Enteroinsular Axis Response in Healthy and Critically Ill Foals

THESIS

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Abstract

Problems of energy dysregulation are frequently encountered when dealing with critically ill foals with up to 70% of foals presenting to a neonatal intensive care unit having a blood glucose value outside of the reference range.[1] The association of hypoglycemia and non-survival has been previously demonstrated,[1–4] as has an association of extreme hyperglycemia and non-survival.[1] Energy metabolism of septic foals has been documented to include hypoglycemia and the endocrine response of elevated glucagon and decreased insulin.[2] Incretin hormones are secreted from the gastrointestinal tract in response to the oral intake of nutrients and enhance insulin secretion in a glucose-dependent fashion. Glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 are the two incretins known to possess insulinotropic effects. While blood glucose remains the most important stimulus for insulin release in the horse, active GLP-1 (aGLP-1) and GIP have been demonstrated to contribute to the overall insulin response in adult ponies following orally administered glucose.[5]

For the first study, we hypothesized that secretion of incretins in healthy foals in response to orally administered carbohydrates (glucose or lactose) would be increased when compared to the same dose of glucose administered intravenously or when compared to a period of fasting. We also hypothesized that this incretin response would be linked proportionately to insulin responses. Thirty-six healthy, Standardbred foals less than 4 days of age were included. After a 60-minute fast, blood samples were collected

immediately prior to the administration of glucose or lactose either orally or intravenously. Blood samples were collected frequently over a 180-minute period in all foals. Foals were not allowed to nurse during the study period. Some foals were additionally sampled following access to free choice nursing. One group of foals assigned to the fasting group experienced this same protocol, but no carbohydrate was administered. Blood glucose was measured using a portable glucometer and plasma insulin, GIP, and GLP-1 were determined by enzyme-linked immunosorbent assays. In this study, we documented that healthy equine neonates have a functional enteroinsular axis (EIA) as evidenced by the rapid and significant increase in both GIP and GLP-1 concentrations following access to free choice nursing. Additionally, we documented that the response of the EIA in equine neonates, in the immediate postpartum period, is highly variable. To our knowledge, this is the first study to investigate the EIA in equine neonates.

For the second study we hypothesized that blood concentrations of insulin, GIP, and GLP-1 would be decreased in septic foals compared with healthy controls. We also expected an association between magnitude of these differences in these hormones and survival status. One hundred five healthy and hospitalized foals of less than 7 days of age were included in this study. Blood samples were collected on admission and then every 24 hours from septic (sepsis score $\geq$ 12 or positive blood culture), sick non-septic (SNS), and healthy foals. Blood glucose was measured using a portable glucometer while plasma insulin, plasma GIP, and plasma GLP-1 were determined by enzyme-linked immunosorbent assays. Septic foals had significantly lower insulin and GIP

concentrations, but higher GLP-1 concentrations at time 0 when compared to healthy foals. Hospitalized foals had significantly lower insulin area under the curve (insulin-AUC) and GIP area under the curve (GIP-AUC) than healthy foals. In healthy foals, a positive correlation existed between insulin and GIP and insulin and GLP-1 at all time points (0, 24, 48, and 72 hours). A positive correlation was also found between GIP and GLP-1 in healthy foals at time 0, 24, and 72 hours. Among septic foals, higher insulin concentrations at time zero were noted in survivors versus non-survivors. There was no difference between survivors and non-survivors with respect to GIP and GLP-1 concentrations at time 0.

Dedication

To the veterinarians who have shaped me along the way: Drs. Rodney Belgrave, Larry Bramlage, Tiffany Marr, Kit Miller and most of all my parents, Marylou and Mike Rings. I would not be here today without your wonderful guidance and unending support. Words cannot express the impact you have had on my career/life and I thank you eternally for showing me what being a truly gifted, dedicated and compassionate veterinarian and human-being looks like. May I find half the success that you each have achieved.

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Chapter 1: Introduction and Literature Review

1.1 The Enteroinsular Axis (EIA) in Humans

Incretin hormones are secreted from the gastrointestinal tract in response to the oral intake of nutrients, which amplifies the magnitude of meal-stimulated insulin secretion from pancreatic islet β-cells in a glucose-dependent manner.[6] Following the discovery of secretin in 1902, it was hypothesized that certain factors/hormones produced by the gastrointestinal tract were capable of regulating the endocrine pancreas.[7] Confirmation of this theory came about in 1906 when it was demonstrated that following oral administration of a homogenized sample of swine duodenal mucosa patients suffering from diabetes mellitus experienced a significant decrease in or even resolution of glucosuria.[8] Insulin was successfully identified in pancreatic extracts in 1921,[9] which also led to the speculation that after nutrients enter the proximal intestine, a gut factor was released into circulation to stimulate insulin secretion and lower glucose concentrations. Purification of this gut factor occurred in 1929 and was given the name "incretin" as an acronym for INtestine seCRETion INsulin.[10] Following scientific advancements in the ability to measure insulin in 1964, it was demonstrated that orally administered glucose produced a significantly greater and more sustained insulin response when compared to the intravenous administration of glucose.[11] This finding has come to be known as the "incretin effect."

Glucose-dependent insulinotropic polypeptide (GIP) was the first incretin identified. Originally named *gastric inhibitory polypeptide* for its initially recognized

ability to inhibit the secretion of gastric acid,[12] this polypeptide was later renamed when its incretin properties were recognized.[13] To date, the only other identified gut hormone that possesses insulinotropic properties is *glucagon-like peptide-1* (GLP-1).[14]

Glucose-dependent insulinotropic polypeptide is a 42-amino-acid hormone that is produced in and secreted from enteroendocrine K-cells primarily in the duodenum and proximal jejunum.[15] Species-specific differences exist as to what nutrient most prominently stimulates the secretion of this hormone. In humans, the most effective stimulator of GIP secretion is the oral consumption of fat while in rodents and swine oral consumption of carbohydrates results in the most significant GIP secretion.[16–19] The GIP sequence is highly conserved among species as demonstrated by greater than 90% homology among human, murine, porcine, bovine, and equine GIP.[20]

Glucagon-like peptide-1 (GLP-1) is a 32-amino-acid hormone that is produced in and secreted from enteroendocrine L-cells located throughout the distal small intestine and colon.[21] GLP-1 is formed from proglucagon via posttranslational processing by prohormone convertase 1/3 (PC1/3).[22] GLP-1 secretion may occur in response to luminal nutrient interaction with intestinal L-cells or from neural or endocrine factors.[15,16] The ingestion of carbohydrates and particularly fats are the primary physiologic stimuli for GLP-1 secretion. GLP-1 exists in multiple forms, the most common of which is GLP-1 (7-36) amide form.[23] Lesser amounts of bioactive GLP-1 (7-37) are also detectable.[24] Both

GLP-1 (7-36) and GLP-1 (7-37) are equipotent in their ability to stimulate the secretion of insulin.[25]

Glucose-dependent insulinotropic polypeptide and GLP-1 exert their insulinotropic effects by binding GIP receptor (GIPR) and GLP-1 receptor (GLP-1R), located on pancreatic β-cells. These receptors are members of the 7-transmembrane-spanning, heterotrimeric G-protein coupled receptor superfamily. This binding leads to the activation of adenylate cyclase and the increase of intracellular cyclic adenosine monophosphate (cAMP).[15,16,21,26] Increased cAMP causes activation of protein kinase A (PKA) and exchange protein activated by cAMP2 (EPAC2). Increased activation of PKA promotes the closure of ATP-sensitive potassium channels (K_{ATP} channel) and facilitates β-cell membrane depolarization. Increased activation of PKA also causes closure of voltage-dependent potassium channels (Kv) and the subsequent reduction in Kv currents which prevents β-cell repolarization.[26] Depolarization of the β-cell opens voltage-gated calcium channels (VDCC) causing an increase in intracellular calcium concentration which ultimately triggers fusion of insulin-containing granules with the β-cell's plasma membrane and results in the secretion of insulin.[27] Increased intracellular calcium also promotes transcription of the proinsulin gene, which increases production of insulin.[21] Activation of EPAC2 secondary to increases in intracellular cAMP levels has also been demonstrated to increase the density of insulin-containing granules within the cell, which further facilitates insulin secretion.[28,29] It is estimated that GIP and GLP-1 account for 50-70% of insulin secretion in humans after consumption of a meal.[16,30]

Additional biological actions of GIP and GLP-1 in regards to the pancreas include stimulation of β-cell and/or progenitor cell proliferation and inhibition of β-cell apoptosis.[31–33] Individually, GLP-1 confers glucose sensitivity to glucose-resistant β-cells. This action is achieved through up-regulation of glucose transporters and glucokinases in the β-cell which improves the cell's capacity to detect and respond to glucose.[34] The effects of the incretin hormones on glucagon secretion from pancreatic α-cells are antagonistic. Glucose-dependent insulinotropic polypeptide enhances the secretion of glucagon while GLP-1 suppresses the release of glucagon during the euglycemic state.[21] During a hypoglycemic state GLP-1 no longer functions to inhibit glucagon secretion. Glucagon-like peptide-1 has been shown to act on pancreatic δ-cells to stimulate pancreatic somatostatin secretion. Evidence strongly suggests that the inhibitory action of GLP-1 on α-cells is indirect and mediated through GLP-1 manipulation of somatostatin.[15]

The incretin hormones are known to have many varied actions in regard to extra-pancreatic tissues. In the central nervous system, both GIP and GLP-1, exert proliferative and anti-apoptotic actions on neural cells in addition to regulating feeding behaviors by promoting satiety.[16,35,36] Both incretins also enhance and promote learning and memory. Inhibition of gastric acid secretion, for which GIP once derived its name, is noted only at supraphysiologic concentrations of GIP.[37] Glucagon-like peptide-1 inhibits gastric emptying, which in addition to its pancreatic actions discussed earlier helps to attenuate the meal-associated rise in blood glucose concentrations as the transit time for nutrient from the stomach to the small intestine is prolonged.[38,39] Glucose-dependent insulinotropic

polypeptide has minimal to no effect on gastric emptying in humans. Cardiovascular effects are seen with GLP-1, as this incretin increases cardiac function and exhibits cardioprotective effects.[40,41] GIP has been reported to promote the secretion of vasoactive substances and has both vasodilatory and vasoconstrictive properties dependent on the specific vascular bed involved.[16] GIP increases lipogenesis and promotes weight gain.[42] GLP-1 decreases lipogenesis and increases glucose uptake and storage in adipose and muscular tissues.[16] Suppression of proinflammatory cytokines is associated with GLP-1. Additionally, GLP-1 has natriuretic and diuretic properties that are associated with increased glomerular filtration rate and inhibition of proximal tubule sodium reabsorption.[16] Glucose-dependent insulinotropic polypeptide affects the skeletal system by promoting new bone formation and decreases bone resorption.[43,44]

GIP and GLP-1 undergo rapid degradation by the ubiquitous proteolytic enzyme dipeptidyl peptidase-4 (DPP-4). Dipeptidyl peptidase-4 is a serine protease that specifically cleaves dipeptides from the amino acid terminus of oligopeptides or proteins that contain an alanine or proline residue in the second position.[45,46] GLP-1 (7-36) peptide is degraded to yield the inactive form GLP-1 (9-36).[26] GIP (1-42) is processed by DPP-4 into GIP (3-42). Tissue expression of this proteolytic enzyme is wide and includes: the intestine, pancreas, liver, kidney, spleen, adrenal glands, central nervous system, immune cells (lymphocytes and macrophages) and endothelial cells.[45] In humans, the half-life of biologically active GLP-1 is less than 2 minutes.[47,48] The half-life of biologically active GIP is approximately 7 minutes in healthy humans[49] and less than 2 minutes in rats.[46]

Greater than 50% of the GLP-1 that enters portal circulation following secretion from enteroendocrine L-cells has already been inactivated by DPP-4 prior to its entry into portal circulation.[16,21] Additional degradation by the liver results in less than one tenth of the secreted GLP-1 from making it to systemic circulation.[50] Renal clearance is the major route of elimination for both GIP (3-42) and GLP-1 (9-36 amide).[51]

Incretin-based therapies have been directed toward the treatment of Type 2 Diabetes Mellitus (T2DM) in the human medical field. Exendin-4, a 39-amino-acid peptide with GLP-1 activity, was first isolated from the venom of the Gila Monster, *Heloderma suspectum*, and shares 53% homology with human GLP-1.[52] Exendin-4 contains the amino acid glycine at position 2 rather than alanine as does GLP-1, making it an unsuitable substrate for inhibition by DPP-4 and subsequently increasing its *in vivo* half-life to approximately 2.4 hours. [16,26,52] The synthetic form of exendin-4 was developed in 2005 and named exenatide. A longer acting formulation called liraglutide gained FDA approval in 2010. Another route for therapy of T2DM is through the inhibition of DPP-4. The inhibition of this protease protein delays the breakdown of active incretin hormones and prolongs their biologic effect. Vildagliptin and sitagliptin are two such drugs and belong to the DPP-4 inhibitor class of drugs. Both medications have been demonstrated to increase plasma levels of GIP and GLP-1 following ingestion of a meal and work to enhance glucose-stimulated insulin secretion and improve β-cell function.[16] Incretin-based therapies offer a promising new adjunctive or even replacement therapy for insulin in the treatment of T2DM.

