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Comparison of endocrine and metabolic responses to oral glucose test and combined glucose-insulin tests in horses

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Summary: Different diagnostic tests to determine the insulin sensitivity in horses are commonly used in veterinary practice. However, endocrine and metabolic responses provoked by physiological processes during the respective test procedures are not well described. In the present study, oral glucose tests (OGTs) and combined iv glucose-insulin tests (CGITs) were employed under standardized conditions. The OGTs and CGITs were performed in twelve healthy warmblood horses of different sex, age (15 ± 6.5 years), weight (567 ± 81 kg) and body condition score (4.8 ± 1.6). Horses were tested under fasting conditions. The OGT was performed with 1 g/kg BW glucose administered via nasogastric intubation and CGIT was performed by injection of 150 mg/kg BW glucose solution and 0.1 IU/kg BW porcine zinc-insulin. Blood samples were taken for three hours at intervals of 15 minutes and were analysed for insulin, glucose, triglyceride, non-esterified fatty acids (NEFA), fructosamine and cortisol concentrations. Glucose concentrations increased in the OGTs and CGITs directly after administration. Insulin concentrations increased significantly in OGTs within 30 minutes and stayed elevated for three hours. Peak concentrations of 493.98 ± 86.84 µIU/mL were measured in the CGITs, followed by a continuous decline. Baseline NEFA concentrations varied between individual horses and declined in a comparable manner to similar minimum concentrations of 93.82 ± 53.22 µmol/L in OGTs and 91.97 ± 56.89 µmol/L in CGITs. Regarding the stress response of the test procedure, cortisol concentrations remained unaffected during CGITs, while the OGT procedure was accompanied by a significant initial rise in cortisol concentrations. To conclude, OGT and CGIT mirror different facets of the metabolic response to a glycemic stimulus, highlighting different aspects of glucose homeostasis and insulin regulation. During the CGIT, insulin dynamics with porcine zinc-insulin differ from insulin dynamics described in reports published previously using short-acting insulins. Furthermore, the antilipolytic effects of insulin during OGTs and CGITs via endogenous secretion or exogenous injection resulted in similar reduction of NEFA concentrations and unaffected triglyceride concentrations. This indicates a saturation of the suppression of lipolysis by insulin with already low concentrations and no induction of re-esterification in liver tissue.

Keywords: horse, insulin, insulin dysregulation, insulin resistance, oral glucose test, combined glucose-insulin test, diagnostic test, physiology

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Introduction

Equine metabolic syndrome (EMS) has been growing steadily in importance in the equine population over the last few years and has been the subject of many studies, workshops and discussions. Alterations in insulin regulation in affected horses are one of the leading laboratory findings, together with predisposition to laminitis and general or regional obesity (*Frank* et al. 2010, *Johnson* et al. 2010, *Frank* and *Tadros* 2014). Obesity prevalence is high in the equine population and studies performed in the UK and the USA published obesity prevalence rates ranging between 30 and 48 % (*Thatcher* et al. 2008, *Wyse* et al. 2008, *Giles* et al. 2014). In addition, horses presenting to a first opinion hospital for evaluation of laminitis were hyperinsulinemic in 86 % of cases (*Karikoski* et al. 2011), indicating the importance of endocrinopathic laminitis. Easy and quick to perform approaches to diagnose insulin dysregulation (ID) or insulin resistance (IR) are required from clinicians. Complex hyperinsulinemic euglycemic clamp tests, which were considered to be the gold standard for assessment of IR (*Rijnen* and *van der Kolk* 2003), are usually reserved for research approaches due to their complex and expensive implementation. In practice, assessment of ID is often based on the measurement of single resting glucose and insulin concentrations, but dynamic diagnostic tests are also performed. Dynamic testing is currently recommended and offers additional information about metabolic responses to certain glucose or insulin, or even combined glucose and insulin stimulations (*Frank* et al. 2010, Equine Endocrinology Group 2016). To date, clinicians have various options of testing protocols for assessment of alterations in insulin regulation, and can chose between protocols based on oral or intravenous (iv) procedures.

The aim of this study was to compare the oral glucose test (OGT) (*Ralston* 2002) and the combined iv glucose-insulin test (CGIT) (*Eiler* et al. 2005) in healthy horses and to obtain physiological information about adaptive metabolic and endocrine processes, with a main focus on insulin and glucose dynamics and their relation to the lipid homeostasis.

It was hypothesized that the OGT and CGIT would give different results in the same horse due to the activation of different

endocrine and metabolic pathways, and addressed the suitability of the tests to be "easy and quick to perform" to diagnose ID or IR in practice.

Materials and Methods

Animals

Twelve healthy warmblood breed horses were included in the study. There were seven mares, two geldings and three stallions, aged (mean ± SD) 15 ± 6.5 years and weighed 567 ± 81 kg. The horses included covered a wide range of body condition scores (BCSs) from 1.9 to 7.5 with a mean BCS of 4.8 ± 1.6 (Table 1). The BCS was determined as the average of five independent assessors according to the scoring system of *Henneke* et al. (1983). The horses were fed average mixed grass/hay twice daily and no supplementary feeding was provided for two weeks prior to the start of the experiment. They were stabled in individual boxes, under standardized feeding and management conditions, and were not exercised, with the exception of two hours on a paddock daily. All horses underwent clinical examinations, laboratory screenings and radiographically examinations of the hooves prior to the start of the study. None of the study horses showed clinical signs of pituitary pars intermedia dysfunction (PPID) or acute or chronic laminitis.

Study design

The horses were fed hay the evening before the test, but food was withheld overnight and in the morning for about 14 hours prior to testing. The horses were muzzled to avoid excessive straw or flex bedding uptake. Water was available continuously before and during the test procedure. Testing of each horse started between 10 and 11a.m. to avoid daily variations in serum insulin concentrations (*Firshman* and *Valberg* 2007). Regarding the seasonal variation of the plasma insulin concentrations described (*Place* et al. 2010, *Funk* et al. 2012), all horses underwent the study examination in autumn and winter during October through January. The horses completed both tests over a two-week period. The trial started for each horse with the OGT, followed by a recovery phase of nine days before the CGIT was performed.

