**Original Article**

**Comparison of fasted basal insulin with the combined glucose-insulin test in horses and ponies with suspected insulin dysregulation**

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**Abstract**

Fasting horses for measurement of basal serum insulin concentration (fasting insulin; FI) has been recommended to standardise testing for insulin dysregulation (ID), yet limited data exist comparing it to dynamic tests. This study aimed to compare FI with the combined glucose-insulin test (CGIT) in horses suspect for ID. We hypothesised that FI would have poor sensitivity for detecting ID compared to CGIT using conventional cut-offs. Records were retrieved from CGITs performed in horses fasted for approximately 8 h. Serum insulin and glucose concentrations were measured before and for 150 min following an IV bolus of glucose followed by insulin. Correlations between FI and CGIT values were assessed. Youden’s index analysis was used to determine the optimal cut-off for FI. Logistic regression and Mann-Whitney *U* tests were used to determine factors affecting the results.

 CGITs (*n*=130) from 62 horses were evaluated. Compared to CGIT, sensitivity and specificity of FI for diagnosis of ID were 14.6% and 100% at a cut-off of 20 µIU/mL and 63.4% and 87.2% at a cut-off of 5.2 µIU/mL, respectively. FI was significantly correlated with insulin at 45 min (*r*s=0.66) and 75 min (*r*s=0.72); area under the curve for insulin (AUCinsulin; *r*s=0.67); glucose at 45 min (*r*s=0.53); and AUCglucose (*r*s=0.50). Obesity was significantly associated with increased odds of a positive CGIT and horses with a positive CGIT were significantly older (*P*<0.05). In conclusion, FI correlated well with CGIT results and had adequate sensitivity and specificity at lower cut-offs, despite poor sensitivity at conventional cut-off values. Further research to derive cut-off values relevant to the fasting period is warranted.

*Keywords***:** Dynamic Testing; Endocrine; Equine Metabolic Syndrome (EMS); Insulin Resistance

**Introduction**

Endocrinopathic laminitis risk is consistently associated with underlying insulin dysregulation (ID; Durham et al., 2019). Laminitis represents a welfare concern because it is painful, has the potential to be severe or recurrent, and is difficult to treat. Therefore, it is important to detect at-risk individuals before the development of clinical laminitis (Bertin and de Laat, 2017).

The detection of ID is central to determining the risk of laminitis in horses with suspected endocrine disease (Frank and Tadros, 2014). Basal insulin testing for hyperinsulinaemia is convenient and practical in clinical practice, yet results can be influenced by feeding. This is particularly relevant in clinical cases, where the non-structural carbohydrate (NSC) content of forage is unlikely to be known by the animal owner. Consequently, measurement of basal fasted insulin (FI) in horses has been recommended to standardise testing (Frank et al., 2010). However, fasting has recently fallen out of favour due to concerns of poor sensitivity (Durham et al., 2019) although data comparing FI to dynamic tests are minimal and not representative of the population at risk of endocrine disease and ID (Dunbar et al., 2016). The combined glucose-insulin test (CGIT) is a dynamic test of insulin dysregulation, primarily providing an indirect measure of tissue insulin resistance (Bertin and de Laat, 2017). The CGIT has been used to successfully demonstrate changes in ID in research (McGowan et al., 2013) and in clinical studies (Morgan et al., 2016). However, it requires at least three blood samples over a 45-150 minute period and, ideally, the pre-placement of an intravenous catheter (Eiler et al., 2005).

Improving the diagnostic value of FI in the detection and monitoring of ID may improve client compliance with testing and reduce the risk of laminitis by facilitating timely interventions. Consensus recommendations are that FI in normal horses should be < 20 μIU/mL when measured by Coat-a-Count insulin radioimmunoassay (RIA), Immulite insulin solid-phase chemiluminescent assay, or DSL-1600 insulin RIA following fasting for at least 6 hours (Frank et al., 2010).

The objectives of this study were to compare FI with the CGIT for the diagnosis of ID in a group of horses and ponies suspected to have endocrine disease and examine factors affecting the results. Should the results for FI and CGIT tests be well correlated, we intended to investigate the most appropriate cut-off value for FI under standardised conditions. We hypothesised that FI would have a low sensitivity for detecting ID compared to the CGIT when using conventional cut-offs. Furthermore, we hypothesised that the diagnostic accuracy of FI could be improved by deriving a lower cut-off value for horses tested in the fasted state.