1.2 The Enteroinsular Axis (EIA) in Horses

The enteroinsular axis as it relates to equine physiology has recently raised attention, in particular as it relates to metabolic conditions and energy regulation. This largely centers around the potential alterations in the EIA leading to elevated insulin concentrations and the connection between laminitis and hyperinsulinemia.[53,54] Hyperinsulinemia can result from a dysregulated insulin response following the ingestion of nonstructural carbohydrates (NSC).[55] Recent studies demonstrating enhanced insulin secretion in response to the oral administration of nutrients in equids may support a connection between the incretin response, hyperinsulinemia and laminitis.[5,56,57] Another promising direction of study is the investigation of the incretin response in neonatal foals as problems of energy dysregulation are common in critically ill equine neonates admitted to intensive care units and are often associated with decreased survival.[1–4,58]

The presence and functionality of the EIA in horses was confirmed by Dühlmeier *et al*. in 2001.[59] Activation of the EIA was demonstrated when a greater insulin response was observed in horses following oral glucose compared to intravenous glucose administration.[59] Also, an increase in plasma GIP concentrations was noted in association with glucose administered orally, but not intravenously.[59] This finding was later observed by de Laat *et al.* in 2016 when intravenous glucose failed to elicit a GIP response, while orally-administered glucose result in a marked elevation of the incretin.[60] One pony within the Dühlmeier *et al*. study had a marked hyperinsulinemic response to both oral and intravenous glucose testing, despite appearing clinically normal. This pony also had a

pronounced increased plasma GIP concentration, which was 2.6 times higher than other animals in the study. The increase in plasma GIP concentration in this outlier pony when compared to the other equines demonstrated a possible connection between the EIA and equine hyperinsulinemia.

Glucagon-like peptide-1 concentrations have been shown to increase in response to oral glucose administration, via an oral sugar test, increasing by a median of 148%.[61] Active GLP-1 (aGLP-1) concentrations also increase over time in response to the oral sugar test.[56,60] A strong association between aGLP-1 and insulin response to oral non-structural carbohydrates (NSC) was also documented.[60] One study indicated that 22.7% of the variation in insulin concentrations was attributable to variation in aGLP-1 concentrations.[60]

Stimulus for the activation of the enteroinsular axis, and more specifically GIP, were undertaken using a cohort of Shetland ponies.[62] In a crossover design, ponies were fed calorically equivalent energy adequate and hypercaloric diets that were either fat-based or carbohydrate-based for a period of 5 weeks per diet. At the conclusion of the feeding periods, an oral glucose test was performed. Ponies fed a fat-based diet had significantly higher plasma GIP concentrations than the ponies fed carbohydrate-based diets.[62] These results suggested that dietary fat rather than carbohydrates may be a more potent stimulus for GIP secretion in equids, which is similar to what occurs in humans and rats.[63,64] This study also demonstrated that ponies fed a hypercaloric fat-based diet had plasma insulin

concentrations that were 25 times greater than basal concentrations.[62] These findings further strengthened the functionality of the EIA in equids.

Genetic factors have previously been demonstrated to influence energy metabolism, and more specifically glucose and insulin dynamics in equids.[65] Breed-related differences in the innate insulin response have also been demonstrated.[59,66] Breed-specific effects on the incretin response were investigated by comparing the postprandial concentrations of glucose, insulin and GLP-1 in Standardbred horses, Andalusian horses and ponies. Glucose-AUC were similar between breeds. Ponies and Andalusians when compared to Standardbred horses exhibited significantly greater insulin-AUC and GLP-1-area under the curve (GLP-1-AUC) when compared to Standardbred horses.[66] Post-prandial insulin concentrations in all breeds had a strong positive correlation with GLP-1 concentrations during the sampling period.[66] A weak correlation between glucose and GLP-1 concentrations indicated that breed-related difference in GLP-1 are not solely related to glycemic response and supported the potential role of the EIA and incretin hormones in the development of postprandial hyperinsulinemia.

Expression of the equine GIP receptor (eGIPR) has been identified in the pancreas, duodenum, liver, kidney, and heart.[67] Equine GLP-1 receptor (eGLP-1R) was found to be expressed in the pancreas, duodenum, heart, liver, kidney, gluteal skeletal muscle, tongue and digital lamella.[68] Immunostaining techniques have localized the pancreatic expression of the eGLP-1R to be limited to the islet cells.[68] Functions of both eGIPR and eGLP-1R in

extra-pancreatic tissue have not been extensively studied. The presence of both of these receptors in extra-pancreatic tissue strengthen the concept that the equine incretin hormones, GIP and GLP-1, like those in humans and in rodents, will have more than just an insulinotropic effect on the β-cells of the pancreas.

While most studies of equine incretin hormones have been centered around the enteroinsular axis' potential link to hyperinsulinemia and laminitis, contrasting information has been presented. Several studies have shown that the metabolic status (normal versus presence of equine metabolic syndrome (EMS)) of the equid has no significant effect on total GLP-1 AUC or aGLP-1-AUC following the administration of an oral sugar test.[56,61] Additionally, metabolic status also appeared not to influence GIP and aGLP-1 concentrations following access to pasture.[69] In contrast to these findings, another study found that aGLP-1 concentrations increased in insulin-dysregulated ponies compared to normal ponies and hypothesized that aGLP-1 played a role in the metabolic dysfunction of these ponies.[5]

Chapter 2: Enteroinsular Axis Response to Carbohydrates and Fasting in Healthy Neonatal Foals

2.1 Materials and Methods:

Experimental Design

Thirty-six Standardbred foals ≤ 4 days of age, owned by a private breeding farm, were included in this prospective, randomized study. Foals were considered healthy based on physical examination, normal complete blood count (CBC) and serum immunoglobulin G (IgG) concentrations (>800 mg/dL). During the study period, foals were stall confined with their dams. Testing took place over two 6-week periods from March to April during the 2017 (n=17) and 2018 (n=19) foaling seasons. Each foal was randomly assigned to either the dextrose, lactose or fasted experimental group. Foals receiving dextrose (n=24) were randomly assigned to oral or intravenous route of administration of a low (300 mg/kg), medium (500 mg/kg) or high (1000 mg/kg) dose in a crossover design, with the alternative route of administration occurring the following day. Foals in the lactose and fasted groups were only sampled on day 1. Care was taken to minimize stress to these foals.

At 60 minutes before initiation of the experimental period, an intravenous catheter[i] was placed in the jugular vein using local anesthesia. Foals were manually restrained and no medications, including α-2 adrenergic receptor agonists, were administered at any point. Foals were muzzled for 240 minutes (-60 to 180 minutes).

This study was approved by the OSU Veterinary Clinical Trials Office, the Institutional Animal Care and Use Committee, and adhered to the principles of humane treatment of animals in veterinary clinical investigations, as stated by the American College of Veterinary Internal Medicine and National Institute of Health guidelines

Oral Glucose Test (OGT)

Foals were administered 50% dextrose solution (VetOne, MWI Animal Health, Boise, ID) at the pre-assigned doses: 300 mg/kg (n=12; OGT-300), 500 mg/kg (n=6; OGT-500) or 1000 mg/kg (n=6; OGT-1000). The dextrose was administered by mouth using a 60 mL catheter-tip syringe and occurred over a period of 1 minute.

Intravenous Glucose Test (IVGT)

Foals were administered 50% dextrose solution (VetOne, MWI Animal Health, Boise, ID) through an intravenous catheter at the pre-assigned doses: 300 mg/kg (n=9; IVGT-300), 500 mg/kg (n=5; IVGT-500) or 1000 mg/kg (n=5; IVGT-1000). Administration occurred over a period of 1 minute and the intravenous catheter was flushed with 20 ml heparinized saline following administration.

Oral Lactose Test (OLT)

Foals were administered lactose (Millipore Sigma, St. Louis, MO) as a 20% solution in water at a dose of 1000 mg/kg (n=6) via nasogastric intubation over 1 minute.

Fasted Group

A group of foals (n=6) did not receive any sugar enterally or parenterally and remained muzzled and unable to nurse for the duration of the study (240 minutes).

Blood Sampling

Blood samples were collected at time 0 (before carbohydrate administration) and at 5, 10, 15, 30, 45, 60, 90, 120, 150 and 180 minutes. After 180 minutes, all foals were unmuzzled, allowed to nurse from the mare *ad libitum*, and additional blood samples in most, but not all foals were collected at 195 and 210 minutes.

Blood samples (4 mL) were placed in pre-chilled EDTA tube containing aprotonin[ii] and diprotin [iii] A. Aprotinin (500 kU/mL of blood) was added to inhibit protease-mediated degradation of peptide hormones and diprotin A (50 µmol/mL of blood) is a dipeptidyl peptidase-4 (DPP-4) protease inhibitor added to reduce degradation of GIP and GLP-1. EDTA-aprotinin-DPP-4 inhibitor tubes were immediately placed on ice for at least 20 minutes. Tubes were centrifuged at 1,000 × g for 10 minutes at 4 °C. Within 6 hours of collection, plasma were aliquoted and stored at -80 °C until analysis. Blood samples for CBC and IgG concentrations were processed within 4 hours of collection.

Sample Analysis

Blood glucose concentrations were measured immediately after collection using a portable glucometer[iv] previously validated for horses.[23] Commercially available enzyme-

linked immunosorbent assays (ELISA) kits previously validated for horses were utilized to measure plasma total GIP[v] and plasma total GLP-1[vi].[60] Plasma insulin concentrations were measured with a human-specific ELISA[vii] that showed linearity up to 1:8 dilutions, inter- and intra-coefficients of variation of less than 10% for equine samples, a working range of 1-300 μIU/mL, and a detection limit of 0.75 μIU/mL.

Data Analysis

Data sets were tested for normality by the Shapiro-Wilk normality test and were not normally distributed. Therefore, median and interquartile ranges (IQR) were calculated. Comparisons between groups were carried out with the Kruskal-Wallis statistic, and Dunn's post hoc test was used to compare each time point individually within the group. Comparisons over time were carried out with the Friedman test. Peak concentrations were measured for each foal (Cmax) and areas under the curve (AUCs) for glucose (glucose-AUC), insulin (insulin-AUC), GIP (GIP-AUC), and GLP-1 (GLP-1-AUC) were calculated using the nonoverlapping trapezoid method. Results are presented as values relative to time 0 (figures) and absolute values (table). Statistical analysis was performed using commercial statistical software[viii,ix]. Statistical significance was set at $P < 0.05$.

2.2 Results

Study Population

A total of 36 Standardbred foals ≤ 4 days of age were included. The median age of foals at the time of study participation was 24 hours with a range of 8 hours to 96 hours. The median IgG concentration was 1700 mg/dL (1295-1957 mg/dL). Twenty-three of 36 foals (64%) were fillies and 13/36 (36%) were colts.

Blood Glucose

Median baseline glucose concentrations for all foals was 140 mg/dL (123-160 mg/dL). The oral administration of 300, 500 or 1000 mg/kg of dextrose did not induce a significant increase in blood glucose concentration at any point during the testing period when compared to baseline values. In the OGT-300 group, median glucose concentration at 180 minutes was significantly lower than at time 0 ($P < 0.01$). Foals in the IVGT-300, IVGT-500, and IVGT-1000 groups had significant increases in blood glucose concentrations at 5-15 minutes compared to time 0 (Figure 2.1; $P < 0.01$). Foals in the OLT had a significant increase in blood glucose concentrations at 30 minutes compared to time 0 (Figure 2.1; $P < 0.05$). Fasted foals had a significant decline in glycemia from 60-180 minutes ($P < 0.05$). However, glucose values remained within the normal range, foals were active, and none showed signs of hypoglycemia.

When glucose concentrations were adjusted to baseline values, concentrations peaked at different time points by 1.8%, 22.5%, 36.8%, and 41.9% for the OGT-300, OGT-

500, OGT-1000, and OLT groups, respectively (Figure 2.2; $P < 0.05$). For the IVGT-300, IVT-500, and IVGT-1000 groups, glucose concentrations increased by 59.2%, 102.2%, and 284.4%, respectively (Figure 2.2; $P < 0.05$). Compared to baseline values, glucose concentrations in fasted foals decreased by 16% at 180 minutes ($P < 0.05$). After foals were allowed to nurse, there was a major and significant increase in glucose concentrations that was evident by 15 minutes (195 and 210 minutes) (Figure 2.2; $P < 0.05$).

