some with a low NSC content.

This is the first study to evaluate GLP-1 response to preserved forages, including soaked and unsoaked hay from the same bale. Soaking hay in water reduces its NSC content (Mack et al., 2014), and results in significantly lower post-prandial glycaemic and insulinaemic responses (Carslake et al., 2017). A significant reduction in mean GLP-1 response resulting from the soaking of hay was also identified in this study, indicating that the difference in insulin response could be driven by any combination of the small but significant difference in the blood glucose response between soaked and unsoaked hay (mean maximum glucose concentration ($Cmax_{glucose}$) 3.7 versus 4.1 mmol/L, respectively), the lower GLP-1 concentration, or other elements of the EIA. The haylage used in this study had a relatively high wet chemistry measured NSC content (Lindase et al., 2018) and elicited a greater GLP-1 response than soaked, but not unsoaked hay. It has been demonstrated in rodents that GLP-1 secretion following oral carbohydrate is dose-dependent (Yoder et al., 2010) and this difference is likely to have been driven, at least in part, by differences in NSC content and availability between the three forages. The fermentation process of haylage also makes it more digestible and available for absorption (Müller, 2012). The intestinal L-cells secrete GLP-1 in response to range of different nutrients in other species (Bodnaruc et al., 2016), and this is likely to have affected the results of this study. Intestinal amino acids and fats result in GLP-1 secretion in humans (Diakogiannaki et al., 2012), and the greater crude protein concentration in the haylage compared to hay and soaked hay (8 %, 6.2 % and 6 %, respectively) might have influenced the GLP-1 response. Additionally, it is possible that GLP-1 secretion is affected by fermentation products such as volatile fatty acids and ethanol that are present in haylage but not hay. As there was no significant difference in mean GLP-1 concentration between hay and haylage, however, this seems unlikely.

Similar to the forages, GLP-1 response following the oral carbohydrate tests (OGT, OST and WEET) was associated with NSC content. The GLP-1 response to the OST was greater than might be expected considering its very low NSC dose compared to all the other interventions, especially the forages. Higher digestibility and availability of the NSC in corn syrup, the bolus dose or variability in the NSC content of corn syrup in the OST could account for this. The duration of GLP-1 secretion for

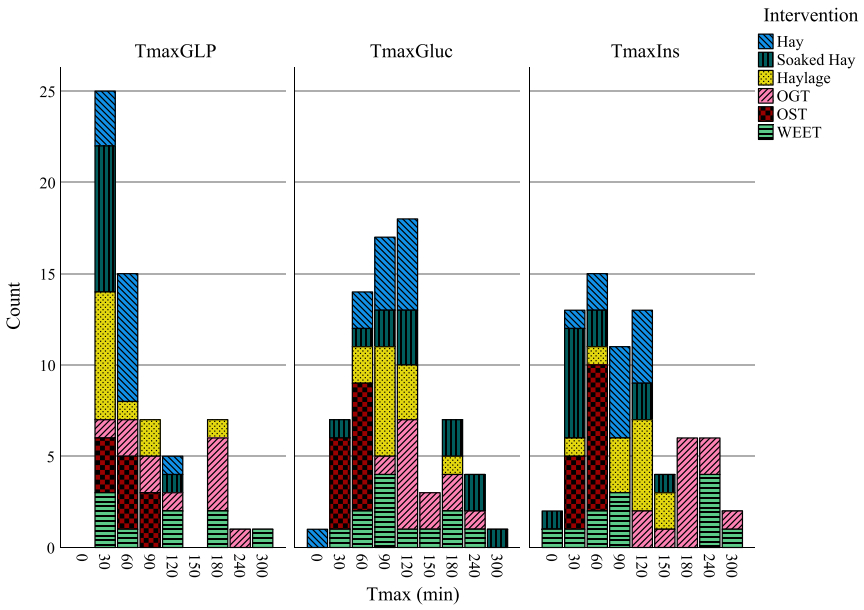

Fig. 2. Stacked bar chart showing time of maximum concentration (min) of glucagon-like peptide - 1 (TmaxGLP, $n = 61$), insulin (TmaxIns, $n = 72$) and glucose (TmaxGluc, $n = 72$) following hay, soaked hay, haylage, oral glucose test (OGT), oral sugar test (OST) and Weetabix test (WEET).

Table 2
Median (IQR) time to maximal concentration (Tmax) for glucagon-like peptide-1 ($Tmax_{GLP1}$), glucose and insulin following six oral carbohydrate interventions. *P* - values for Wilcoxon signed-rank test comparing $Tmax_{GLP1}$ with Tmax for insulin and glucose.