Oral Glucose Test (OGT)

An iv indwelling catheter size 12 G (EquiCath™ Fastflow, Braun Vet Care GmbH, Tuttlingen, Germany) was aseptically implanted in the left or right jugular vein of the horses for the collection of blood samples twelve hours before the start of the OGT. An amount of 1 g/kg body weight (BW) glucose powder (Glukose, WDT, Garbsen, Germany) was dissolved in two litres of water and administered by nasogastric intubation (*Ralston* 2002). Prior to the administration of glucose solution, a first basal blood sample was extracted via a jugular vein catheter. After administration of the glucose solution, blood samples were obtained at 15, 30, 45, 60, 75, 90, 105, 120, 135, 150, 165 and 180 minutes. The catheter was flushed with saline solution (NaCl; 0.9 %; B. Braun Melsungen AG, Germany) after each blood sample removal.

Combined Glucose-Insulin Test (CGIT)

The horses underwent a modified version of the CGIT procedure first described by *Eiler* et al. (2005). Two iv indwelling catheters size 12 G (EquiCath™ Fastflow, Braun Vet Care GmbH, Germany) were aseptically implanted in each jugular vein of the horses twelve hours before the start of the CGIT. One catheter was used for the administration of glucose solution and insulin, and the second one for the collection of blood samples during the testing procedure. An amount of 150 mg/kg BW glucose solution (Glucose 500 mg/mL, B. Braun Melsungen AG, Germany) were injected intravenously as a bolus within 1 minute, immediately followed by 0.1 IU/kg BW porcine zinc-insulin (Caninsulin® 40 I.E./ml, MSD, Unterschleißheim, Germany). Due to legal restrictions, veterinarians in Germany are legally bound to pharmaceuticals labelled for veterinary use before drug formulations for human purposes can be used, with the exception that the human formulation provides an essential advantage for the specific application. Therefore, we decided to test porcine zinc-insulins suitability for implementation of the CGIT. In contrast to

Table 1 Age, sex, body weight and body condition score of all study horses

Horse	Age (years)	Sex	Body Weight (kg)	Body Condition Score (BCS)
1	19	mare	465	2.9
2	19	mare	440	1.9
3	24	gelding	480	3
4	4	stallion	540	6
5	15	mare	619	5.1
6*	16	mare	663	6.5
7	15	stallion	515	5.1
8	25	mare	570	4.5
9	4	stallion	560	4
10	11	gelding	640	5.9
11	20	mare	537	4.7
12	13	mare	695	7.5

* no combined glucose-insulin test (CGIT) due to technical reasons

the original protocol developed by *Eiler* et al. (2005) that employed a fast-acting human recombinant insulin formulation. An amount of 20 mL saline solution (NaCl; 0.9%; B. Braun Melsungen AG, Germany) were used to flush the catheter after the insulin injection. A first blood sample was taken via the jugular catheter prior to the administration of glucose and insulin. Subsequently, blood samples were taken at 3, 6, 9, 12, 15, 30, 45, 60, 75, 90, 105, 120, 135, 150, 165 and 180 minutes. The catheter was flushed with saline solution (NaCl; 0.9%; B. Braun Melsungen AG, Germany) after each blood sample removal.

Blood samples and analyses

An amount of 5 mL of blood were withdrawn through the catheter and discarded before 20 mL blood were taken for measurements. The catheter was flushed with 10 mL saline solution after each sampling step. Blood samples were placed into tubes containing fluoride oxalate for the determination of glucose concentrations and into plain tubes for serum preparation. Blood samples for serum preparation were incubated at room temperature for sixty minutes, centrifuged at 1000 × g for 6 minutes, and stored at -80 °C until further analysis. Glucose concentrations were measured immediately after finishing the diagnostic test by using a colorimetric assay (GLUC3, Cobas, Roche Diagnostics GmbH, Mannheim, Germany) on an automated discrete analyser (Cobas Mira, Roche Diagnostics GmbH, Mannheim, Germany). Serum insulin concentrations were analysed by using a commercially available equine-optimized enzyme-linked immunosorbent assay (ELISA; Equine Insulin ELISA, Mercodia, Uppsala, Sweden) previously validated for use in horses (*Öberg* et al. 2012, *Warnken* et al. 2016). Samples with insulin concentrations greater than the highest value of the standard curve from the ELISA (>1.5 µg/L) were diluted with Diabetes Sample Buffer (Diabetes Sample Buffer, Mercodia, Uppsala, Sweden). The conversion factor 115 is suggested by the manufacturer (*Ab* 2017) to convert the results supplied in µg/L by the ELISA into the SI unit mU/L or µIU/mL used commonly. Triglycerides (TRG) and non-esterified fatty acids (NEFA) concentrations were measured with commercial kits (ABX Pentra Triglycerides CP, HORIBA ABX, Montpellier, France; Wako NEFA-HR(2), Wako Chemicals GmbH, Neuss, Germany) for enzymatic colorimetric measurements on an automated discrete analyser (ABX Pentra 400, HORIBA ABX SAS). Cortisol was measured by a commercial chemiluminescent immunoassay kit with an automated analyser (IMMULITE Cortisol, Siemens Medical Solutions, Bad Nauheim, Germany) and fructosamine concentrations were measured with a commercially available kit (FRA, Roche Diagnostics GmbH, Mannheim, Germany) for automatic analysers (Cobas Mira, Roche Diagnostics GmbH, Mannheim, Germany).

Statistics and calculations

Data analysis was performed using GraphPad Prism software (version 7.02; GraphPad Inc. La Jolla, CA, USA). Data were tested for normality using the Shapiro-Wilk normality test. Time courses were compared with RM One-Way ANOVA followed by Tukey's multiple comparisons test, as appropriate. Comparisons between basal metabolite and hormone concentrations on OGT and CGIT days were performed with student's paired t-tests. Areas under the curve (AUC) were calculated for insulin, glucose, NEFA, TRG and cortisol with the trapezoid method. Pearson and Spearman correlations were used to compare the laboratory parameter with individual background data, i.e. gender, age, BW and BCS. Maximal and minimal concentrations (Cmax and Cmin) for insulin, glucose, NEFA, TRG and cortisol were calculated, as well as metabolite and hormone concentrations at specific time points. Comparison between tests and groups was performed with RM two-way ANOVA or two-way ANOVA followed by Sidak's multiple comparisons test. Statistical significance was accepted at $P < 0.05$. All values are expressed as mean ± SD, unless otherwise indicated.