**Materials and methods**

*Study design*

Clinical records were obtained from horses and ponies (referred to collectively as ‘horses’) that had undergone CGIT testing at the University of Liverpool’s Equine Hospital as part of investigation into suspected endocrine disease. Horses were either presented directly by their owners, referred by veterinary surgeons (Morgan et al., 2016) or were being screened or tested for experimental studies (McGowan et al., 2013; Carslake et al., 2018). Clinical cases were investigated under institutional ethical approval VREC248 (Approved 04.09.2014) and the experimental cases under The Animals (Scientific Procedures) Act 1986, project licence number PPL 40/3715. Following the trials, the experimental horses were assessed as healthy and were re-homed.

Information on signalment, bodyweight, diet and previous and current laminitis was obtained where it was included in the clinical records. Partial datasets were retained, with missing values being excluded during analysis. Diets were recorded in the clinical notes contemporaneously as reported by the owner.

*CGIT protocol*

All endocrine testing was performed according to an established, standardised hospital protocol. The day prior to the CGIT, an intravenous catheter was placed in a jugular vein and flushed with heparinised saline periodically overnight. A meal of approximately 1% of body mass (as dry matter) of grass hay was fed between 3.00 and 4.00 p.m. and CGITs commenced at 8 AM the following morning. Based on typical consumption rates, animals were fasted for 8 - 12 h. A CGIT was then performed as previously described (Eiler et al., 2005). Briefly, 150mg/kg glucose was administered intravenously as a 40%(Dextrose 40% w/v solution, Dechra) or 50%(Glucose 50% w/v solution, Fresenius Kabi) glucose solution in less than 1 minute immediately followed by a 0.1 IU/kg bolus of insulin (Humulin-S, Lilly) and heparinised saline. Serum insulin concentrations were measured at 0 min (FI), 45 min (insulin45) and 75 min (insulin75) using a chemiluminescent immunoassay (Immulite insulin solid-phase chemiluminescent assay; Immulite 1000 or Immulite 2000, Siemens) validated for use in horses (Carslake et al., 2017). Whole blood glucose concentration was measured at 0, 1, 5, 15, 25, 35, 45, 60, 75, 90, 105, 120, 135 and 150 min (glucose0 – glucose150) using a validated handheld device utilising glucose dehydrogenase enzyme and colourimetric principles (Alphatrak, Zoetis; Hackett and McCue, 2010) or a benchtop analyser utilising a glucose oxidase enzymatic method (YSI 2300, YSI), with the same analyser being used throughout each individual CGIT test. Results from the YSI 2300 were highly correlated with those of the Alphatrak (*r*2=0.99; median coefficient of variance 2.2%, interquartile range 1.0-3.5%), when transformed using linear regression according to the formula -

Alphatrak = (YSI 2300 x 1.52) – 0.46.

Therefore, results from the YSI 2300 device were transformed and retained for analysis.

*Case definition*

Horses were classified as positive for ID using CGIT if insulin45 was ≥ 100μIU/mL and/or glucose45 was above baseline glucose (Frank et al., 2010) by at least 1 mmol/L, in order to account for the precision of the glucometer. A FI cut-off of ≥ 20μIU/mL was used as positive for ID (Frank et al., 2010). Horses were classed as obese where their body condition score was recorded as ≥ 4/5 (Carroll and Huntington, 1988) or ≥ 7/9 (Henneke et al., 1983). For the purpose of statistical analysis, breeds were categorised into native ponies; cobs and cold bloods; and other horse breeds. Animals were classed as previously laminitic where this was reported by the owner or referring vet and/or where there was unequivocal evidence on examination of the hooves of previous laminitic changes (divergent growth rings, convex sole; Karikoski et al., 2015). Horses were classed as currently laminitic when they were recorded to show lameness that had been diagnosed at the hospital to be laminitis, graded as Obel grade ≥ II/IV (Obel, 1948). Feeding was classified in a binary fashion according to access to pasture/high NSC feed/supplementary feeding. Horses were considered to have access to high NSC feed if they had access to unlimited pasture, received supplementary feeding, or were fed unsoaked haylage (Carslake et al., 2018).