Insulin

Baseline plasma insulin concentration for all foals was 10.7 μIU/ml (6.0-16.70 μIU/mL). No statistically significant increase in plasma insulin concentrations from baseline concentrations occurred during the OGT-300, OGT-500, OGT-1000 or OLT (Figure 2.3). However, a trend toward increased insulin concentrations was noted between 10 and 60 minutes for the OGT (all doses) and OLT groups. A significant decrease in insulin concentrations was noted for the OGT-300 group between 120-150 minutes and the fasted group from 60-180 minutes ($P < 0.05$). The intravenous administration of dextrose produced significant increases in insulin concentrations between 5 and 15 minutes compared to time 0.

As a relative percent change from baseline values, insulin concentrations increased by 10.7%, 33.4%,110.6%, and 220.3% for the OGT-300, OGT-500, OGT-1000, and OLT groups, respectively (Figure 2.4; $P < 0.05$). For the IVGT-300, IVT-500, and IVGT-1000 groups, insulin concentrations increased by 802%, 309.9%, and 4238%, respectively

(Figure 2.4; $P < 0.05$). Compared to baseline values, insulin concentrations in fasted foals decreased by 72.5% at 180 minutes. After foals were allowed to nurse, there was a major and significant increase in insulin concentrations that was evident by 15 minutes (195 and 210 minutes) (Figure 2.4; $P < 0.05$).

Glucose-dependent insulinotropic polypeptide (GIP)

Baseline GIP concentration for all foals was 238.2 pg/mL (137.4-391.3 pg/mL). From baseline, plasma GIP concentrations decreased significantly from 90-180 minutes for the OGT-300 and OGT-500 groups and from 120-180 minutes for the OGT-1000 group (Figure 2.5; $P < 0.05$). In the IVGT-300, IVGT-500 and IVGT-1000 groups, GIP concentrations decreased earlier and longer (60-180 minutes.) (Figure 2.5; $P < 0.05$)

As a relative percent change from baseline, GIP concentrations decreased by 67.83%, 73.4%, 53.4%, 68% for the OGT-300, OGT-500, OGT-1000, and OLT groups, respectively at 180 minutes (Figure 2.6; $P < 0.05$). For the IVGT-300, IVGT-500, and IVGT-1000 groups, GIP concentrations decreased by 74.3%, 74.6%, and 59.4%, respectively (Figure 2.6; $P < 0.05$) at 180 minutes. Compared to baseline, GIP concentrations in fasted foals decreased by 75.5% at 180 minutes. After foals were allowed to nurse, there was a major and significant increase in GIP concentrations that was evident by 15 minutes (195 and 210 minutes) (Figure 2.6; $P < 0.05$).

Glucagon-like peptide 1 (GLP-1)

Baseline GLP-1 for all foals was 113 pM (57.30-173.5 pM;). The only group that elicited a statistically significant increase in GLP-1 was the OGT-1000 at 15 minutes. Plasma GLP-1 concentrations showed a statistically significant decrease in the OGT-300 (120 minutes) and OGT-500 (180 minutes) groups ($P < 0.05$). Plasma GLP-1 concentrations also decreased in the IVGT-300 and IVGT-500 from 30 to 180 minutes (Figure 2.7; $P < 0.05$). Foals in the OLT had significantly lower GLP-1 concentrations from 120-180 minutes (Figure 2.7). In the fasted and IVGT-1000 groups, there was no significant change in GLP-1 concentrations throughout the testing period.

As a relative percent change from baseline values, GLP-1 concentrations decreased by 12.4%, 46.38%, 7.8%, 34.8% for the OGT-300, OGT-500, OGT-1000, and OLT groups, respectively at 180 minutes (Figure 2.8). For the IVGT-300, IVT-500, and IVGT-1000 groups GLP-1 concentrations decreased by 31.6%, 22.3%, and 16.6%, respectively (Figure 2.8; $P < 0.05$) at 180 minutes. Compared to baseline values, GLP-1 concentrations in fasted foals decreased by 14.6% at 180 minutes. After foals were allowed to nurse, there was a major and significant increase in GLP-1 concentrations that was evident by 15 minutes (195 and 210 minutes) (Figure 2.8; $P < 0.05$).

Figure 2.11 provides an overall picture of the relative changes in glucose, insulin, GIP, and GLP-1 concentrations.

Area under the curve for glucose, plasma insulin, plasma GIP and plasma GLP-1

The glucose-AUC and insulin-AUC were statistically different between study groups (Figure 2.9). Foals in the OGT-300, OGT-1000, and IVGT-1000 groups had significantly greater glucose-AUC compared to fasted foals ($P < 0.05$). Insulin-AUC was not different among groups, except for OTL which had a higher AUC compared to the fasted group ($P < 0.05$). For GIP, the AUC was not different between treated and fasted foals, however, when comparing equivalent oral and intravenous doses, the AUC for oral glucose was larger ($P < 0.05$). No significant difference for GIP-AUC existed between fasted foals and the other study groups.

Access to free choice nursing

Access to free choice nursing resulted in a rapid and significant increase in blood glucose (34.6% at 195 minutes and 76% at 210 minutes), insulin (551% at 195 minutes and 637% at 210 minutes), GIP (137% at 195 minutes and 268% at 210 minutes) and GLP-1 (21% at 195 minutes and 39.8% at 210 minutes) concentrations compared to 180 minutes (Figure 2.10). These increases were greater than most rises seen following oral or lactose administration. The increase in GIP concentrations was more evident than for GLP-1 concentrations.

2.3 Discussion

In the current study, using oral and intravenous dextrose, oral lactose, and fasting, we documented that healthy equine neonates have a functional EIA. We also found that the response of the EIA in equine neonates in the immediate postpartum period is highly variable. To our knowledge, this is the first study to investigate the EIA in healthy newborn foals.

While the EIA response to lactose was the most evident, we were surprised with the minimal increase in insulin, GIP, and GLP-1 concentrations despite high oral carbohydrate doses. This is a novel and major finding in equine neonatal and comparative endocrinology that deserves further investigation. For horses and ponies, oral glucose doses of 150 mg/kg are considered appropriate to assess pancreatic insulin secretion[71–73] and, in general, higher doses are unnecessary. Insulin and incretin concentrations in the foals of this study followed a similar pattern. Despite high oral glucose and lactose dosing, both GIP and GLP-1 concentrations continued to decrease until the foals were allowed to nurse. The minimal incretin response to enteric carbohydrates is the most likely explanation fort eh negligible to absent insulin secretion in these foals. This is further supported by marginal changes in glycemia with oral glucose and lactose administration; however, intravenous glucose administration induced a rapid and significant insulin secretion.

The rapid insulin increase without an incretin response after intravenous dextrose administration provides further validation to the functionality of the EIA in equine neonates, and is in line with similar findings in ponies, where enteral glucose provoked GIP and GLP-1 secretion, but intravenous administration did not.[60]

In contrast to the minimal incretin response to oral glucose or lactose, once foals were allowed to nurse, there was a rapid and significant increase in both GIP and GLP-1 concentrations over their respective values at baseline and at 180 minutes. This indicates that the EIA in newborn foals is functional and highly responsive to nutrients contained in mare's milk, other than those we administered. Mare's milk is composed of approximately 1% fat, 2% protein and 7% lactose.[74] Lactose is a disaccharide composed of glucose and galactose in equal parts. Thus, foals in the lactose group (1000 mg/kg) received an equivalent glucose dose as foals in the OGT-500. The difference noted between these two study groups would be 500 mg/kg of galactose or another undetermined variable. Fat-based diets in adult ponies have previously been demonstrated to elicit increased plasma GIP responses following an oral glucose test when compared to ponies maintained on a carbohydrate-based diet that had the same testing performed.[62]

Despite glucose, insulin, GIP and GLP-1 following a similar trend after nursing, we notice that compared to 180 minutes, the GIP response at 210 minutes was stronger (~300%) than for GLP-1 (~40%). Potential explanations for this increase could be that

GLP-1 is degraded faster by DPP-4 or that GIP is a more important incretin in the early neonatal period.

It is also possible that insulin and incretin dynamic changes are different in older foals and result from the transition from *in utero* to extrauterine life. Maturation of the energy endocrine axis in foals continues in the post-partum period. Foals are inherently insulin resistant in the first days after birth and this has been attributed to activation of the hypothalamic-pituitary-adrenal axis and increased cortisol concentrations.[75–77] This could be a reason for individual variation among the foals in this study. In addition to incretins, other factors that may contribute to glucose dynamics and insulin secretion in the foals of this study include other gastrointestinal hormones (e.g., gastrin, secretin, and ghrelin), somatostatin and glucagon.

Regarding clinical relevance, this research brings light on the importance of constant exposure of intestinal cells to nutrients to maintain a functional EIA, as impaired incretin secretion can further complicate glucose regulation in critically ill foals. In addition, conditions that damage intestinal epithelial cells such as ischemia and infections (viral, bacterial) can indirectly disturb the endocrine pancreas and energy metabolism. It is possible that many foals admitted to equine hospitals with evidence of energy dysregulation have a dysfunctional EIA, which goes unnoticed because these factors are not measured clinically. In support of this statement, we recently found that foals with

severe sepsis tend to have lower insulin and GIP concentrations (L. Rings/R. Toribio, personal communication).

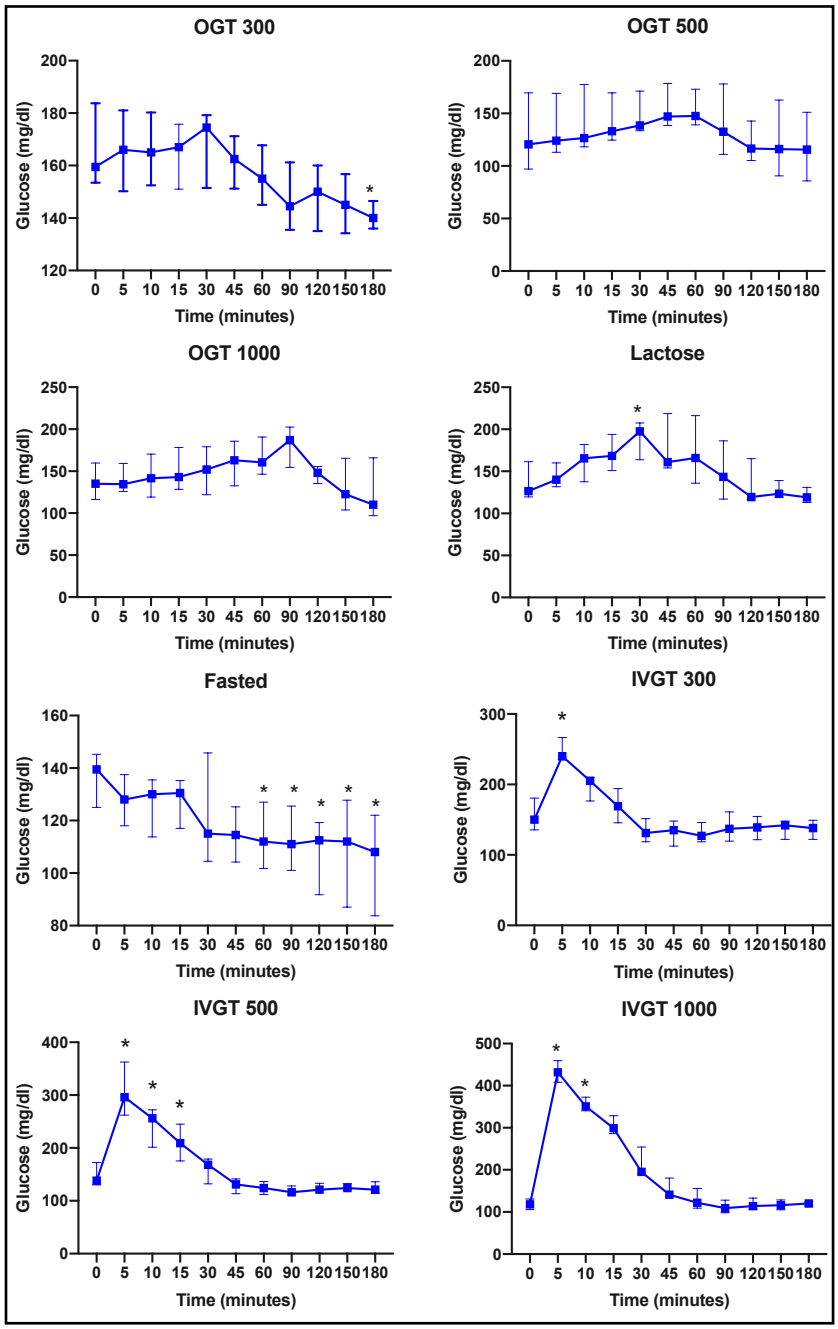
Another point of interest regarding neonatal endocrinology is the big difference in GLP-1 and GIP concentrations between the foals of this study and values reported for ponies and horses using the same assay. This indicates that equine enteroendocrine cells have a high capacity to produce incretins in the early neonatal period, perhaps as an evolutionary adaptation to a diet rich in fat, carbohydrates, and protein. The dynamics of incretin secretion over time in healthy foals remains to be investigated; however, preliminary work from our lab indicates that 3-day-old foals have lower GLP-1 concentrations (L. Rings/R. Toribio, personal communication).