Results

All horses tolerated the experiments well. Neither clinical signs of hypoglycaemia were observed during CGIT, nor did horses discover signs of laminitis during the whole study period. One of the twelve horses exhibited slight colic symptoms during the washout phase between OGT and CGIT, whereby the CGIT was postponed by one week. One horse (horse 6)

Table 2 Hormone and metabolite concentrations during oral glucose test (OGT). Mean±SD, n = 12.

	insulin (µIU/mL)	glucose (mmol/L)	NEFA (µmol/L)	TRG (mmol/L)	cortisol (ng/mL)
baseline	6.42 ± 3.72	4.61 ± 0.31	402.08 ± 216.30	0.28 ± 0.22	41.37 ± 17.53
C_{min}	6.23 ± 3.81	4.59 ± 0.31	93.82 ± 53.22	0.20 ± 0.24	34.35 ± 17.10
C_{max}	69.10 ± 39.07	8.46 ± 0.89	469.08 ± 224.24	0.34 ± 0.27	80.35 ± 29.85
C_{120min}	54.05 ± 32.02	7.98 ± 1.09	133.86 ± 61.57	0.24 ± 0.26	47.59 ± 25.88

Table 3 Hormone and metabolite concentrations during combined glucose-insulin test (CGIT). Mean±SD, n=11.

	insulin (µIU/mL)	glucose (mmol/L)	NEFA (µmol/L)	TRG (mmol/L)	cortisol (ng/mL)
baseline	5.33 ± 2.07	4.63 ± 0.23	492.00 ± 230.26	0.27 ± 0.11	41.57 ± 14.71
C_{min}	5.12 ± 2.20	3.81 ± 0.65	91.97 ± 56.89	0.17 ± 0.08	33.24 ± 14.38
C_{max}	493.98 ± 86.84	10.49 ± 0.93	613.91 ± 224.69	0.29 ± 0.10	57.04 ± 23.21
C_{45min}	51.96 ± 34.78	6.35 ± 1.07	205.30 ± 139.99	0.28 ± 0.26	
$C_{60min\ cortisol}$					45.38 ± 26.56

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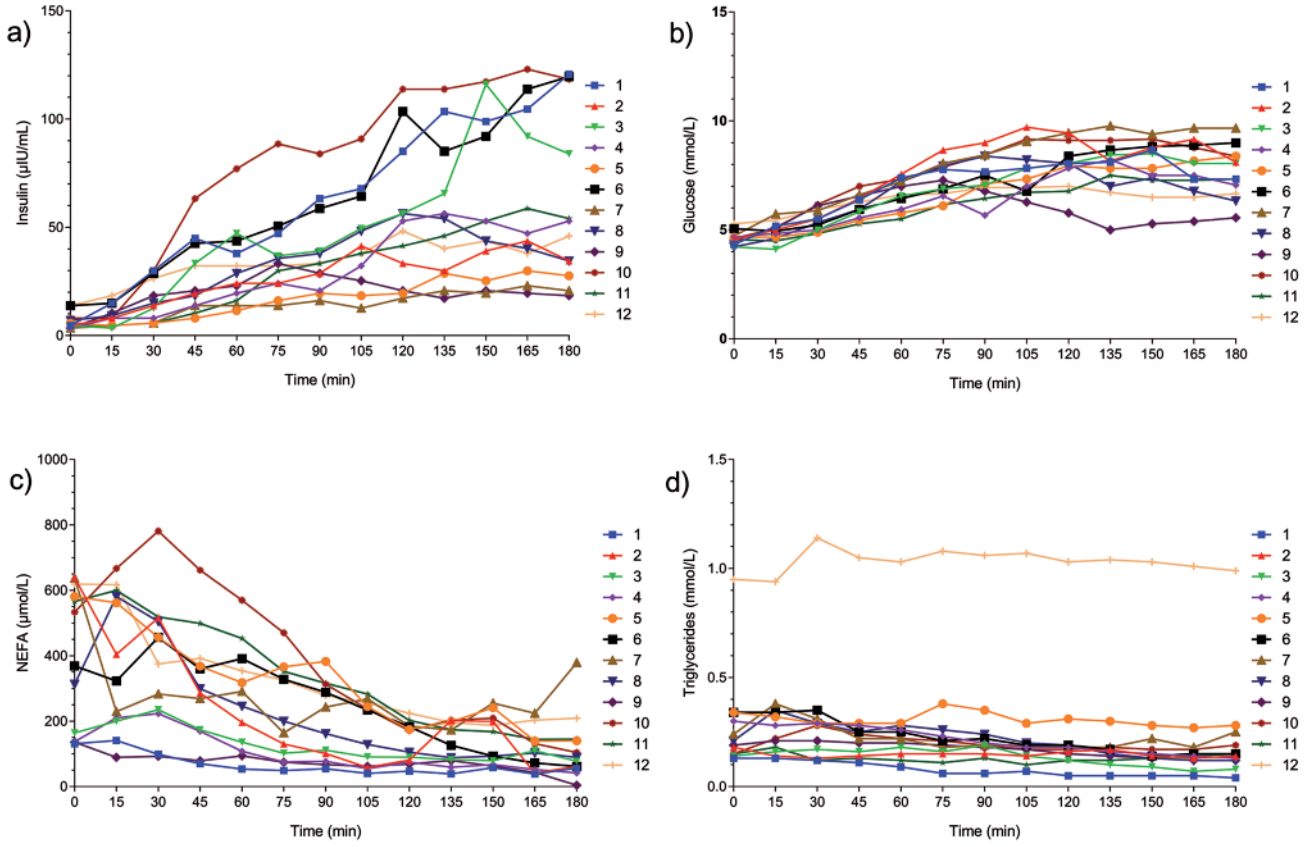

Fig. 1 Hormone and metabolite concentrations of twelve study horses during oral glucose tests (OGTs). a) Insulin (µIU/mL), b) glucose (mmol/L), c) non-esterified fatty acids (NEFA) (µmol/L) and d) triglycerides (TRG) (mmol/L).