*Data analysis*

For comparison of FI and CGIT results, data included multiple datasets from the same horse but at different stages of treatment (i.e. following manipulation of diet and exercise). Normality of data was tested using Shapiro-Wilk analysis. Sensitivity and specificity of FI was calculated with reference to different outcome variables of the CGIT. Area under the curve (AUC) for insulin and glucose were calculated using the trapezoid method. Spearman’s rank correlation coefficient was used to compare FI with insulin45, glucose45, insulin75, AUCinsulin and AUCglucose. Receiver operating characteristic (ROC) curve and Youden’s index analysis were used to calculate optimal cut-off values for FI with reference to the CGIT result.

For investigation of horse level factors associated with test outcome, only datasets from the first CGIT on each horse were used. Mann-Whitney *U* tests were used to investigate the association between test outcome and continuous variables of age and weight. The effect of signalment (age category/ breed category/ sex), bodyweight category, diet (access to supplementary feed/ access to pasture/ access to high NSC feed) and laminitis status (history of laminitis/ currently lame/ history of laminitis but currently sound) on the likelihood of a positive CGIT result and the likelihood of a false negative FI result (positive CGIT result and negative FI result) were explored with univariable logistic regression. Data analysis was performed using Microsoft Office Excel (2016), SPSS version 24(IBM) and Ausvet Epi Tools[[1]](#footnote-1) and *P*<0.05 was considered significant.

**Results**

A total of 130 datasets were included from 62 animals. The majority (105 datasets) were from client-owned animals, 13 datasets were from preliminary screening of experimental animals and 12 datasets were from experimental animals. Twenty-four geldings contributed 42 datasets and 38 mares contributed 88 datasets. The median age at time of sampling was 10 years (interquartile range (IQR) 8.0 – 12.2 years; range 4 to 21 years). Pony breeds (*n*=36) included Welsh Section A ponies (*n*=11); other Welsh pony breeds or crosses (*n*=7); Shetlands (*n*=5); other UK native pony breeds (*n*=12); and other pony breeds (*n*=1). Cobs and cold bloods (*n*=21) included cobs (*n*=13); Welsh Section D cobs (*n*=7); and Clydesdales (*n*=1). Other horse breeds (*n*=5) included Arab; Anglo Arab; Cleveland Bay; Dutch Warmblood; and Quarter Horse (all *n*=1).

Values for glucose0; glucose45; and AUCglucose were normally distributed however the values for insulin0 (FI); insulin45; insulin75 and AUCinsulin were not normally distributed, and therefore non-parametric tests were used. Median (IQR) FI was 4.6 (2.0 to 11.6) μIU/mL; insulin45 was 79.4 (43.5 to 182.0) μIU/mL; and insulin75 was 29.7 (11.2 to 79.3) μIU/mL.

Using the CGIT test cut-offs, 63.8% (*n*=83/130; 95% confidence interval (CI) 55.6-72.1%) of results were positive for ID. Only 9.3% (*n*=12/129; 95% CI 4.3-14.3%) of results were positive for ID when classified by FI using a cut off of ≥ 20 µIU/mL. This resulted in sensitivity of FI of 14.6% (95% CI 7.8-24.2%) but specificity of 100% (95% CI 92.5-100%) compared to the CGIT test (Table 1), with area under the ROC curve 0.79 (95% CI 0.71 to 0.86).

Basal FI concentration was moderately to highly correlated with insulin45 (Fig. 1), insulin75 (Fig. 1) and AUCinsulin with Spearman's rank correlation coefficients of 0.66, 0.72 and 0.67 respectively (all *P*<0.001). However, FI was only moderately correlated with glucose45 (Fig. 1) and AUCglucose with Spearman's rank correlation coefficients of 0.53 and 0.50 respectively (all *P*<0.001).