Limitations of this study include the relative small sample size considering variations in hormone concentrations in the first 48 hours after birth, different methods of oral carbohydrate administration, as well as concentration and volume of oral solutions (50% dextrose vs 20% lactose). Pancreatic β-cell response to glucose is low immediately post-partum compared to foals 5-7 days of age[77,78] while foals of the study reported here were < 4 days of age. A narrower age range of foals would have been ideal, however, foal access was dictated by the farm. It would be valuable to investigate incretin dynamic changes that occur in the first of week after birth. Considering that foals under normal circumstances, do not consume pure glucose in addition to a more profound activation of the EIA observed when foals consumed milk compared to oral glucose or lactose, it will

be important to evaluate other substrates (e.g., amino acids, fats) to better characterize the response of the EIA in equine neonates. Plasma aGLP-1 concentrations were not measured in the foals of this study but will be considered in future studies.

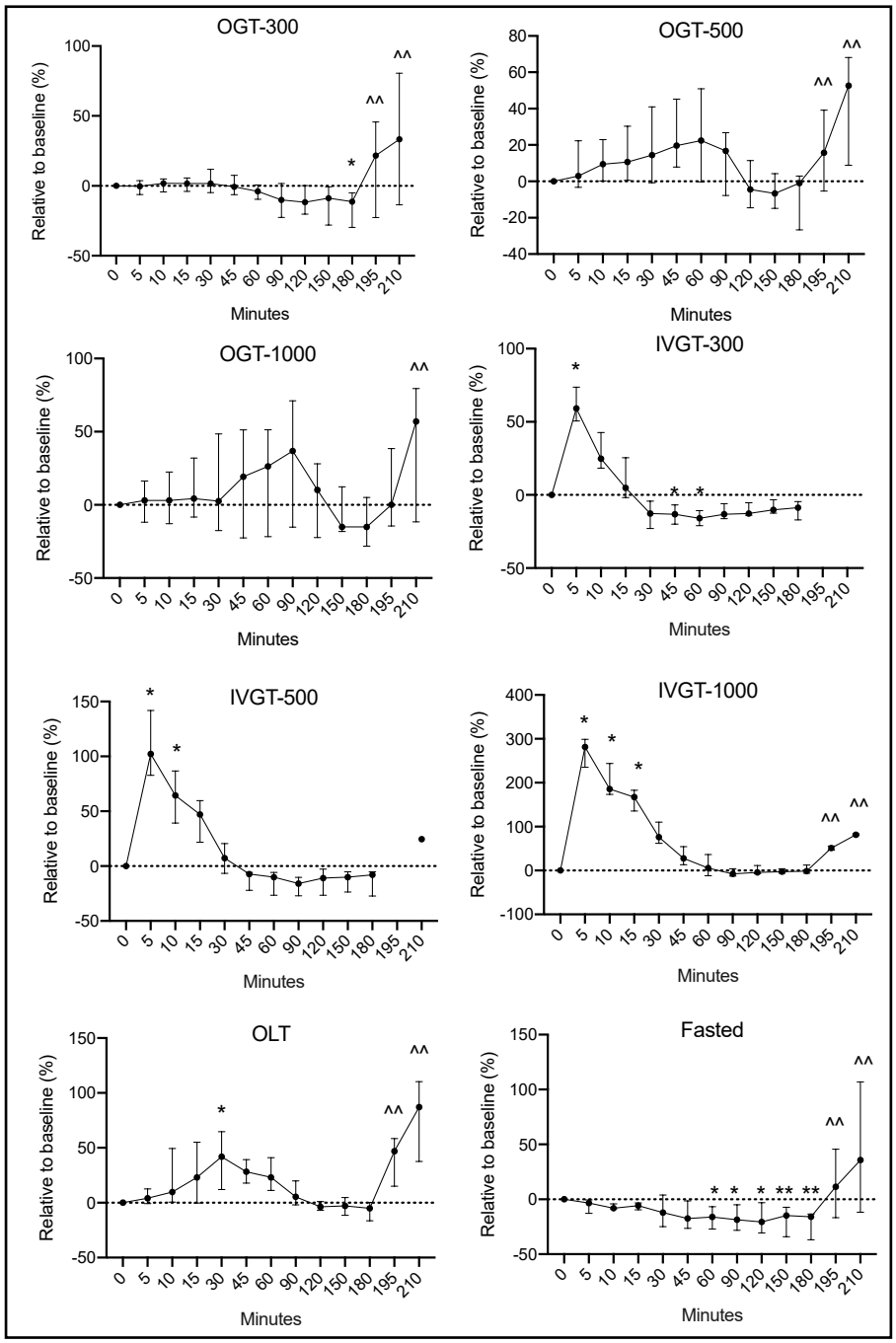
In conclusion, we documented that healthy newborn foals have a functional EIA. While activation of the EIA was minimal in response to orally administered glucose and lactose at doses up to 1000 mg/kg, rapid and significant increases in GIP and GLP-1 concentrations were noted when foals were allowed to resume nursing *ad libitum*. Future research on the EIA in foals should focus on other substrates that may stimulate incretin release and their potential therapeutic implications.

Figure 2.1. Blood Glucose concentrations by study group. Values expressed as median and IQR.



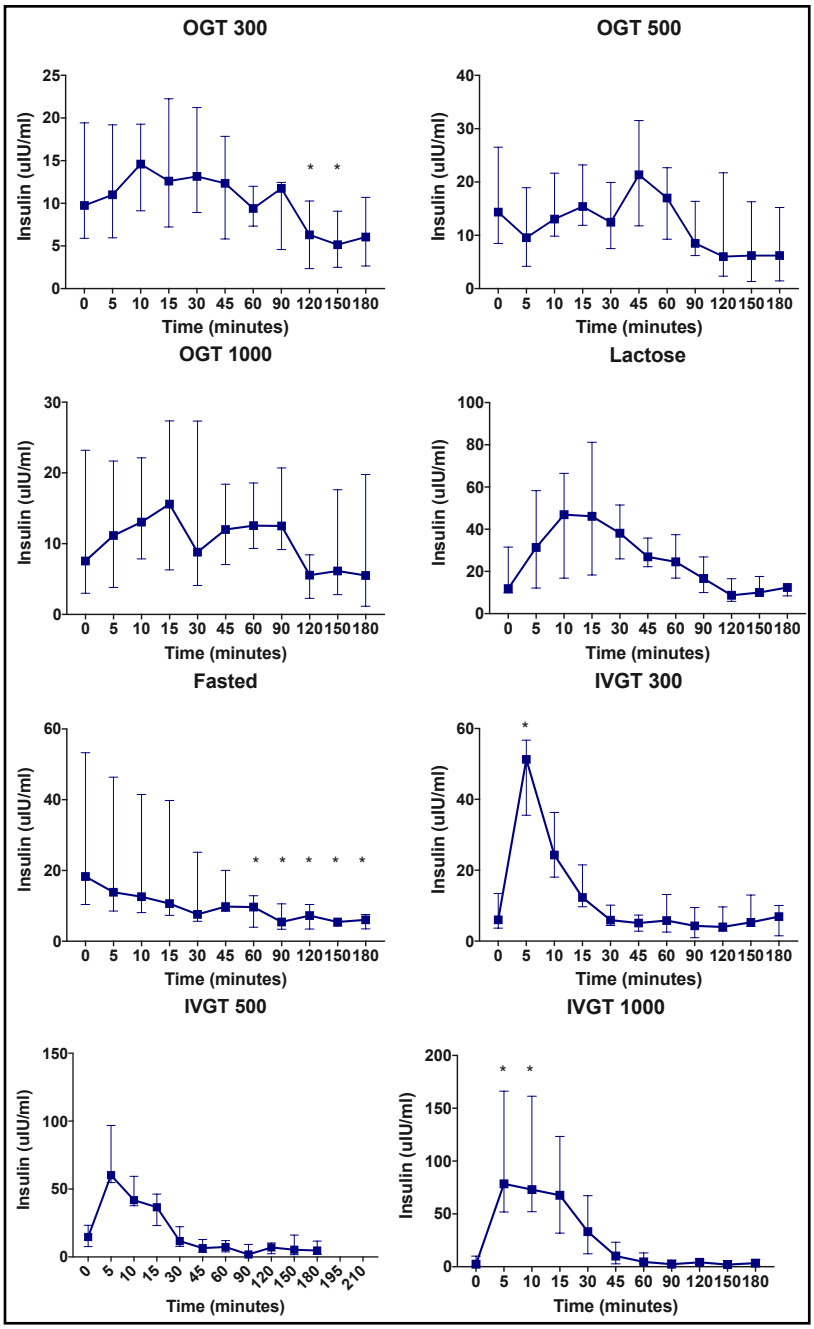
OGT, oral glucose test; IVGT, intravenous glucose test
* = P < 0.05 compared to time 0

Figure 2.2 Relative changes in blood glucose concentrations by study group. Values (median, IQR) presented relative (%) to time 0.



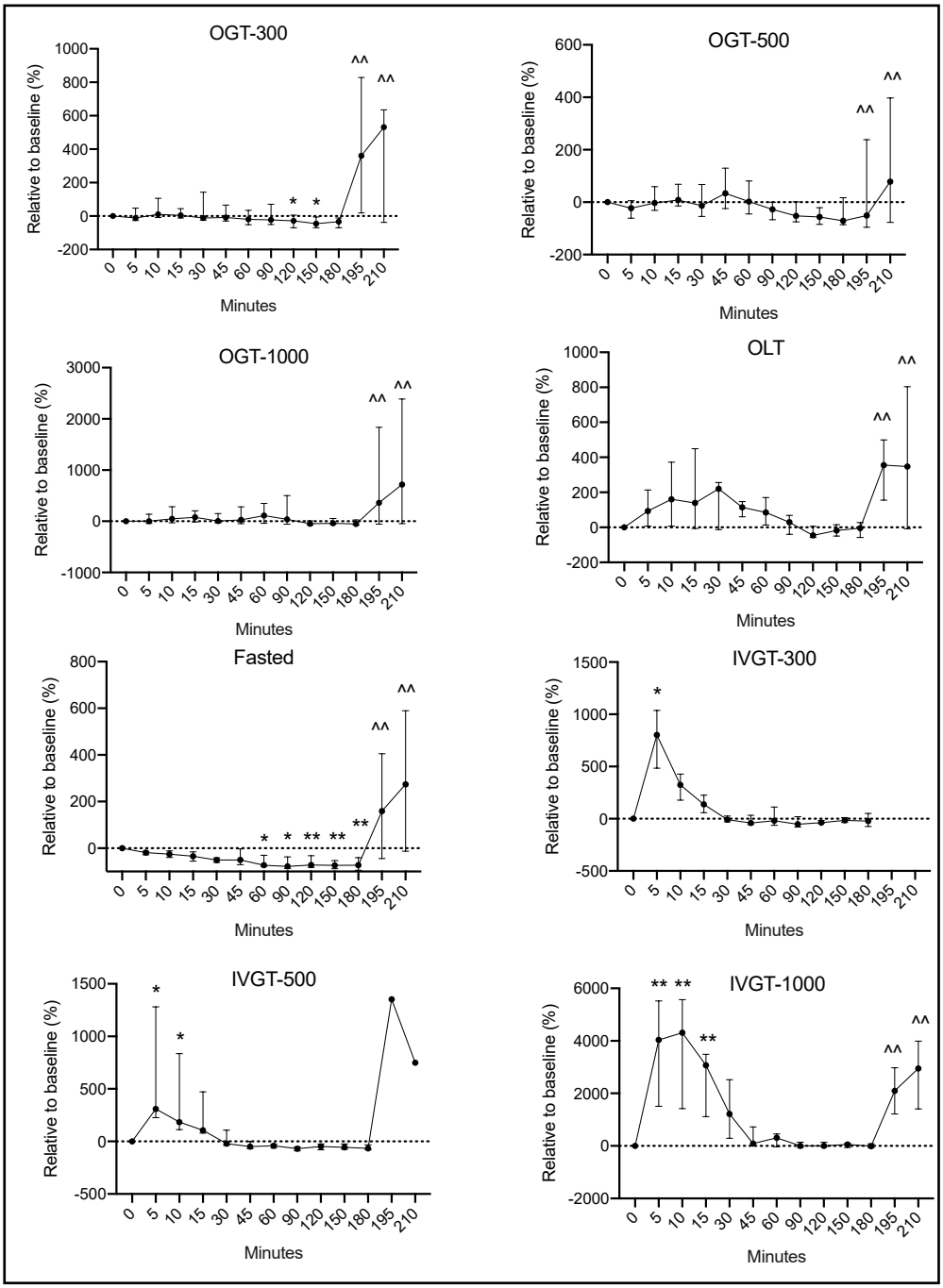
OGT, oral glucose test; IVGT, intravenous glucose test; OLT, oral lactose test
* = P < 0.05; ** = P < 0.01 compared to time 0; ^ = P < 0.05, ^^ = P < 0.01 compared to 180 minutes.