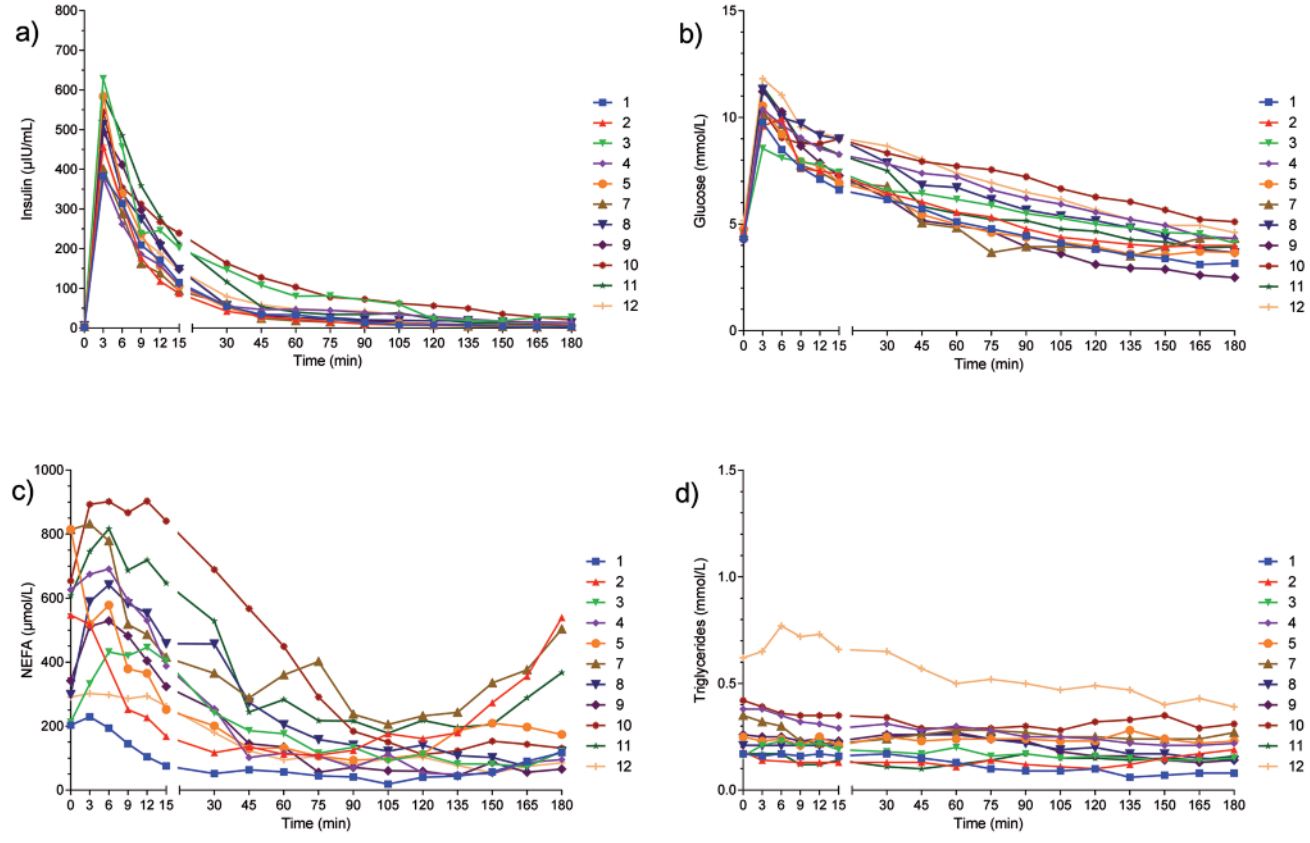

Fig. 2 Hormone and metabolite concentrations of eleven study horses during CGITs. a) Insulin (µIU/mL), b) glucose (mmol/L), c) NEFA (µmol/L) and d) TRG (mmol/L).

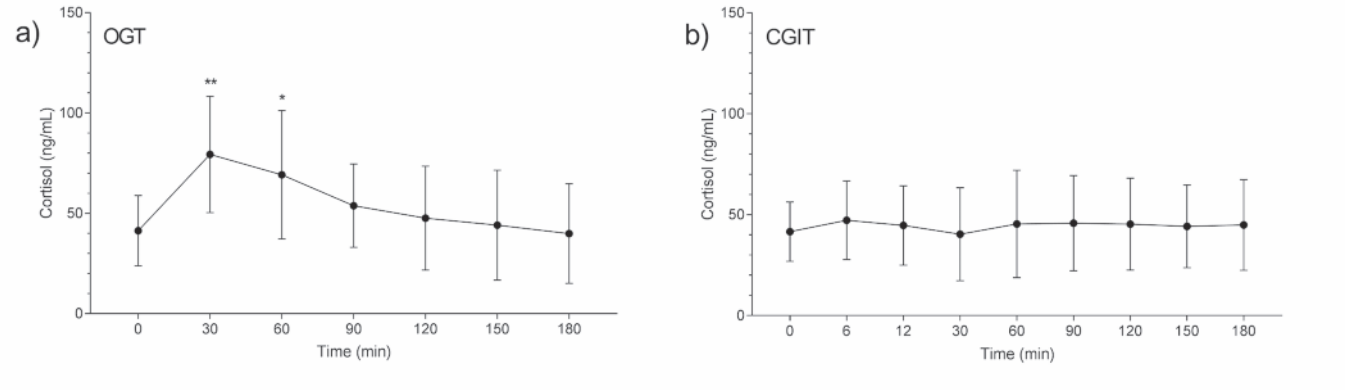

Fig. 3 Cortisol concentrations (ng/mL) during a) OGT and b) CGIT. Given are means ± SD; one-way ANOVA; * $P < 0.05$ ** $P < 0.01$.