Using Youden’s index to maximise test efficiency, the optimal cut-off value for FI was 5.2µIU/mL, with an associated sensitivity of 63.4% (95% CI 52.6-73.0%) and specificity of 87.2% (95% CI 74.8-94.0%; Fig. 2).

*Effect of signalment, weight, diet and laminitis status on test outcomes*

At the time of initial presentation, median bodyweight was 430kg (IQR 274-506kg), and 85.0% of horses (*n*=51/60; 95% CI 76.0-94.0%) were recorded as obese. Overall, 67.7% of horses (*n*=42/62; 95% CI 56.1-79.4%) had previously had laminitis and 50.0% of horses (*n*=31/62; 95% CI 37.6-62.4%) had laminitis at the time of initial testing. Fifty-six percent of horses (*n*=34/61; 95% CI 43.3-68.2%) had access to pasture; a further 36.1% (*n*=22/61; 95% CI 24.0-48.1%) had no or only muzzled pasture access and the remaining 9.8% of horses (*n*=5/61; 95% CI 2.4-17.3%) had access to bare pasture. Only 4.9% of horses (*n*=3/61; 95% CI 0-10.3%) were reported to receive supplementary feed. Overall, 85.2% of horses (*n*=46/54; 95% CI 75.7-94.7%) were categorised as having access to high NSC feed at the time of testing.

 The age of animals with a positive CGIT result was significantly greater (median 11.5 years) than those with a negative CGIT (median 10.0 years; *P*=0.04). Weight was not significantly different between animals with a positive CGIT result versus those with a negative CGIT result (*P*=0.65). Using univariable logistic regression, the presence of obesity at the time of initial presentation was associated with increased odds of a positive CGIT result (odds ratio (OR) 26.0; 95% CI 2.9-229.4; *P*=0.003). There were no significant associations between a positive CGIT result and weight, breed category, sex, history of laminitis, current lameness, history of laminitis but currently sound, supplementary feeding, pasture access, or access to high NSC feed (all *P*>0.05).

At the time of initial presentation, age and weight were not significantly different between horses with a false negative FI result (i.e. positive CGIT result and negative FI result) versus those without. Using univariable logistic regression, the presence of obesity was associated with increased odds of a false negative FI outcome (OR 9.7; 95% CI 1.1-83.7; *P*=0.04). There were no significant associations between a false negative FI result and weight, breed category, sex, history of laminitis, current lameness, history of laminitis but currently sound, supplementary feeding, pasture access, or access to high NSC feed (all *P*>0.05).

**Discussion**

This study confirmed poor sensitivity of FI for diagnosis of EMS in horses and ponies suspected to have ID when the conventional cut-off of ≥ 20 µIU/mL on a chemiluminescent assay is used, in line with recent opinion moving away from the use of fasted sampling conditions (Durham et al., 2019). A previous study also showed a poor sensitivity of FI in a group of twelve horses not suspected to have ID; however, these comparisons were made against an arbitrary single cut-off value for the frequently sampled intravenous glucose tolerance test and further examination of the cut-off itself was not performed (Dunbar et al., 2016). Depending on the population sampled, the sensitivity and specificity of a test is affected by the cut-off value used; reducing the cut-off usually improves the sensitivity but reduces the specificity. In this study, there were moderate to high correlations between FI and dynamic test results. Furthermore, a lower cut-off value derived by Youden’s index markedly improved sensitivity whilst maintaining high specificity. Our study, therefore, demonstrated that the poor sensitivity is related to the cut-off value used rather than FI being an intrinsically inappropriate test.

Lowering the cut-off for FI to 5.2 µIU/mL improved sensitivity from 15% to 63% and lowered the specificity from 100% to 87%. We acknowledge that the decreased specificity would mean increased false positive diagnoses of ID, with the potential consequence of management interventions being recommended when ID is not actually present. However, management of ID primarily involves reduction of obesity and incorporation of increased exercise into the treatment plan. Furthermore, obesity is known to cause a range of problems and is considered detrimental to the health of horses even without ID (McGregor-Argo, 2009). Since obesity and age are risk factors for Equine Metabolic Syndrome (EMS), which consistently features ID (Morgan et al., 2014; Durham et al., 2019), treatment using diet and exercise may help prevent development of EMS, i.e. of ID and consequent clinical laminitis. Thus reducing the cut-off is likely to be overall beneficial to the welfare of the equine population.