Intervention (n)	Median (IQR) $Tmax_{GLP1}$ (min)	Median (IQR) Tmax (min)		*P* – value for comparison to $Tmax_{GLP1}$
All oral tests (61)	60 (30–90)	Glucose	90 (60–120)[a]	0.01
		Insulin	90 (90–135)[a]	<0.001
Hay (11)	60 (30–60)	Glucose	90 (60–120)	0.070
		Insulin	60 (90–120)	0.051
Soaked Hay (9)	30 (30–30)	Glucose	120 (75–240)[a]	0.024
		Insulin	30 (30–60)	0.85
Haylage (11)	30 (30–90)	Glucose	90 (90–120)	0.055
		Insulin	120 (90–120)[a]	0.031
OGT (11)	120 (60–180)	Glucose	120 (120–180)	0.86
		Insulin	180 (150–180)[a]	0.027
OST (10)	60 (30–90)	Glucose	60 (30–60)	0.23
		Insulin	60 (30–60)	0.26
WEET (9)	120 (30–180)	Glucose	90 (75–180)	1.0
		Insulin	90 (45–240)	0.29

OGT, Oral glucose test; OST, Oral sugar test; WEET, oral test using proprietary breakfast cereal.
[a]Indicates significant difference to $Tmax_{GLP1}$

OGT and WEET lasted longer than for OST and other interventions, even when the different rates of consumption are accounted for. The higher dose of NSC, other nutritional components or differences in digestibility between the different interventions could have resulted in a greater persistence of the nutrients in the intestine and a longer duration of GLP-1 secretion. Expression of GLP-1 has been demonstrated in all areas of the equine intestine (Fitzgerald et al., 2024), and persistence of nutrients in the intestinal lumen might have stimulated secretion from the more distal regions. There was no discernible GLP-1 response to intravenously administered glucose, consistent with results from other studies on GLP-1 (de Laat et al., 2016) and GIP (Duehlmeier et al., 2010). The exogenous insulin bolus administered as part of the CGIT shortens the period of hyperglycaemia and could have altered the GLP-1 response.

Contrary to our hypothesis, GLP-1 concentration was unable to differentiate ID and IR status, as determined by the OGT and CGIT respectively. This was despite the IR- and ID- positive animals showing greater hyperinsulinaemic responses to all oral interventions (previously published (Carslake et al., 2018, Carslake et al., 2023)) and a strong positive correlation identified between insulin and GLP-1 concentrations, both at individual time points and as AUC. This positive correlation has been supported by other studies (Bamford et al., 2015; de Laat et al., 2016). Other studies have found variable associations between ID status and GLP-1 concentration, with one study even showing a negative correlation between GLP-1 and insulin concentrations and a lower median GLP-1 concentration in the ID group, when measured at two time points following an OST (Frank and Walsh, 2017). GLP-1 concentration following an OGT did not predict the development of laminitis in a group of ponies subsequently exposed to a high NSC dietary challenge (Meier et al., 2020), and in another study there was no difference in GLP-1 response to grazing according to ID status, as determined by an OGT (Fitzgerald et al., 2019). The results of the current study are in contrast to de Laat et al. (2016), which showed different GLP-1 concentrations following an OGT acording to ID status. This inconsistency could be accounted for by differenes in the populations from which study groups were taken, handling of GLP-1 samples, or doses of glucose used in the OGT. The metabolic derangements leading to