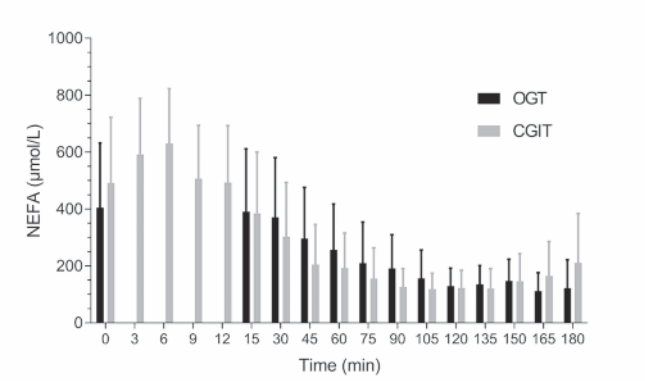

Fig. 4 NEFA concentrations during OGT (black) and CGIT (grey). Means ± SD are given.

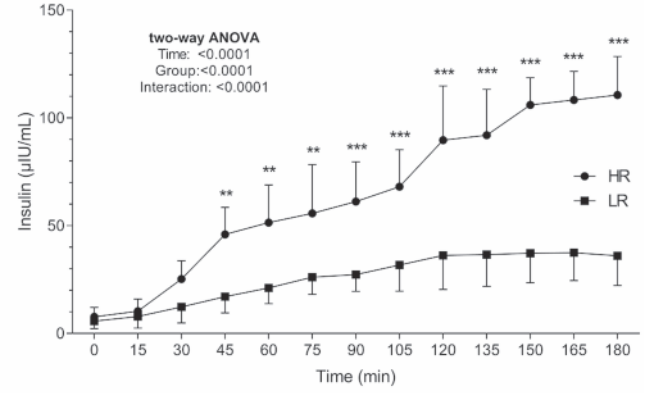

Fig. 5 Serum insulin concentration (µIU/mL) during the OGT in high responding (HR) and low responding (LR) animals. Means ± SD are given; two-way ANOVA; ** $P < 0.01$ *** $P < 0.001$. HR: n = 4; LR: n = 8.

excluded from the analysis of CGIT because of technical reasons.

Baseline concentrations in all metabolites and hormones examined did not differ significantly between OGT and CGIT days (Tab. 2 and 3; Fig. 1 and 2). The fructosamine concentration was 323.73 ± 25.2 µmol/L prior to OGT and 319.6 ± 26.0 µmol/L prior to CGIT and remained unaffected during both testing procedures.

The horses had basal cortisol concentrations of 41.37 ± 17.53 ng/mL prior to OGT and responded to glucose administration via nasogastric intubation with an increase in cortisol to 79.34 ± 28.95 ng/mL after 30 minutes ($P = 0.0020$), the first sample which was analysed for cortisol. Cortisol concentration decreased consistently during the test procedure which followed and did not differ significantly from basal concentrations 90 minutes after intubation (Fig. 3, Tab. 2). Basal cortisol concentrations were 41.57 ± 14.71 ng/mL prior to CGIT and remained unaffected during the testing procedure (Fig. 3b, Tab. 3).

Insulin, glucose and NEFA dynamics during OGT and CGIT

OGT

Insulin concentration started to increase after 30 minutes ($P = 0.0330$) during the OGT, with a huge variation in individual horses, and remained elevated until the end of the sampling period, i.e. 180 minutes ($P = 0.0141$) (Fig. 1a). A $C_{max\ insulin\ OGT}$ of 69.10 ± 39.07 µg/L was reached after 75 to 180 minutes (mean: 148 ± 32 minutes), but C^{max} insulin OGT varied from 23 to 123.05 µIU/mL. Four out of twelve horses exhibited maximal insulin concentrations above 100 µIU/mL, whereas the remaining eight horses ranged between 23 µIU/mL and 58.65 µIU/mL (Fig 1a). The $C_{120min\ insulin\ OGT}$

Table 4 Hormone and metabolite area under the curves (AUC) during oral glucose test (OGT) and combined glucose-insulin test (CGIT). Mean±SD; OGT n = 12; CGIT n = 11.

area under the curve (UC)	OGT	CGIT
insulin (µIU/mL x min)	6991.62 ± 3773.34	10362.75 ± 4242.51
glucose (mmol/L x min)	1249.42 ± 120.13	1006.27 ± 156.40
NEFA (µmol/L x min)	40428.42 ± 19791.13	36807.73 ± 17147.87
TRG (mmol/L x min)	46.32 ± 45.51	41.71 ± 20.51
cortisol (ng/mL x min)	10349.45 ± 4549.50	8269.90 ± 3935.52

was 54.05 ± 32.02 µIU/mL (Tab. 2). Blood glucose concentration increased immediately after the administration of glucose in all horses and no decrease back to baseline levels was observed during the whole OGT testing period in any horse (Fig. 1b). The increase in serum glucose concentration was significantly different after 30 minutes compared to baseline levels (P = 0.0051). The basal NEFA concentration (402.08 ± 216.30 µmol/L) had a wide variety after fasting conditions at the beginning of the test prior to glucose application, and declined during OGT procedure in all horses (P < 0.0001) (Fig. 1c). The NEFA concentration (199.60 ± 116.20 µmol/L) decreased significantly (P < 0.05) within 90 minutes after glucose administration. The mean $C_{min\ NEFA\ OGT}$ was 93.82 ± 53.22 µmol/L and was reached in 153 ± 33 minutes (range 75 to 180 minutes). The horses reached a plateau phase with only marginal changes in NEFA concentration towards the end of the sampling period from 120 to 180 minutes.

CGIT

Serum insulin concentration increased from 5.33 ± 2.07 µIU/mL at baseline to $C_{max\ insulin\ CGIT}$ 493.98 ± 86.84 µIU/mL three minutes after injection in the CGIT (Fig. 2a, Tab. 3). All horses showed an even decline in insulin concentration immediately after the initial peak. The peak serum insulin concentration was nearly halved (243.80 ± 64.40 µIU/mL) after nine minutes. Only three out of twelve horses returned to baseline insulin concentrations after 150 or 165 minutes (Fig. 2a). The mean serum insulin concentration ($C_{45min\ insulin\ CGIT}$) was 51.96 ± 34.78 µIU/mL 45 minutes after injection of glucose and insulin. The glucose concentration increased from physiological baseline concentrations of 4.63 ± 0.23 mmol/L to peak concentrations of 10.49 ± 0.93 mmol/L at the first sampling time point three minutes after injection (P < 0.0001) (Fig. 2b, Tab 3). Thereafter, plasma glucose concentration declined and returned to baseline concentrations 123 ± 37 minutes after the beginning of insulin and glucose injection. Decline continued slightly, with minimal glucose concentrations of 3.81 ± 0.65 mmol/L (Fig. 2b). Clinical signs of hypoglycaemia were not observed in any of the horses during the test procedure. The $C_{45min\ glucose\ CGIT}$ was 6.35 ± 1.07 mmol/L.