Multiple research studies have utilised fasting following a set evening meal to standardise testing conditions, although, due to the experimental designs, the results focussed on the effects of interventions rather than specific cut-off values (McGowan et al., 2013; Morgan et al., 2016; Carslake et al., 2018). Fasting was also recommended in clinical ID testing for the same reason (Frank et al., 2010). The cut-off value of 20 μIU/mL may however have been based on unfasted or very briefly fasted animals, based on work published prior to this recommendation. In ponies removed from pasture for only 1-3 hours prior to sampling, plasma insulin concentrations (mean ±SE) in previously laminitic ponies were 21.6 ±2.2 μIU/mL and in non-laminitic ponies 10.7 ±0.8 μIU/mL using an RIA (Treiber et al., 2006). In the same herd of pastured ponies, a basal insulin cut-off of 32 μIU/mL adequately predicted laminitis in unfasted ponies (area under the ROC curve 0.88 [95% CI 0.80-0.96]) using the same RIA (Carter et al., 2009). In addition, the RIA reads consistently higher at lower insulin values than the chemiluminescent assay more commonly used in the UK (Carslake et al., 2017) so these higher cut-offs may have been inappropriately applied if extrapolated to laboratories utilising the chemiluminescent assay.

Following a diagnosis of laminitis, recommended management changes to reduce forces on the damaged lamellae include stabling and diet restriction (van Weeren et al., 2016). Under these conditions, a standardised fast can be incorporated practically into a horse’s routine, limiting the variations in insulin concentrations that can occur after feeding of different types of preserved forage (Carslake et al., 2018). However, FI concentrations of < 5 μIU/mL are close to the limits of detection of the chemiluminescent assay, so a shorter period of fasting or feeding of a standardised forage may be ideal and further research to determine the optimal period of fasting or feed withholding is warranted.

FI was better correlated with CGIT insulin outcome variables than with CGIT glucose outcome variables in this study, consistent with previous work demonstrating superior repeatability for insulin responses compared to glucose responses during the CGIT (Eiler et al., 2005; Bröjer et al., 2013). FI was also better correlated with insulin75 than insulin45. The additional insulin measurement at 75 min is part of the CGIT protocol used at the centre where this study was undertaken and was based on the original research on the CGIT where insulin returned to baseline values in normal horses by 75 min (Eiler et al., 2005). The return of glucose to baseline by 45 min was derived from the original research on the CGIT (Eiler et al., 2005) while the cut-off for insulin45 of 100 μIU/mL was only ever derived by expert opinion (Frank et al., 2010).

Obesity at the time of initial presentation was associated with increased odds of a positive CGIT result, which is unsurprising given obesity is an important predisposing factor for EMS (Durham et al., 2019). The association of obesity with increased odds of a false negative FI result may reflect the poor sensitivity of FI at the 20 μIU/mL cut-off or could potentially be due to interventions undertaken by owners prior to presentation that reduced the degree of fasting hyperinsulinaemia (e.g. restricting feed intake). Horses with a positive CGIT were older than those with a negative result, which supports previous research showing that age is a significant risk factor for hyperinsulinaemia (Morgan et al., 2014). The lack of statistically significant associations between test outcomes and breed, diet and laminitis status may reflect a Type II statistical error due to the small sample size (*n*=62) and/or the difficulties in accurately classifying diet based on historical clinical records. The effect that these variables have on test outcome may be better initially explored in experimental studies altering only one variable at a time, rather than in a retrospective case series, in order to optimise the test performance of basal insulin.

The inclusion of datasets from experimental studies as well as from client-owned animals presenting for investigation of suspected endocrine disease could be considered a study limitation. However, the experimental animals were acquired for the purpose of investigating suspected endocrine disease and were all native breeds acclimatised to pasture management prior to acquisition and managed either at pasture or stabled with access to grass hay or soaked grass hay during studies, thus mirroring conditions for many client-owned animals with endocrinopathic laminitis, such that results can reasonably be extrapolated to client-owned horses. Conversely, investigation of the effect of baseline diet on test performance may be limited by difficulties in retrospectively categorising diet based on potentially biased owner reporting. The horses included in the study could have altered insulin dynamics compared to ponies, however the breed distribution is likely to be similar to the population seen for suspected EMS in clinical practice and univariable regression did not identify a significant difference in odds of a positive CGIT in cobs and cold bloods or other horse breeds compared to ponies.