Figure 2.3. Plasma insulin concentrations by study group. Values expressed as median and IQR.



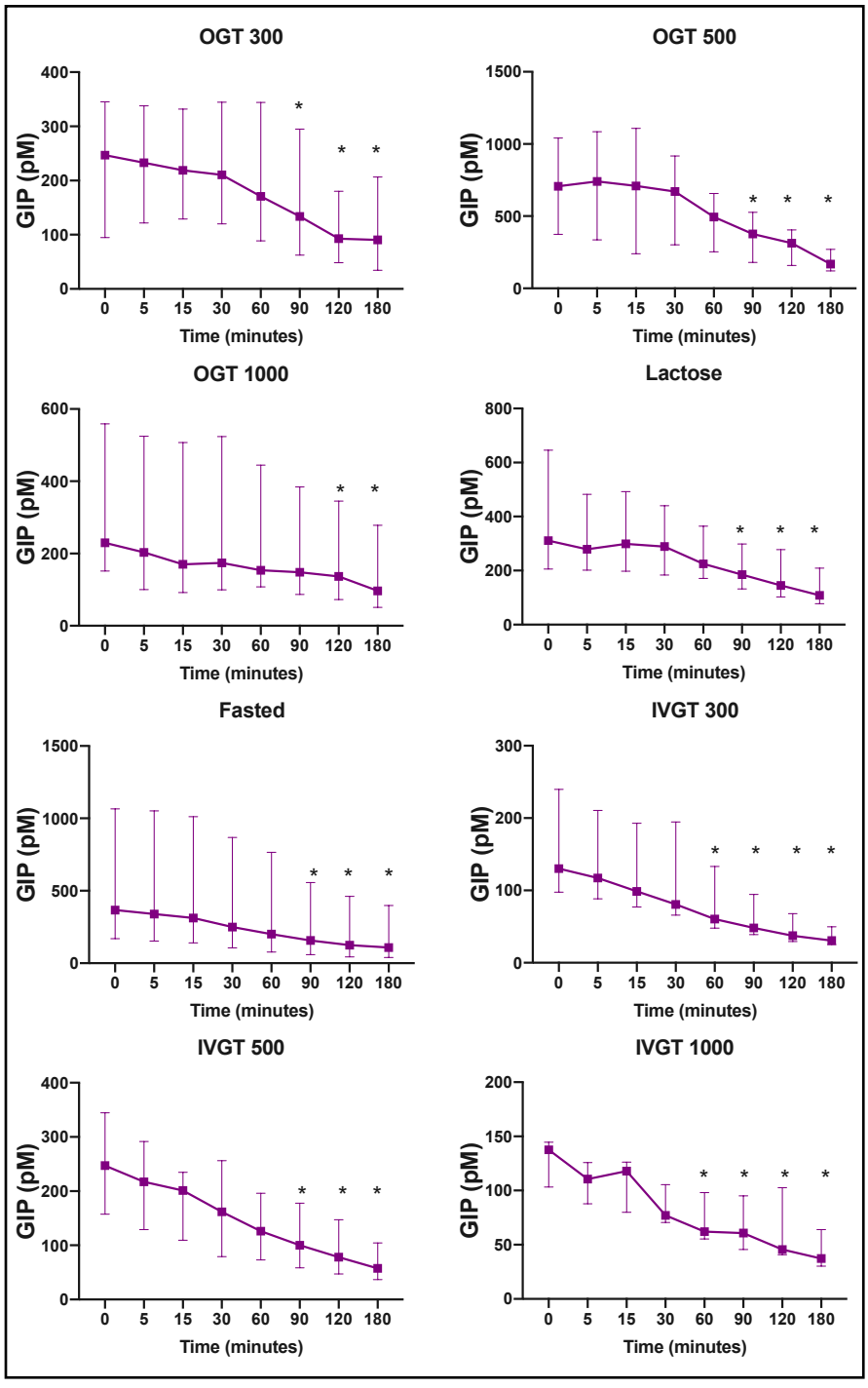
OGT, oral glucose test; IVGT, intravenous glucose test
* = P < 0.05 compared to time 0

Figure 2.4. Relative changes in plasma insulin concentrations by study group. Values (median, IQR) presented relative (%) to time 0.



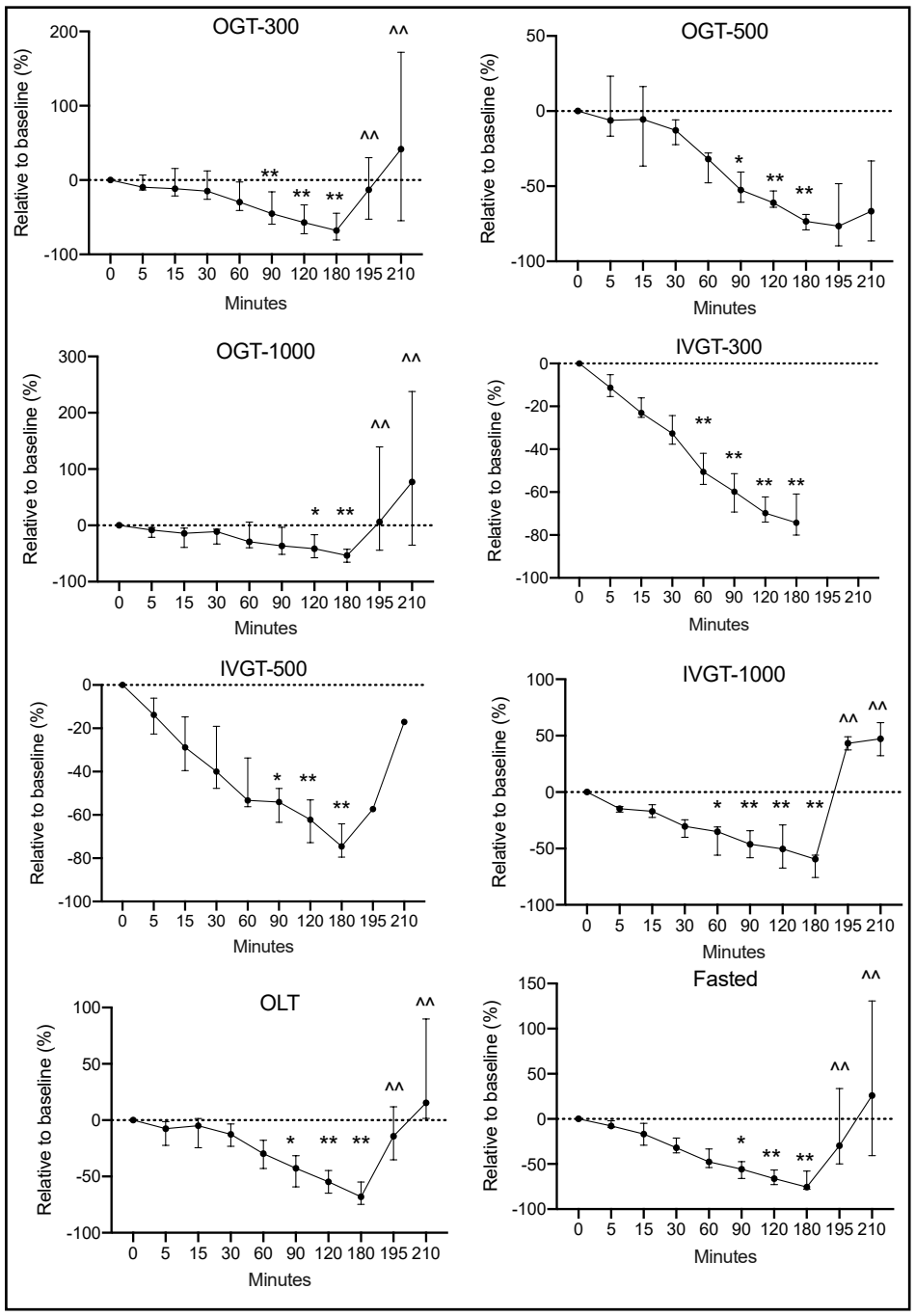
OGT, oral glucose test; IVGT, intravenous glucose test; OLT, oral lactose test
* = P < 0.05; ** = P < 0.01 compared to time 0; ^ = P < 0 .05, ^^ = P < 0.01 compared to 180 minutes.

Figure 2.5. Plasma GIP concentrations by study group. Values expressed as median and IQR.



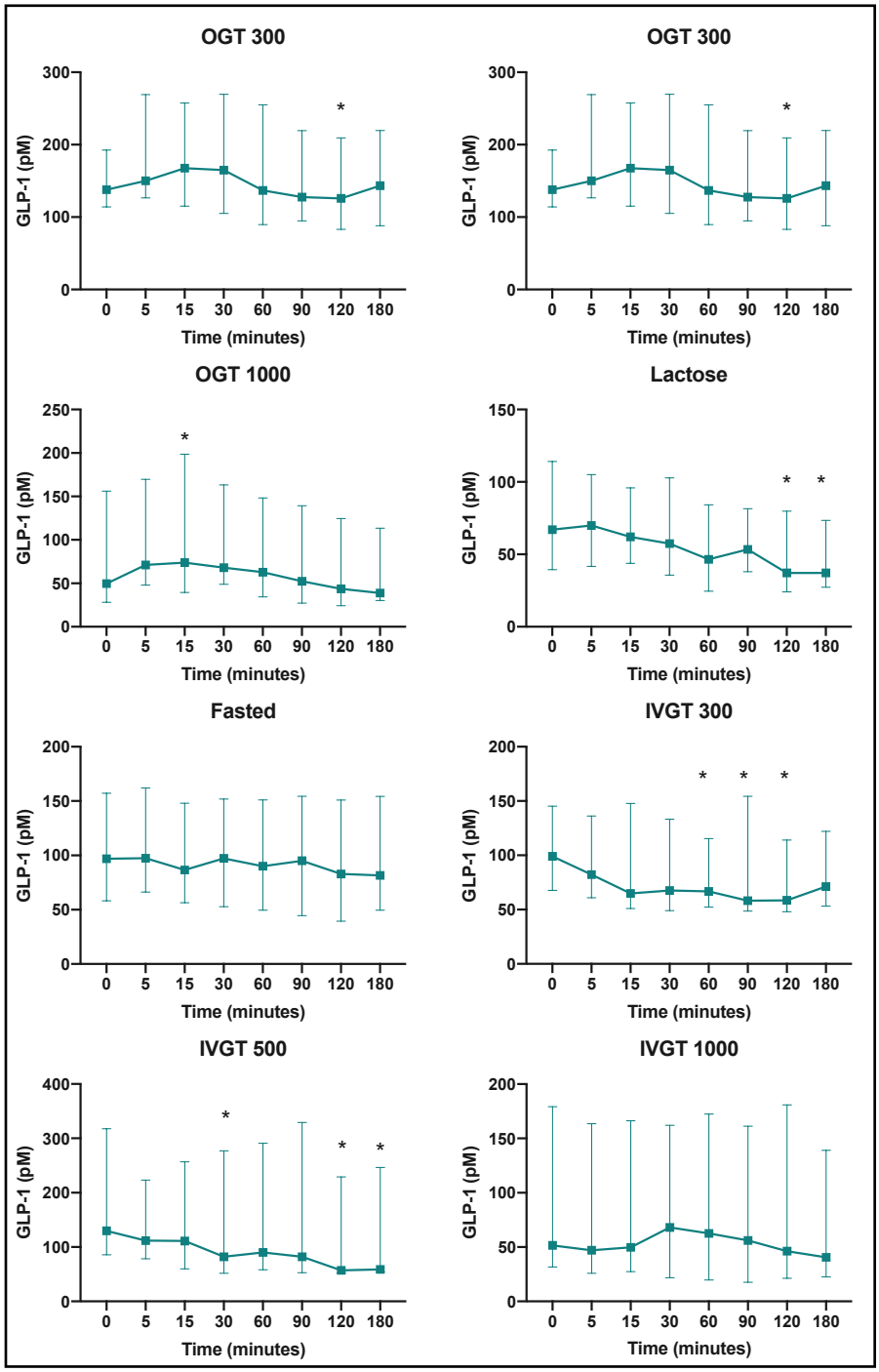
OGT, oral glucose test; IVGT, intravenous glucose test
* = P < 0.05 compared to time 0

Figure 2.6. Relative changes in plasma GIP concentrations in by study group. Values (median, IQR) presented relative (%) to time 0.