The basal NEFA concentration varied greatly, similar to that already observed prior to OGT. The baseline concentrations were 492.00 ± 230.26 µmol/L and NEFA concentrations decreased in all horses with the first statistically significant change compared to baseline concentrations after 75 minutes (P = 0.0186). The $C_{min\ NEFA\ CGIT}$ was 91.97 ± 56.89 and was reached after 123 ± 30 minutes (Fig. 2c, Tab. 3). The NEFA concentrations during OGTs and CGITs were comparable with similar decrease and comparable $C_{min\ NEFA}$ concentrations (OGT 93.82 ± 53.22 µmol/L; CGIT 91.970 ± 56.89 µmol/L) (Fig. 4).

Individual variations in endocrine and metabolic response

The twelve horses were clustered into two groups based on these findings and the underlying variations in insulin concentrations (absolute values, AUC) in the OGT. Four horses (high responder – HR) exhibited significantly higher insulin concentrations over time (P < 0.0001) in response to the oral glucose load compared to the eight horses remaining (low responders – LR) (Fig. 5). The four HR horses exhibited significantly higher values for AUCinsulin OGT (10289.05 ± 1070.65 µIU/mL × min) compared to the LR horses (3685.75 ± 424.35 µIU/mL × min), while comparing AUC-glucose OGT did not reveal any differences. The HR and LR did not differ either in basal insulin concentrations, nor in any other metabolite or hormone concentrations analysed at any time. None of the hormone or metabolite concentrations or responses to the test procedures showed any correlation with gender, age or BCS of the horses.

Discussion

Testing horses for ID is challenging work in routine practice. Obesity prevalence is high in the equine population (*Thatcher* et al. 2008, *Wyse* et al. 2008, *Giles* et al. 2014) and owners are becoming more aware of the potential cross-links between obesity or reginal adiposity, ID and laminitis. Therefore, owners' request for EMS testing has increased in recent times. No standard testing protocol has been established to date in equine medicine to determine ID in equine patients. However, recently published recommendations (Equine Endocrinology Group 2016) provide useful information for targeted diagnostic approaches. Insulin resistance is generally defined as a pathologic condition in which the biological tissue responsiveness to insulin is decreased (*Kahn* 1979). Tissue IR, especially in ponies, is often compensated by an increased pancreatic secretion of insulin, resulting in hyperinsulinemia (*Jeffcott* et al. 1986). Recent research has highlighted the importance of incretins, gastrointestinal tract hormones that effect insulin regulation and glucose homeostasis (*de Laat* et al. 2016). The term ID is used more commonly to consider the complexity related to variations in insulin dynamics and to shift the focus from only peripheral IR to a more complex pattern of impairments of insulin and glucose homeostasis. The term ID highlights the combination of fasting hyperinsulinemia and postprandial hyperinsulinemia assessed, for example, by oral glucose challenge procedures (Equine Endocrinology Group 2016).

Variations in dynamic insulin responses and plasma insulin clearance

Oral stimulation tests include assessment of functions of the gastrointestinal tract. It has recently been shown that, similar to other mammals, glucagon-like peptide-1 (GLP1) and gastric inhibitory peptide, also known as glucose-dependent insulinotropic polypeptide (GIP), are secreted in response to oral glucose or food and may enhance the glucose-dependent insulin secretion in the horse (*Chameroy* et al. 2010, *Bamford* et al. 2014, *de Laat* et al. 2016). The importance of the gastrointestinal tract, i.e. incretins, has been shown by several studies where insulin secretion after oral glucose intake or application was higher than after isoglycaemic iv glucose application (*Dühlmeier* et al. 2001, *de Laat* et al. 2016). Furthermore, it has been shown that ID can occur independently from tissue IR and that iv and oral tests gave different results regarding the insulinemic state being abnormal or nor-

mal in ponies being obese and/or laminitic (*de Laat* et al. 2016). Measuring glucose and insulin increase in plasma and time-dependent disappearance from plasma is the net result of intestinal absorption, glucose uptake by insulin-sensitive and insulin-independent tissues, and urinary glucose spilling. This rate of disappearance is influenced by all glucose-related adaptive physiological responses of peripheral tissues. Therefore, results from oral testing protocols could be influenced by several physiological pathways, thus, oral testing protocols can be used to assess ID and are considered to be indirect methods to assess IR. Oral glucose tests simulate the physiological way of ingestion of meals containing starches and reflect the physiological conditions followed by food intake. The way of application and dosages of glucose or other sugars in OGTs vary between protocols and range from administration of corn syrup with low glucose content (*Carter* et al. 2009, *Schuver* et al. 2014) through in-feed variations, with 0.5 or 1.0 g glucose powder mixed in low-glycemic meals (*Smith* et al. 2015), to glucose application via nasogastric intubation (*Ralston* 2002). In contrast to oral stimulation tests, none of the gastro-intestinal tract-related functions are assessed during iv stimulation tests, such as the CGIT, where glucose and insulin were applied directly into the bloodstream (*Eiler* et al. 2005). Therefore, no absorption from the GIT is necessary, the enteroinsular axis is bypassed and even pancreatic response is thought to be depressed due to the immediate application of supra-physiological doses of insulin. Consequently, besides urinary glucose spilling and insulin clearance by liver passage, only the capacity of exogenous insulin to shift the injected glucose into the insulin-sensitive tissues is assessed with this test.