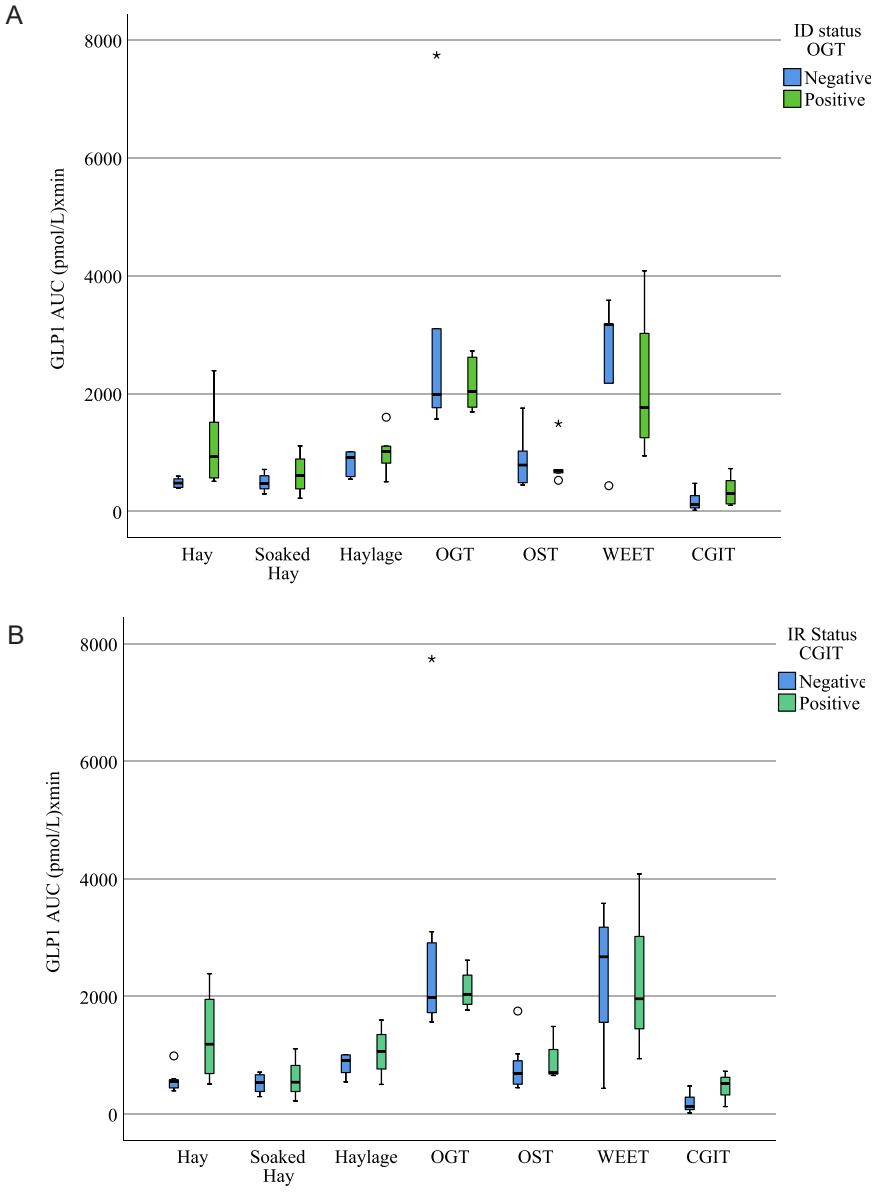

Fig. 3. Box and whisker plots of area under the curve for glucagon-like peptide-1 (AUC GLP-1, min*pmol/L) following seven different interventions separated according to insulin dysregulation (ID) status determined by an oral glucose test (OGT, positive [$n = 6$, green] and negative [$n = 6$, blue] animals) (A), and insulin resistant (IR) status determined by a combined glucose-insulin tolerance test (CGIT, positive [$n = 4$, green] and negative [$n = 8$, blue] animals) (B). OST, oral sugar test; WEET, Weetabix test. Area under the curve was calculated using GLP-1 measurements over 300 min for all interventions except CGIT which was only measured over 180 min. * = outliers >3 times IQR from the end of a box; o = outliers 1.5 – 3 times IQR from end of the box.

Table 3
Mean and standard error (SE) $\log_{10}$ Area under the curve for glucagon-like peptide-1 ($AUC_{GLP\text{-}1,}$ min*pmol/L) following six oral carbohydrate interventions, adjusted for within horse clustering, with *P* value for comparisons.

Intervention	Mean Log_{10} $AUC_{GLP\text{-}1}$	SE	*P* value of comparison				
			Hay	Soaked hay	Haylage	OGT	OST
Hay	2.87^{ac}	0.063	-	-	-	-	-
Soaked hay	2.70^{abd}	0.068	0.023	-	-	-	-
Haylage	2.93^{bf}	0.063	0.363	0.002	-	-	-
OGT	3.37^{abe}	0.063	<0.001	<0.001	<0.001	-	-
OST	2.91^{de}	0.065	0.545	0.005	0.780	<0.001	-
WEET	3.27^{cdf}	0.068	<0.001	<0.001	<0.001	0.174	<0.001

OGT, oral glucose test; OST, oral sugar test; WEET, Weetabix test.
[a – f] Values with the same superscript letter are significantly different.

hyperinsulinaemia and laminitis are complex and likely vary between individual horses (Frank and Tadros, 2014). Intravenous tests such as the CGIT assess insulin resistance only and do not capture the effect of the EIA. This might account for the current study's failure to detect a difference in GLP-1 secretion between the IR and non-IR groups. Oral tests such as the OGT include the contribution of the EIA to insulin concentration, but post-prandial glucose absorption has a greater insulinaemic effect than incretins (de Laat et al., 2016). For these reasons, using OGT and CGIT results as a reference standard will not fully reflect the role of GLP-1 in hyperinsulinaemia and laminitis risk.