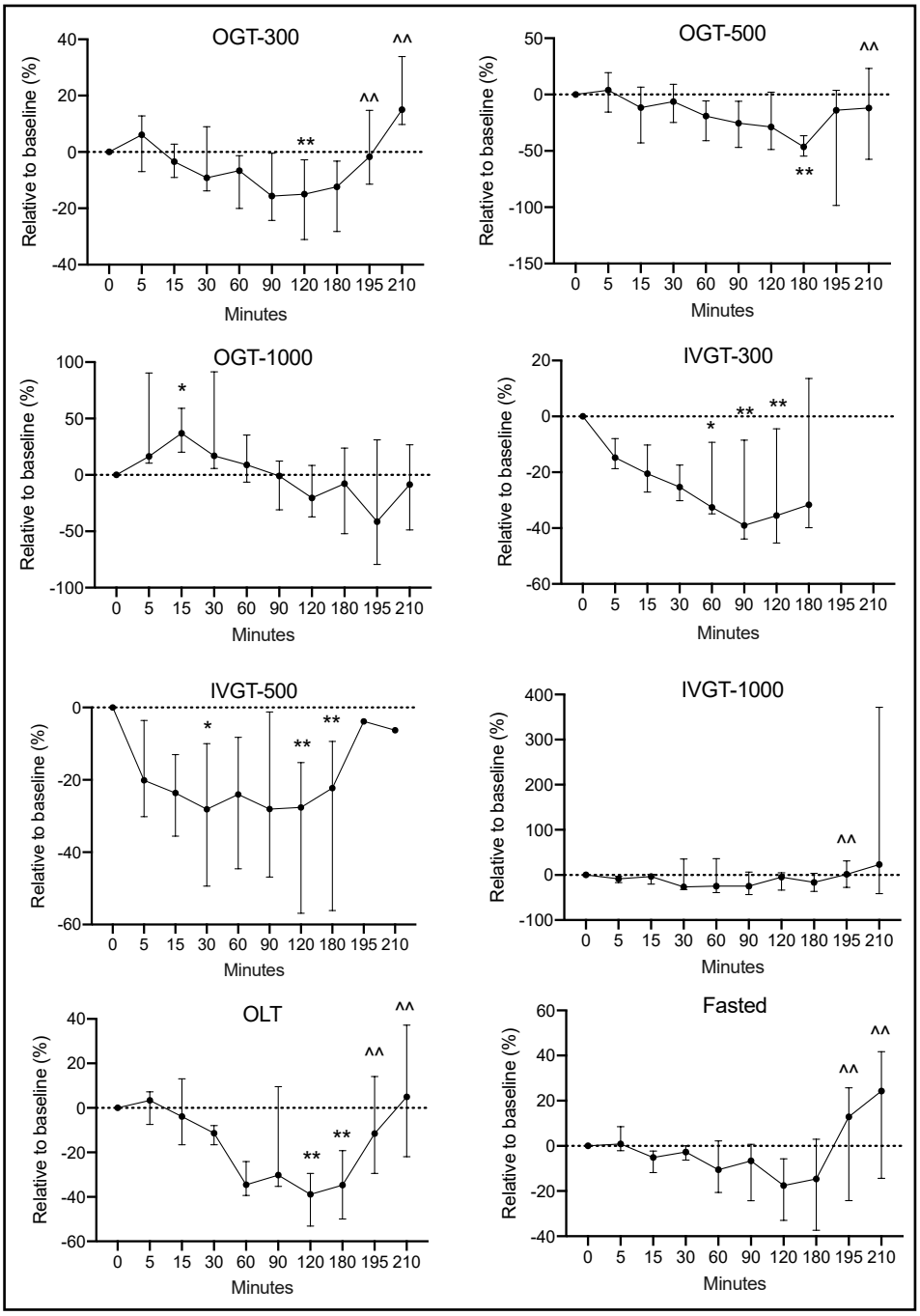
OGT, oral glucose test; IVGT, intravenous glucose test; OLT, oral lactose test
* = P < 0.05; ** = P < 0.01 compared to time 0; ^ = P < 0 .05, ^^ = P < 0.01 compared to 180 minutes.

Figure 2.7. Plasma GLP-1 concentrations by study group. Values expressed as median and IQR.



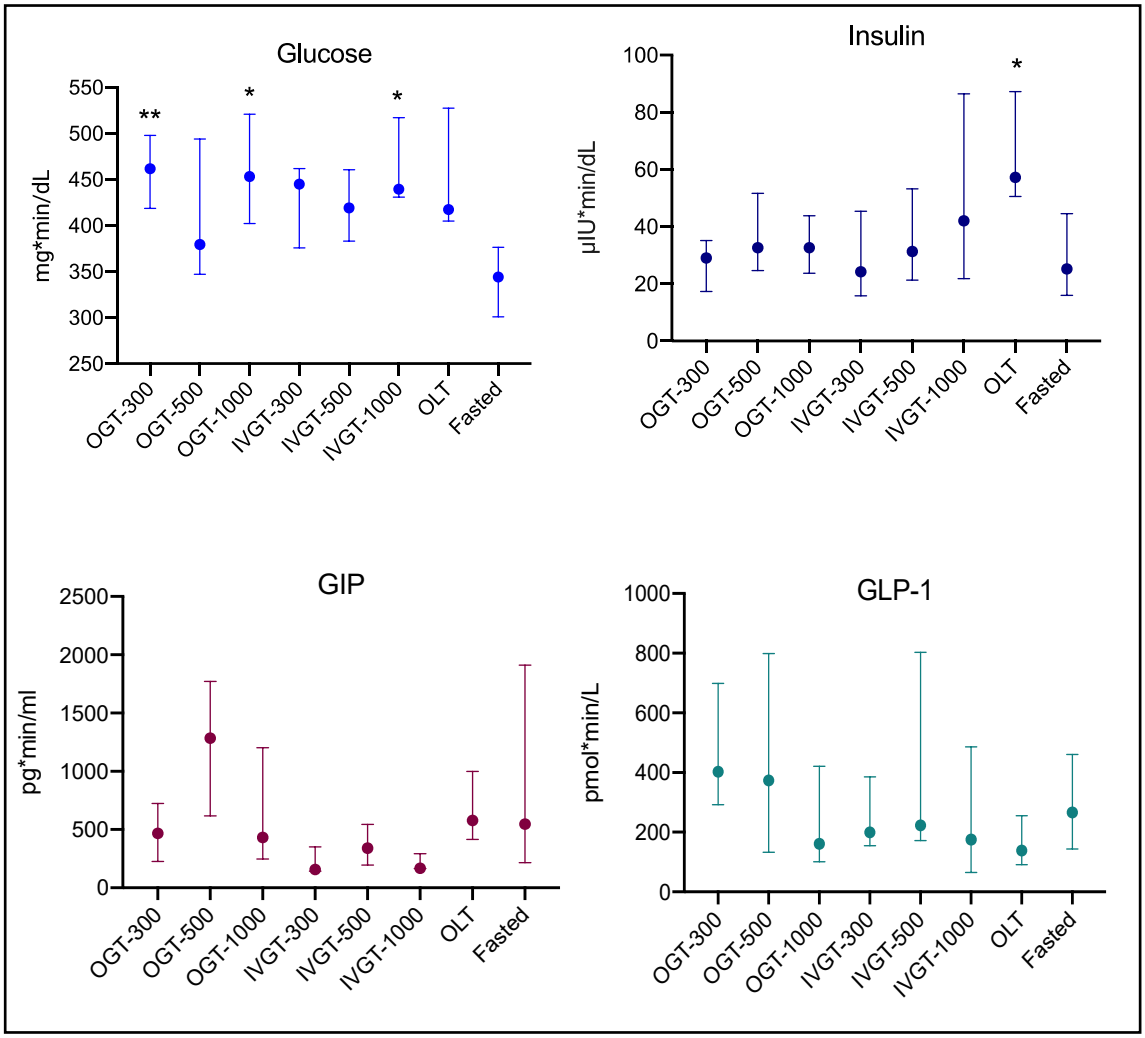
OGT, oral glucose test; IVGT, intravenous glucose test
* = P < 0.05 compared to time 0

Figure 2.8. Relative changes in plasma GLP-1 concentrations by study group. Values (median, IQR) presented relative (%) to time 0.



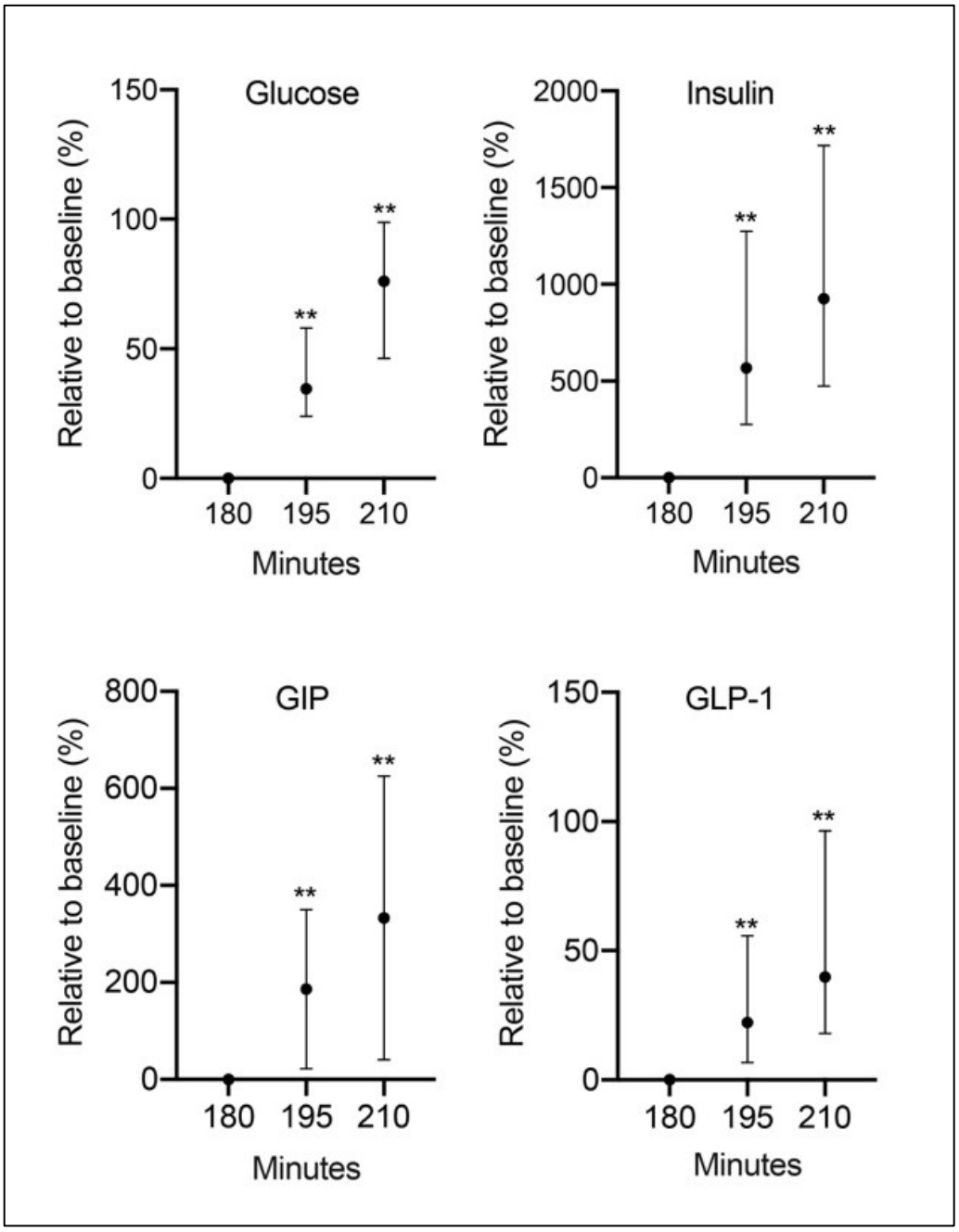
OGT, oral glucose test; IVGT, intravenous glucose test; OLT, oral lactose test
* = P < 0.05; ** = P < 0.01 compared to time 0; ^ = P < 0 .05, ^^ = P < 0.01 compared to 180 minutes.

Figure 2.9. Area under the curve values for blood glucose (mg*min/dL), plasma insulin (µIIU*min/mL), plasma GIP (pg*min/mL) and plasma GLP-1 (pmol*min/L). Values expressed as median and IQR.