The OGT in the present study could identify four horses that had a clearly exaggerated insulin response (HR) compared to the other eight horses (LR). The AUCinsulin OGT differed, whereas the AUCglucose OGT did not differ between HR and LR animals, suggesting that HR horses secrete more insulin to maintain their glucose homeostasis after the oral glucose challenge. According to recently published reference ranges for OGT procedures performed by nasogastric intubation and quantification of equine insulin with the ELISA used in the present study, insulin concentrations above 110 µIU/mL insulin at 120 minutes are indicative for ID (*Warnken* et al. 2018). If this cut-off is supplied to the oral sugar test (OGT) data only one horse (horse 10) has slightly elevated insulin response in OGT with 114 µIU/mL. It has been reported that insulin responses after OGT show wide variation and fluent transition from IS to ID (*Warnken* et al. 2018). However, it remains unclear whether these clear differences between horses were normal physiological variation or already indicative of subclinical abnormalities which may predispose these four individuals to develop metabolic pathologies. Differences between animals may also be explained by variations in gastric emptying rate, intestinal glucose absorption, hepatic extraction or urinary glucose spilling if renal threshold was exceeded after enteral glucose absorption or iv glucose.

Variations between the twelve horses due to variable feeding and management regimes were unlikely, since all horses were kept and fed under equal conditions for a fourteen-day acclimatization period in the clinic. Several studies investigated the effect of feeding and different diets on basal glucose and insulin, as well as their dynamics during diagnostic procedure with increased insulin concentrations and decreased insulin sensitivity (*Pratt* et al. 2006, *Bailey* et al. 2007, *Borer* et al. 2012). However, the horses in the present study were all maintained on average grass-hay diet without additional supplement feeding for at least 14 days prior to the start of the study. Besides variations due to feeding managements, seasonal changes in blood glucose and insulin concentrations and dynamics have also been reported (*McIntosh* 2006, *Bailey* et al. 2008, *Borer* et al. 2010, *Banse* and *McFarlane* 2014). In the present study, all horses underwent the study examination in autumn and winter during October through January to minimize seasonal variations in insulin and glucose dynamics.

Frank and colleagues (2006) showed that horses with a moderate BCS of 4 to 6 on a nine-point scale had lower insulin levels compared to horses with a BCS of 7 to 9. *Pleasant* and colleagues (2013) detected that horses aged 17 to 20 years had higher insulin levels and lower insulin sensitivity compared to younger horses in a random sample of 300 light breed horses. Comparably, *Vick* and colleagues (2007) found that age was negatively correlated with insulin sensitivity in a population of horses aged 3 to 29 years. Moreover, this negative correlation was not influenced by BCS, leading to the conclusion that older horses might be generally at a higher risk of developing IR. In this study, we were not able to show associations between BCS or age and insulin concentrations, either in basal insulin concentrations or in dynamic insulin response in OGTs or insulin clearance in CGITs.

Influence of insulins formulation used for CGITs

In contrast to the original protocol developed by *Eiler* et al. (2005) due to legal restrictions, a porcine zinc-insulin formulation was used in the present study to perform the CGIT instead of a fast-acting human recombinant insulin formulation. The porcine zinc-insulin formulation is an intermediary insulin consisting of highly sanitized amorphous insulin and crystalline insulin. According to the results of the present study, this formulation results in higher concentrations and a slower elimination of the insulin after injection compared to short-acting insulins. *Fleeman* et al. (2009) reported two-step responses in blood insulin concentration after subcutaneous porcine zinc-insulin injection in dogs. Mean peak concentrations where observed after three and nine hours, whereas median duration of insulins action was approximately 14 hours. By contrast, blood insulin concentration following subcutaneous injection of regular human insulin peak faster in humans and are reported to reach mean peak concentration after 117 minutes (*Hompesch* et al. 2011). However, reliable pharmacokinetic and pharmacodynamics data following iv or subcutaneous injection in horses are lacking and subject of current research. In the present study, none of the twelve horses returned back to baseline glucose concentrations at the suggested clinical-relevant time-point 45 minutes (C^{45min}) after glucose and insulin administration. Moreover, two horses (horse 3 = 108 µIU/mL and horse 10 = 127 µIU/mL) remained with insulin concentrations above the cut-off value of 100 µIU/mL at 45 minutes published previously (*Eiler* et al. 2005). Nonetheless, adjustment of reference ranges and cut-off values when used in clinical settings for assessment of IR by CGIT with porcine zinc-insulin in patients would be necessary.

A possible advantage of porcine zinc-insulin for CGITs is that the porcine insulin molecule and the equine insulin molecule only differ by one amino acid at position A9, whereas the human insulin molecule differs from the equine insulin molecule at position A9 and position B30 (*Ho* et al. 2008). Position B30 is especially considered to be important for the three-dimensional structure of the protein (*Conlon* 2001) and, thus, might influence receptor binding. Therefore, porcine insulin may act more comparably to the physiological endogenous equine insulin. It was noteworthy that none of the horses in our study exhibited a clinically relevant hypoglycaemia in response to the iv injection of exogenous insulin, either during the sampling period or after sampling had finished. This is a commonly observed adverse effect in CGITs (*Funk* et al. 2012). Thus, the use of a porcine-zinc insulin may be a safer and more physiological alternative to the use of short-acting human insulin preparations in CGITs.