A further limitation of this study was that both FI and CGIT represent predominantly tissue insulin resistance rather than enteroinsular axis derived insulinaemic responses, as can be measured with oral dynamic testing. The clinical and pathophysiological relevance of investigating the enteroinsular axis doesn’t, however, eliminate the requirement for tests of tissue insulin resistance. Previous work has shown that the CGIT has superior sensitivity and specificity to the oral sugar test (OST; Dunbar et al., 2016) and is more repeatable than oral tests (Bertin and de Laat, 2017). The CGIT has also been used in numerous experimental and clinical studies previously (Eiler et al., 2005; Bröjer et al., 2013; McGowan et al., 2013; Morgan et al., 2016; Carslake et al., 2018). It has been estimated that the incretin derived component of ID accounts for less than 23% of the total insulinaemic response (de Laat et al., 2016).

Previous work has identified non-fasted basal insulin to be significantly associated with the future development of laminitis in the next 1, 2 and 3 years in a large group of ponies under variable owner controlled management conditions (Menzies-Gow et al., 2016). This study has demonstrated the value of utilising ROC to identify cut-off values appropriate to the testing conditions, rather than extrapolating from observational studies. We identified that a cut-off of 5.2µIU/mL is more appropriate than a cut-off of 20µIU/mL when using a chemiluminescent assay to measure FI in order to diagnose ID. This contests the concern that FI is a poorly sensitive test, by demonstrating this previous conclusion to be a consequence of an inappropriate cut-off value. However, as this cut-off value is close to the limit of detection of the chemiluminescent assay, increasing the cut-off value by using a shorter fasting period may be beneficial. Further work is needed to investigate whether basal insulin test performance is improved when performed on fed or fasted horses and, if fed, how to standardise the constituents, volume and time frame of feeding and sampling.

**Conclusions**

Measurement of FI using a chemiluminescent assay is insensitive for the diagnosis of ID at a cut-off of 20 μIU/mL, but more sensitive whilst remaining specific at a cut-off of 5.2 μIU/mL. Based on these results, screening for ID can be performed using fasted conditions whilst achieving adequate sensitivity by lowering the cut-off. Further work is needed to investigate optimal sampling conditions for basal insulin testing and to identify which factors may affect the interpretation of results.

**Conflict of interest statement**

 None of the authors has any other financial or personal relationships that could inappropriately influence or bias the content of the paper.

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**Table 1.**
Fasted basal insulin (FI) and combined glucose-insulin test (CGIT) results.

|  |  |  |  |
| --- | --- | --- | --- |
|  | CGIT positive | CGIT negative | Total |
| FI positive | 12 | 0 | 12 |
| FI negative | 70 | 47 | 117 |
| Total | 82 | 47 | 129a |

a One animal was excluded from this analysis due to absence of FI measurement on the third CGIT test

**Figure legends**

Fig. 1. Scatterplots of fasted basal insulin with A) insulin at 45 min of the combined glucose-insulin test (CGIT; *r*s=0.66); B) insulin at 75 min of the CGIT (*r*s=0.72) and C) glucose at 45 min of the CGIT (*r*s=0.53). Triangles represent samples with a positive result using CGIT cut-offs (insulin ≥100 μIU/mL and/or glucose > baseline + 1mmol/L at 45 min); ovals represent samples with a negative CGIT result.

Fig. 2. Plot showing sensitivity (solid line) and specificity (wider dotted line) of fasted insulin at a range of cut-off values, with reference to the combined glucose-insulin test (CGIT) result. The solid black line indicates the recommended cut-off of 20 µIU/mL and the dashed black line indicates the cut-off derived in this study of 5.2 µIU/mL.

1. See: Epitools epidemiological calculators. <http://epitools.ausvet.com.au> (Accessed 17 January 2019) [↑](#footnote-ref-1)