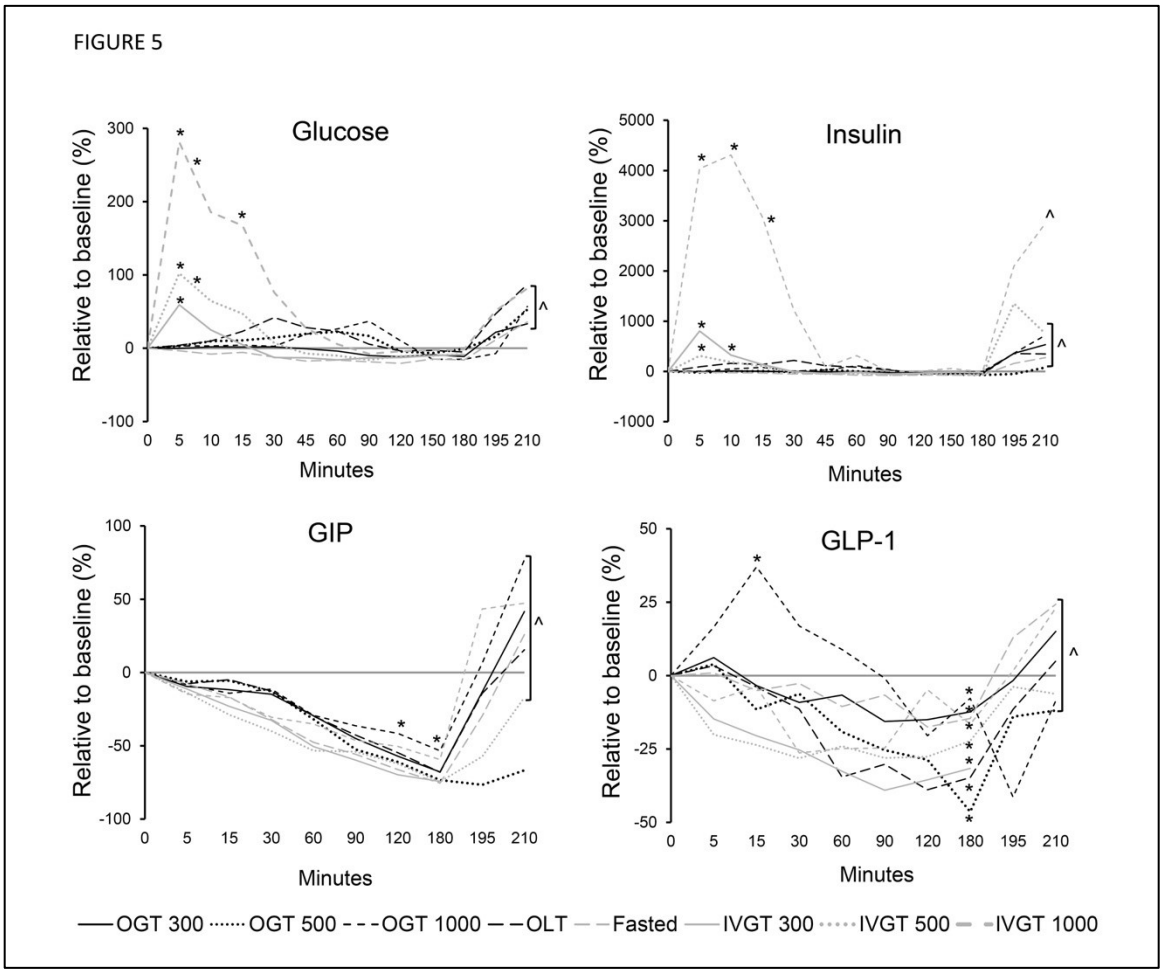
OGT, oral glucose test; IVGT, intravenous glucose test
* = P < 0.05; ** = P < 0.01 compared to fasted group

Figure 2.10. Blood glucose, plasma insulin, plasma GIP and plasma GLP-1 concentrations after being unmuzzled following the 180-minute blood sample and allowed *ad libitum* nursing from their dam. Values expressed as median and IQR.



** = P < 0.01 compared to 180 minutes

Figure 2.11. Relative changes in blood glucose, plasma insulin, plasma GLP-1, and plasma GIP concentrations in healthy newborn foals. Values presented relative (%) to time 0. IQR and statistical significance have been omitted.



OGT, oral glucose test; IVGT, intravenous glucose test; OLT, oral lactose test
* = $P < 0.05$; ^ = $P < 0.05$, compared to 180 minutes.

Chapter 3: Insulin and Incretins Over Time in Healthy and Critically Ill Foals

3.3 Materials and Methods

Animals

Foals ≤7 days of age, of any breed or sex admitted to 3 equine hospitals over 3 foaling seasons (2016-2018) were included. Hospitalized foals were classified into 2 groups: septic and sick non-septic (SNS) foals. Foals in the septic group had a sepsis score of ≥12, a positive blood culture, or both.[79] Foals in the SNS group were hospitalized for illnesses other than sepsis (e.g., hypoxic ischemic encephalopathy, failure of transfer of passive immunity, meconium impaction, flexural deformities) requiring hospitalization. These foals had negative blood cultures and a sepsis score of ≤11. The healthy control group consisted of 12-72-hour-old foals examined on a routine basis at breeding farms in Ohio. Healthy foals were classified as such based on physical exam, a normal complete blood count (CBC), serum biochemistry, a serum immunoglobulin G (IgG) concentration >800 mg/dL and a sepsis score of ≤4. Survival was defined as being discharged from the hospital alive. Foals that died or were euthanized due to a grave medical prognosis were defined as non-survivors. Foals euthanized for other reasons such as financial constraints were excluded from the study.

This study was approved by the OSU Veterinary Clinical Trials Office, the Institutional Animal Care and Use Committee, and adhered to the principles of humane

treatment of animals in veterinary clinical investigations, as stated by the American College of Veterinary Internal Medicine and National Institute of Health guidelines.

Data Collection

Clinical history obtained upon presentation included expected foaling date, duration of pregnancy, parity of the mare, maternal illnesses, premature lactation, observed or assisted parturition, dystocia, passing and appearance of fetal membranes, and medications (mare and foal). Clinical data collected from the foal included signalment (sex, gestational and actual age, breed), physical examination findings, CBC, biochemistry profile, IgG concentrations, and blood culture results. Endocrine measurements included plasma insulin, plasma GIP, and plasma GLP-1 concentrations. The sepsis score was calculated by a single evaluator for each foal, based on recorded history, physical examination, and laboratory findings.[79]

Sampling

Blood samples for hormone assays were collected within one hour of foal admission (time 0) via sterile jugular venous catheterization. Subsequent samples were obtained at 24, 48, and 72 hours if the foal survived and remained hospitalized. Samples from healthy foals were obtained during routine examination of newborn foals via jugular venipuncture with subsequent samples obtained following 24, 48, and 72 hours. Blood was collected in serum clot tubes and pre-chilled EDTA tubes which contained aprotinin[iv] and dipeptidyl peptidase-4 (DPP-4) protease inhibitor[v]. Aprotinin was added to inhibit

protease-mediated degradation of hormones (500 kU/mL of blood). A DPP-4 inhibitor was added to inhibit the degradation of the incretin hormones (GIP and GLP-1). Clot activator tubes were left to clot at room temperature for 30 minutes while the EDTA-aprotinin-DDP4 inhibitor tubes were immediately placed on ice for at least 10 minutes. Both samples were centrifuged at 1,000 × g for 10 minutes at 4 °C. Serum and plasma were aliquoted and stored at -80°C until analyzed. Blood samples for CBC, serum biochemistry, and IgG concentrations were processed immediately.

Hormone Concentrations

Commercially available enzyme-linked immunosorbent assays (ELISA) kits previously validated for horses were utilized to measure plasma total GIP[viii] and plasma total GLP-1[ix].[60] Plasma insulin concentrations were measured with a human-specific ELISA[vii] that showed linearity up to 1:8 dilutions, inter and intra-coefficient of variation of less than 10% for equine samples, a working range of 1-300 µIU/mL, and a detection limit of 0.75 µIU/mL.

Statistics

Shapiro-Wilk statistic was used to assess the data normality and the data was not normally distributed. Therefore, median and interquartile ranges (IQR) were calculated. Nonparametric comparisons between groups were carried out with the Kruskal-Wallis statistic, and Dunn's post hoc test was used to compare each time point individually within the group. Comparisons over time were carried out with the Friedman test. Areas under

the curve (AUCs) for insulin (insulin-AUC), GIP (GIP-AUC), and GLP-1 (GLP-1-AUC) were calculated using the nonoverlapping trapezoid method.

The Mann-Whitney-U test was applied to compare survivors with non-survivors within each group. Linear correlations were determined between plasma insulin, plasma GIP and plasma GLP-1 using a Spearman's rank order correlation (ρ). Statistical analysis was performed using commercial statistical software.[x,xi] Statistical significance was set at $P < 0.05$.

3.2 Results

Study Population

A total of 105 neonatal foals (89 hospitalized; 16 healthy) of ≤ 7 days of age were included. The median age of all hospitalized foals at admission was 12 hours (0 hours to 144 hours). For healthy controls, the median age was 24 hours (8-72 hours). Fifty-five percent of hospitalized foals (49/89) were classified as septic and 45% (40/89) as SNS. The survival rate in septic foals was 51% (25/49) and in SNS foals was 97.5 (39/40). Forty-seven percent (23/49) of septic foals had positive blood culture results. Median sepsis scores for septic and SNS foals were 14 and 7, respectively. Breeds represented in the hospitalized group included: Thoroughbreds (n=60), Standardbreds (n=12), Quarter Horses (n=11), Arabians (n=2), Belgians (n=2), Gypsy Vanner (n=1), and Percheron (n=1). Breed of healthy foals included: Standardbreds (n=13), Quarter Horses (n=2), and Welsh Pony (n=1).

Insulin, GIP and GLP-1 concentrations upon admission

Plasma insulin, plasma GIP and plasma GLP-1 concentrations were determined for each time point (Table 3.1). Septic foals had significantly lower insulin and GIP concentrations than healthy foals at time 0 ($P = 0.012$ and $P < 0.001$, respectively). Septic foals had significantly higher GLP-1 concentrations ($P = 0.001$) when compared to healthy foals at time 0. Foals in the SNS group had lower GIP concentrations ($P < 0.001$) than healthy foals at time 0 but did not have statistically different insulin or GLP-1

concentrations at baseline. Insulin, GIP and GLP-1 concentrations were not different between hospitalized foals (septic and SNS) at time 0.

Insulin, GIP and GLP-1 concentrations over time

Insulin concentrations among healthy and septic foals did not differ over time from baseline values. SNS foals had lower insulin concentrations at 24 and 48 hours compared to baseline. Plasma GIP concentrations did not vary from baseline values over time. Healthy foals had significantly lower GIP values at 48 hours compared to baseline. Septic foals had significantly lower GIP concentrations at 72 hours compared to time 0. Both Septic and SNS foals' GLP-1 concentrations were significantly decreased at 48- and 72-hours compared to baseline. Healthy foals followed this trend but GLP-1 values only differed significantly at 72 hours in this group.

Area under the curve for insulin, GIP and GLP-1

For healthy, septic and SNS foals the area under the curve (AUC) was determined for glucose (glucose-AUC), plasma insulin (insulin-AUC), plasma GIP (GIP-AUC) and plasma GLP-1 (GLP-1-AUC) concentrations (Table 3.2). Hospitalized foals had significantly lower insulin-AUC and GIP-AUC compared to healthy foals ($P < 0.001$). There was no statistical difference in insulin-AUC and GIP-AUC between septic and SNS foals; however, septic foals tended to have lower AUCs compared to SNS foals. No difference existed between healthy, SNS or septic foals in regard to GLP-1-AUC.

Insulin, GIP and GLP-1 correlations

Correlations between plasma insulin, plasma GIP and plasma GLP-1 were determined for healthy, SNS and septic foals (Table 3.3). A positive correlation existed in healthy foals between insulin and GIP, insulin and GLP-1, at all time points ($P < 0.05$), and between GIP and GLP-1 at 0, 24 and 72 hours. Sick non-septic foals had a positive correlation between insulin and GIP at 24 hours, and GIP and GLP-1 at 72 hours. In septic foals, GIP and insulin, and GIP and GLP-1 were positively correlated at 48 and 72 hours.

Association of insulin, GIP and GLP-1concentrations and survival

Surviving septic foals had significantly higher plasma insulin concentrations on admission at time 0 compared to non-survivors ($p=0.019$) (Table 3.4). Insulin AUC was larger in surviving compared to non-surviving foals; however, this difference was not statistically significant (Table 3.5). There was no difference in GIP and GLP-1 concentrations between survivors and non-survivors at time 0. Additionally, there was no difference between survival and non-survival when evaluating GIP-AUC and GLP-1-AUC. A positive correlation between insulin and GIP at time 24, 48 and 72 hours was noted in septic foals that survived. A positive correlation in these foals was also noted between GIP and GLP-1 at time 48 and 72 hours. No correlation was found between these hormones and septic, non-surviving foals.