Variations in insulin-dependent NEFA suppression

The concert of maintaining an organism's metabolism is complex. In addition to major aspects in the control of glucose homeostasis, insulin also affects lipid metabolism. Results of the present study clearly illustrate insulin effects on lipid metabolism. Basal NEFA concentrations, determined prior to both tests, showed high interindividual variation. The TRG stored in adipose tissue undergo lipolysis in a state of fasting and NEFA and glycerol are released into the circulation. Previous studies investigating the effect of withholding feed on insulin and very low-density lipoprotein concentrations found significantly increased NEFA concentrations in horses which were subjected to 36 hours of withholding feed compared to fed control horses. The mean NEFA concentrations of 513 µmol/L observed in the study by *Frank* et al. (2002) were comparable to the values of the present study. Fasting horses prior to assessment of IS is discussed controversial. Recent research investigated the optimal fasting period prior to dynamic testing for ID and IR and reported different periods for specific tests. Knowles et al. (2017) reported significant higher insulin responses in oral sugar test (OST) after fasting compared to non-fasted conditions. Currently, a three hour fast is recommended for OST (*Bertin* et al. 2016), whereas overnight fasting is recommended for in-feed OGT and a 14 hours fast has been described prior to CGIT (*Bröjer* et al. 2013). In order to provide similar metabolic conditions prior to testing, horses included in the present study were fasted for 14 hours before OGT and CGIT were started. Euglycemic hyperinsulinemic clamp studies in horses have reported reduced NEFA concentrations during clamp procedures (*Suagee* et al. 2011, *Urschel* et al. 2014). Similar reduction of NEFA during clamp studies in humans occurred and is due to a reduced plasma rate of appearance of fatty acids (*Shadid* et al. 2007). The reduction of plasma NEFA concentration is believed to be due to the inhibitory effects of insulin on the lipolytic enzyme hormone-sensitive lipase, resulting in reduced release of NEFA from adipose tissue (*Meek* et al. 1999). It was shown recently that insulin in the horse also seems to deactivate hormone-sensitive lipase (*Warnken* et al. 2017). Furthermore, recent literature suggested that, in addition to the suppression of lipolysis in adipose tissue, insulin also stimulates free fatty acid uptake from the plasma into peripheral tissues in humans (*Ramos-Roman* et al. 2012). However, whether similar conditions occur in horses is not known so far. Interestingly, the NEFA dynamics were very similar in OGTs and CGITs, despite the different insulin dynamics in both tests. In the OGT, the insulin concentrations continuously increased, whereas in CGIT, there was an initial increase followed by a continuous decrease. Despite these differences, both led to a comparable decrease in plasma NEFA concentrations. There was no statistically significant difference in NEFA concentration between OGT and CGIT. The C_{min} NEFA was nearly similar during the OGT and CGIT procedure with 93.82 ± 53.22 µmol/L and 91.97 ± 56.89 µmol/L, respectively. This might indicate a saturation of the suppression of lipolysis by insulin already with insulin concentrations, provoked by the OGT after enteral resorption of oral-applied glucose. In accordance with these findings, hyperinsulinemic euglycemic clamp studies indicated that insulin concentrations similar to postprandial insulin concentrations were sufficient for maximal antilipolytic effects (*Urschel* et al. 2014). Moreover, the present study revealed that the anti-lipolytic effect seems to be independent of the insulin's origin. The exogenous porcine zinc-insulin seems to be as equally effective as the endogenous equine insulin.

Absent increase in TRG concentrations during OGT and CGIT in our study indicates that re-esterification of NEFA in liver tissue is unlikely and, thus, insulin seems to suppress lipolysis predominantly in adipose tissues in the horse.

Several studies reported partly conflicting results of correlations between NEFA and/or TRG concentrations and weight gain, obesity or gender (*Frank* et al. 2006, *Carter* et al. 2009, *Pleasant* et al. 2013). In our study, lipid parameters or their response to the challenges did not correlate with age, gender, weight or BCS.

Effect of short-term hyperglycaemia on fructosamine

Fructosamine is used as a marker for the evaluation of abnormal glycaemic control in humans (*Goldstein* et al. 2004) and companion animals (*Reusch* et al. 1993, *Reusch* and *Haberer* 2001) suffering from diabetes for long-term monitoring. Hyperglycaemia, which may affect fructosamine concentrations, is often reported in horses diagnosed with pituitary pars intermedia dysfunction (PPID) (*McFarlane* 2011). However, the potential clinical applicability of fructosamine as a marker in horses is questionable due to a significant overlap of concentration ranges obtained from PPID horses and those horses not suspected of PPID (*Knowles* et al. 2014). In the present study, fructosamine concentrations did not differ significantly pre and post OGT or CGIT, or during the experimental time period. Horses exhibited higher or lower concentrations after glucose challenges with no consistent trend during the testing procedure or the experimental period. Thus, even repeated challenging with both tests and two times of unphysiological hyperglycaemia did not affect fructosamine concentrations in our twelve study horses.

Stress response in OGT and CGIT

Manipulation by nasogastric intubation for the administration of glucose solution into the stomach of the horse may lead to stress in most of the animals. Variability in blood glucose concentrations due to stress during nasogastric intubation is a

commonly discussed issue (*Firshman* and *Valberg* 2007). Furthermore, stress hormones can impact glucose dynamics and alter test results. A corresponding rise in serum cortisol concentrations after manipulations was observed. Nevertheless, horses returned to baseline levels after a short period of time. Protocols for oral glucose tests with oral application or feeding have been used successfully in several studies (*Schuver* et al. 2014, *Smith* et al. 2015, *de Laat* and *Sillence* 2016), but it has been shown that meal size and compositions as well as feed consumption time alter gastric emptying and small intestine motility (*Metayer* et al. 2004). Therefore, these disruptive factors, which may influence test results, are minimized when glucose is administered directly into the stomach of the patient. However, further studies are required to investigate stress response during OGT procedure with nasogastric intubation and examine the interference of test results.

The results of the present study underline that OGT and CGIT mirror different facets of glucose homeostasis and insulin regulation and sensitivity. While CGIT focuses on tissue insulin sensitivity, OGT reflects more facets of glucose homeostasis and insulin regulation, leading to the conclusion that oral testing protocols are superior to iv protocols to assess ID because of their physiological mode of action. Furthermore, insulin dynamics during CGIT with porcine zinc-insulin differ from insulin dynamics described in reports using short-acting insulins published previously, and necessitates adjustment of clinical interpretation when used for the assessment of IR in patients. On the other hand, our data indicate that intermediary insulins may have the advantage of being safer, i.e. have a lower risk of inducing hypoglycaemia than short-acting insulins.

Abbreviations

BCS: body condition score; CGIT: combined glucose-insulin test; EMS: equine metabolic syndrome; Fig: figure; ID: insulin dysregulation; IR: insulin resistance; ELISA: enzyme-linked immunosorbent assay; NEFA: non-esterified fatty acids; OST: oral sugar test; PPID: pituitary pars intermedia dysfunction; SD: standard deviation; Tab: Table; TRG: triglycerides

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Animal Welfare Statement

The study has been approved by the ethics committee within the University of Veterinary Medicine, Hannover, and the State Office for Consumer Protection and Food Safety in accordance with the German Animal Welfare Law (LAVES – Reference number: 33.14 42502-04-13/1259).

Competing interests

The authors declare that they have no competing interests.

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