For all oral interventions together, median $Tmax_{GLP-1}$ was earlier than $Tmax_{Glucose}$ and $Tmax_{Insulin}$. When interventions were analysed individually, median $Tmax_{GLP-1}$ was earlier or there was no significant difference (Figs. 1 and 2, Table 2). This finding is consistent with other equine studies (de Laat et al., 2016; Fitzgerald et al., 2019), which showed that Tmax for GLP-1 preceded glucose and insulin following oral carbohydrate. Post-prandial secretion of GLP-1 is biphasic in rats and humans, the first phase occurring within 10–15 min and predominantly mediated by neuroendocrine mechanisms rather than direct contact of nutrients on the secretory L-cells which are mostly located in the distal small intestine (Baggio and Drucker, 2007). These mechanisms could also account for the similar timing of the first GLP-1 peak between the different interventions, in contrast to the glucose and insulin peaks which were more variable. Although not present for all interventions, a biphasic GLP-1 response was discernible for most, as reported in humans (Herrmann et al., 1995) and horses (de Laat et al., 2016).

Glucagon-like-peptide-1 was the only incretin measured in this study, for financial reasons and because it has been shown to have a greater effect on post-prandial insulin secretion compared to GIP (de Laat et al., 2016). The active form of GLP-1 is rapidly degraded (Bodnaruc et al., 2016), and an assay measuring both the active form and its metabolite (total GLP-1) has been described in horses (de Laat et al., 2016). In this study, only the active form of GLP-1 was measured to indicate dynamic secretion in response to the oral interventions and the associated stimulation of insulin secretion. Pilot data indicated low concentrations of GLP-1 were likely, meaning that the high sensitivity version of the assay was used. Another insulinotropic peptide, glucagon-like peptide 2 (GLP-2) has been identified in horses (de Laat et al., 2018) and measurement of this and GIP together with total and active GLP-1 could offer a greater insight into the EIA and its association with ID. Difficulties in sample handling are likely to limit the diagnostic use of GLP-1 in clinical practice.

There were missing samples in this study, which may have weakened the power of the study to detect any differences and significant interactions between ID and intervention. Due to missing values, multilevel modelling adjusted for repeated measures within horses was used for analysis instead of repeated measures ANOVA. Multiple imputation was not used as the data were missing completely at random, it was only the dependent variable missing, and it was a small and non-normally distributed dataset. A prospective sample size calculation was not performed for this study, as data relating to likely GLP-1 responses, and clinically significant differences were lacking. This might have resulted in the study being more susceptible to insufficient statistical power and type-2 error. Pony breeds were chosen for this study as they are known to be at increased risk of hyperinsulinaemic laminitis (Jeffcott et al., 1986; Pollard et al., 2019) and are commonly presented for veterinary investigation. Ponies and Andalusian horses had a greater GLP-1 response to feed compared to standardbreds in a different study (Bamford et al., 2015) and the data presented here might not be applicable to other breeds. The inter-assay CV calculated for the GLP-1 assay used in this study was 15.6 %, likely caused by operator error or imprecision of the equipment used during assays, and a possible source of measurement error. This inter-assay CV was, however, close to that reported in the assay characteristics from the manufacturer, which gave inter-assay CVs of 13 % and 10 % for mean concentrations of 4.5pmol/L and 21.4pmol/L, respectively (Millipore, 2012).

Conclusions

In summary, consumption of preserved forages and three oral dynamic tests resulted in a detectable mean GLP-1 response within 30 min. Different carbohydrate feeds altered GLP-1 responses, and soaking hay caused a marked attenuation. Contrary to our hypothesis, an association between GLP-1 response and ID or IR status, as defined by the OGT and CGIT respectively, was not detected, indicating that GLP-1 has limited use as an adjunctive test for these conditions. There was a strong positive correlation between GLP-1 and insulin responses, however, and further work is warranted to determine if GLP-1 is a suitable diagnostic and therapeutic target for endocrinopathic laminitis susceptibility in horses.

CRediT authorship contribution statement

Person Catherine McGowan: Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Supervision, Writing – review & editing. **Alex A. H Dugdale**: Investigation, Supervision, Writing – review & editing. **Caroline M Argo**: Conceptualization, Investigation, Writing – review & editing. **Gina L Pinchbeck**: Data curation, Formal analysis, Methodology, Supervision, Validation, Writing – original draft, Writing – review & editing. **Harry B Carslake**: Conceptualization, Data curation, Formal analysis, Investigation, Project administration, Visualization, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

None of the authors has a financial or personal relationship with other people or organisations that could inappropriately influence or via the content of the paper.

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