3.3 Discussion

In the current study, we documented that changes in the EIA in critically ill foals are characterized by decreased insulin, decreased GIP and increased GLP-1 concentrations. Mortality in septic foals was associated with decreased insulin at time 0, while GIP and GLP-1 values at presentation were not different between survivors and non-survivors. The positive correlation between insulin and GIP, insulin and GLP-1, and GIP and GLP-1 at all time points in healthy foals is a major finding of this study, supporting the presence of a functional EIA in newborn foals.

Hospitalized (septic and SNS) foals had significantly lower insulin concentrations compared to healthy foals at time 0. This finding was in agreement with previous work from our laboratory that found critically ill foals were hypoglycemic, hypertriglyceridemic, with decreased insulin and high glucagon concentrations.[2] Decreased insulin concentrations at time 0 in hospitalized foals could be interpreted as an appropriate physiologic response to hypoglycemia, a common finding in hospitalized equine neonates[1–4] or this finding might be secondary to dysfunction of the EIA in critically ill foals. It was further demonstrated that the insulin-AUC was significantly lower in hospitalized foals compared to healthy controls.

The lack of correlation between insulin and incretin hormones in hospitalized compared to healthy foals indicates that dysregulation of the EIA is a potential player in

energy dyshomeostasis in critically ill foals. This is further supported by the persistent positive correlations between insulin and GLP-1, and GIP and GLP-1 during hospitalization in surviving foals. Non-surviving foals tended to have lower insulin-AUC than surviving foals though this difference was not statistically significant.

Sepsis is the most common cause of mortality in foals and often leads to problems with energy dysregulation.[4,80] Septic foals are often hypoglycemic upon admission and require rapid intervention. Enteral feeding in these foals is often restricted due to concerns of gastrointestinal tract intolerance to oral nutrition. This is particularly valid for foals that present for enteritis or colitis. Removing the enteral route of nutrition necessitates that the foal's energy requirement be met through parenteral nutrition. However, it is important to mention that in order to maintain a functional gastrointestinal tract, enterocytes and neuroendocrine cells must be exposed to certain amounts of luminal nutrients (tropic stimuli).

Hyperglycemia was reported in up to 89% of foals that received parenteral nutrition and can make their management challenging.[81] Previous work in adult equids has demonstrated that orally administered glucose produces a greater insulin release than intravenously administered glucose administered at an equivalent dose.[60] There is a similar finding in humans that has been termed the incretin effect.[16,82]

It has been estimated that while glucose is the main stimulus for insulin secretion in the horse, the incretin hormones account for almost 25% of the variation in insulin secretion.[60] Perhaps with the findings of our study, the practice of preventing enteral nutrition in critically ill foals should be re-evaluated, as it may be a potential cause or contributor to the decreased insulin concentrations that was seen in the critically ill foals of this study.

Values for GIP and GLP-1 collected at time 0 did not vary between septic and SNS foals, but were significantly different from healthy foals. This may represent dysfunction of the EIA secondary to systemic disease.

One limitation of this study was that hospitalized foals included in this study continued to receive varied treatments and nutritional support at the preference of the attending clinician while enrolled. It was challenging to account for various therapies and management techniques when forming our study groups. Healthy foals were largely Standardbreds (13/16), while hospitalized foals were of different breeds. Breed-related differences in glucose and insulin dynamics have been identified for Standardbreds, ponies and Andalusian horses;[66] however, not between Standardbreds, Quarter Horses, and Thoroughbreds, which were the bulk of the foals in this study. In the future, a larger and more varied healthy control group would help to strengthen our findings.

This study provides evidence that dysfunction of the EIA may occur in critically ill equine neonates and can lead to problems with energy regulation. The value of insulin to distinguish between septic survivors and non-survivors should continue to be investigated as this may offer valuable prognostic information in the future. Our data suggests that the EIA may play a role in the disease pathology of hospitalized equine neonates and that further study of this topic is warranted.

Table 3.1. Plasma insulin, GIP and GLP-1 concentrations. Values expressed as median and IQR.

	Hour	n	Insulin (µIU/ml)	GIP (pg/ml)	GLP-1 (pM)
Healthy	0	16	22.5 (9.1-34.4)	420.8 (264.8-637.4)	55.2 (29-89.5)
	24	16	15.4 (6.1-41.1)	337.5 (236.3-480.5)	56.2 (30-71.0)
	48	16	24.8 (11.6-29.7)	329 (220.0-382.7)*	45.0 (22.9-54.8)
	72	15	30.0 (21.7-42.4)	323.4 (252.5-514.8)	31.2 (18.3-61.9)*
SNS	0	40	9.9 (6.0-22.4)	127.4 (56.0-297.4) **	98.1 (51.1-166.5)
	24	40	9.1 (6.5-12.9)*	128.4 (55.9-181.4)	36.5 (22.3-82.7)
	48	39	9.2 (6.2-12.1)*	141.8 (72.1-269.3)	24.3 (17.0-41.9)*
	72	31	11.0 (8.7-13.6)	175.5 (58.5-316.9)	24.8 (17.3-38.6)*
Septic	0	49	8.6 (0.7-13.4) **	167.9 (82.5-364.7) **	139.3 (70.5-250) **
	24	38	8.5 (5.3-17.2)	116.1 (51.8-248.2)	50.0 (24.2-116.3)
	48	32	6.8 (3.0-10.5)	97.7 (34.7-320.1)	29.8 (16.0-74.2)*
	72	25	7.0 (3.1-22.1)	71.8 (38.1-283.2)*	31.4 (13.2-69.7)*

SNS, sick non-septic
* = P < 0.05 compared to hormone concentration at time 0
** = P < 0.01 compared to hormone concentration in healthy foals at time 0

Table 3.2. Area under the curve for plasma insulin, GIP and GLP-1. Values expressed as median and IQR.

	n	**Insulin** (µIIU*hour/mL)	**GIP** (pg*hour/mL)	**GLP-1** (pmol*hour/L)
Healthy	16	66.0 (41.5-102.2)	1061.0 (726.0-1411.0)	140.4 (93.1-196.4)
SNS	40	28.5 (21.7-42.3)*	396.4 (272.2-664.9)**	131.0 (83.7-187.0)
Septic	49	23.4 (10.8-44.0)**	230.7 (158.2-761.9)**	152.5 (106.7-230.4)

SNS, sick non-septic
** = P < 0.01 compared to healthy foals

Table 3.3. Correlations (ρ) between insulin and incretin hormones in foal groups during over time. A) Healthy foals, B) Sick non-septic foals (SNS), C) Septic foals

A)

Healthy Foals	T0 GIP	T24 GIP	T48 GIP	T72 GIP	T0 GLP-1	T24 GLP-1	T48 GLP-1	T72 GLP-1
T0 Insulin	0.506*	0.409	0.44	0.547	0.679**	0.291	0.371	0.604
T24 insulin	0.365	0.512*	0.527	0.672	0.159	0.753**	0.615	0.786
T48 insulin	0.518	0.541	0.508*	0.763	0.344	0.635	0.606*	0.743
T72 Insulin	0.286	0.457	0.625	0.663**	0.229	0.432	0.521	0.571*
T0 GIP					0.538*	0.276	0.394	0.546
T24 GIP					0.482	0.594*	0.471	0.718
T48 GIP					-0.00589	0.177	0.369	0.336
T72 GIP					0.347	0.361	0.363	0.524*

B)

SNS Foals	T0 GIP	T24 GIP	T48 GIP	T72 GIP	T0 GLP-1	T24 GLP-1	T48 GLP-1	T72 GLP-1
T0 Insulin	0.181	-0.0624	0.0939	-0.0383	0.0992	-0.157	-0.148	-0.0635
T24 insulin	-0.195	0.375*	-0.0646	-0.0938	0.0915	0.057	0.00192	-0.115
T48 insulin	-0.0512	0.252	0.207	-0.074	-0.0687	0.174	0.13	0.129
T72 Insulin	0.102	0.38	0.192	0.134	-0.404	-0.174	-0.0961	0.218
T0 GIP					-0.0208	0.0602	-0.137	0.0817
T24 GIP					-0.114	0.118	0.0374	-0.0143
T48 GIP					-0.0457	0.191	0.291	0.469
T72 GIP					-0.096	0.154	0.163	0.374*

Continued

Table 3.3 continued.

C)

Septic Foals	T0 GIP	T24 GIP	T48 GIP	T72 GIP	T0 GLP-1	T24 GLP-1	T48 GLP-1	T72 GLP-1
T0 Insulin	-0.0885	-0.104	-0.0163	0.00077	0.0382	-0.307	-0.064	0.0851
T24 insulin	-0.094	0.245	0.328	0.234	-0.124	-0.236	0.00532	0.095
T48 insulin	0.0825	0.464	0.498**	0.377	-0.0147	-0.0957	0.0948	0.12
T72 Insulin	-0.278	0.233	0.259	0.396*	-0.11	-0.278	-0.0746	0.142
T0 GIP					-0.084	0.235	0.315	0.522
T24 GIP					-0.203	0.226	0.189	0.283
T48 GIP					-0.137	0.0638	0.379*	0.418
T72 GIP					-0.0385	0.152	0.422	0.541**

T0, time 0; T24, time 24 hours; T48, time 48 hours; T72, time 72 hours
* = $P < 0.05$; ** = $P < 0.01$

Table 3.4. Plasma insulin, GIP and GLP-1 concentrations at time of admission (time 0) in surviving and non-surviving septic foals. Values expressed as median and IQR.

	n	Insulin (μIU/ml)	GIP (pg/ml)	GLP-1 (pM)
Survivor	25	10.0 (5.35-13.85)	178.2 (111.4-372.0)	132.8 (82.1-181.5)
Non-survivor	24	2.95 (0.1-11.2)**	145.7 (73.0-366.0)	165.7 (37.0-278.4)

** = $P < 0.01$

Table 3.5. Area under the curve for plasma insulin, GIP and GLP-1 in surviving and non-surviving septic foals. Values expressed as median and IQR.

	Insulin (μIU*hour/mL)	GIP (pg*hour/mL)	GLP-1 (pmol*hour/L)
Survivors	27.5 (14.75-44.2)	340.3 (159.1-718.1)	152.5 (120.3-201.1)
Non-survivors	12.5 (8-33.3)	220.2 (144.2-893.3)	147.5 (61.3-338.1)

Table 3.6. Correlations (ρ) between insulin and incretin hormones in survivors and non-survivors over time. A) Survivors, B) Non-survivors

A)

Variable	T0 GIP	T24 GIP	T48 GIP	T72 GIP	T0 GLP-1	T24 GLP-1	T48 GLP-1	T72 GLP-1
T0 Insulin	0.0331	0.188	0.11	0.0755	0.248	-0.143	-0.0503	-0.0974
T24 insulin	-0.0232	0.441*	0.316	0.29	0.0509	0.0455	0.237	0.246
T48 insulin	0.0548	0.428	0.458*	0.427	0.0949	-0.212	0.202	0.292
T72 Insulin	-0.0509	0.542	0.332	0.467*	-0.184	-0.121	0.221	0.279
T0 GIP					-0.248	0.324	0.589	0.619
T24 GIP					-0.234	-0.00494	0.418	0.525
T48 GIP					-0.0999	0.0525	0.497*	0.602
T72 GIP					-0.181	0.0754	0.53	0.628**

B)

Variable	T0 GIP	T24 GIP	T48 GIP	T72 GIP	T0 GLP-1	T24 GLP-1	T48 GLP-1	T72 GLP-1
T0 Insulin	-0.22	-0.418	-0.0552	-0.232	-0.0532	-0.367	0.0184	0.493
T24 insulin	-0.114	-0.05	0.418	0.0857	-0.218	-0.298	-0.224	-0.0857
T48 insulin	0.164	0.552	0.539*	0.2	-0.309	0.219	-0.297	-0.486
T72 Insulin	0.143	0.257	0.6	0.371	-0.657	-0.0286	-0.371	-0.371
T0 GIP					0.0374	0.122	-0.176	0.486
T24 GIP					-0.136	0.495*	-0.309	-0.486
T48 GIP					-0.321	0.146	0.0303	-0.2
T72 GIP					0.314	0.714	0.371	0.371

T0, time 0; T24, time 24 hours, T48, time 48 hours, T72, time 72 hours
* = $P < 0.05$; ** = $P < 0.01$

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[i] Terumo SURFLO® EFTE IV Catheter 14G x 2", Terumo Medical Corp, Somerset, NJ

[ii] Aprotinin, Goldbio, St, Louis, MO

[iii] Diprotin A, Bachem, Torrance, CA

[iv] AlphaTRAK blood glucose monitoring system meter, Abbott Animal Health, Chicago, IL

[v] Human GIP (Total) (EZHGIP-54K), ELISA, Millipore Sigma

[vi] Multi species total glucagon-like peptide-1 (EZGLP1T-36K) ELISA, Millipore Sigma

[vii] Insulin Microplate ELISA, MP Biomedicals, Orangeburg, NY

[viii] Prism 8.0, GraphPad Software Inc, San Diego, CA

[ix] SigmaPlot 14, Systat Software, Chicago, IL