Title: The between-day reproducibility of fasting, satiety-related analytes, in 8 to 11 year-old boys.

Authors: Susan Allsop[a*], Penny L. S. Rumbold[a], Benjamin P. Green[a].

Institutional affiliations:

[a] Faculty of Health and Life Sciences, Department of Sport, Exercise and Rehabilitation, Northumbria University, Northumberland Building, Newcastle upon Tyne, NE1 8ST, UK.

[*]**Corresponding author:** Susan Allsop Tel: +44(0)1913719562, fax: +44(0)191 227 3190, email: s.allsop@northumbria.ac.uk.

Abstract

The aim of the present study was to establish the between-day reproducibility of fasting plasma GLP-1_{7-36}, glucagon, leptin, insulin and glucose, in lean and overweight/obese 8-11 y boys. A within-groups study design was utilised wherein the boys attended two study days, separated by 1 week, where a fasting fingertip capillary blood sample was obtained. Deming regression, mean difference, Bland-Altman limits of agreement (LOA) and typical imprecision as a percentage coefficient of variation (CV %), were utilised to assess reproducibility between-days. On a group level, Deming regression detected no evidence of systematic or proportional bias between-days for all of the satiety-related analytes however, only glucose and plasma GLP-1_{7-36} displayed low typical and random imprecision. When analysed according to body composition, good reproducibility was maintained for glucose in the overweight/obese boys and for plasma GLP-1_{7-36}, in those with lean body mass. The present findings demonstrate that the measurement of glucose and plasma GLP-1_{7-36} by fingertip capillary sampling on a group level, is reproducible between-days, in 8-11 y boys. Comparison of blood glucose obtained by fingertip capillary sampling can be made between lean and overweight/obese 8-11 y boys. Presently, the comparison of fasting plasma GLP-1_{7-36} according to body weight is inappropriate due to high imprecision observed in lean boys between-days. The use of fingertip capillary sampling in the measurement of satiety-related analytes, has the potential to provide a better understanding of mechanisms that affect appetite and feeding behaviour in children.

Keywords

GLP-1_{7-36}; Insulin, Glucagon; Leptin; Glucose; Satiety.

1. Introduction

Human appetite and the regulation of feeding behaviour are sophisticated processes. Emerging evidence confirms the control of appetite and regulation of feeding behaviour is primarily governed through interaction between the nervous and digestive systems, via the enteric nervous system (ENS) [1]. There are numerous analytes which elicit episodic (short-term) and tonic (long-term) properties, relaying information through the gut-brain axis to regulate satiety. The present work will focus on glucagon-like-peptide 1 (GLP-1_{7-36}), glucagon, glucose (episodic analytes), insulin and leptin (tonic analytes). The aforementioned analytes represent several commonly measured metabolic variables documented as having fundamental roles in satiety signalling [2] and thus contribute to human energy balance [3-6].

During consumption of a meal, GLP-1_{7-36} is released by the endocrine L-cells as nutrients are detected in the duodenum. Appearance of GLP-1_{7-36} in the circulation is bi-phasic, occurring within 10 to 15 minutes [7, 8] and at 30 to 60 minutes [9] following ingestion. The effects of GLP-1_{7-36} via the ENS are to inhibit gastric emptying and intestinal motility, a process termed the 'ileal brake' which brings about meal termination [6]. Levels of circulating GLP-1_{7-36} can be elevated for more than 3 h following a meal [10]. It has been suggested that GLP-1_{7-36} not only acts to restrict food intake but also functions to extend the time before any further eating episode can occur [10]. Recent evidence indicates that glucagon is able to signal the brain through vagal afferent neurons, to effect meal termination and may also decrease meal size [11]. Primarily glucagon opposes the actions of insulin [11, 12] which is released due to the detection of glucose in the blood. The main function of insulin in satiety therefore, is to enable uptake of glucose and reduce levels of the blood sugar [13] in accordance with the 'Glucostatic theory' [14]. The short-term actions of GLP-1_{7-36}, insulin and glucagon, are in contrast to leptin which has long term anorectic properties. Leptin is an adipokine largely produced by adipocytes and is correlated with white adipose tissue [15]. When an individual is in a positive energy balance state, circulating plasma leptin is increased which facilitates a reduction in food intake, until energy balance is restored [12]. Leptin also has specific short term functions that bring about a reduction in meal size. It appears to do this by acting on taste sensitivity through the hyperpolarization of taste buds on the tongue [16] which reduces the positive reinforcing effects of food ingestion on the brain [17].

In England, 19.1% of children are currently obese [18] and it appears to have greater prevalence in boys during mid-to-late childhood (8-11 y) [19]. Assessment of the aforementioned analytes in paediatric populations could provide essential information in relation to the regulation of appetite and feeding behaviour in children. To the author's knowledge, appetite research that quantifies glucose, GLP-1_{7-36}, insulin, glucagon and leptin in healthy paediatric populations, particularly 8-11 y boys is sparse and is likely due to the sampling methods invariably utilised.

Generally, in research and clinical practice, blood is obtained by antecubital-venous or arterio-venous sampling. For research with vulnerable populations such as children, antecubital-venous sampling is invasive and may even be deemed as unethical. Recent research from our laboratory has examined the agreement and reproducibility of plasma GLP-1_{7-36}, glucagon, leptin and insulin, between fingertip capillary blood and antecubital-venous sampling in healthy adults [20]. Green and colleagues (2014) [20] demonstrated that fingertip capillary blood sampling provided a comparable and reproducible alternative to antecubital-venous, to quantify glucagon and to lesser degree, GLP-1_{7-36}, leptin and insulin. Such a method is far less invasive than venous sampling, and thus represents a more suitable procedure for use in paediatric populations [20].

To the best of our knowledge, evidence of between-day reproducibility in fasted plasma GLP-1_{7-36}, glucagon, leptin and insulin exists only for healthy adults, for traditional methods of blood sampling [10, 21, 22] and fingertip capillary sampling [20]. Currently, there is no understanding of between-day reproducibility of fasted plasma GLP-1_{7-36}, glucagon, leptin and insulin obtained from fingertip capillary blood in children. In view of the less invasive nature of fingertip capillary sampling, prior to short-term intervention in appetite-related studies with children, it seems prudent to establish between-day reproducibility in fasted levels of these analytes of interest. Knowledge of the between-day reproducibility will inform researchers whether changes are due to intervention and not imprecision related to sample handling, analytical procedures and equipment, or disparity in biological responses. Consequently, the aim of the present study is to establish the between-day reproducibility of fasting plasma GLP-1_{7-36}, glucagon, insulin, leptin and blood glucose obtained by fingertip capillary sampling, in 8-11 y lean and overweight/obese boys.

2. Methods

2.1. Study design

A within-groups study design was utilised to establish between-day reproducibility in fasting plasma GLP-1_{7-36}, glucagon, insulin and leptin and blood glucose obtained from fingertip capillary blood, in 8-11 y old boys.

The study was conducted according to 2013 Declaration of Helsinki (World Medical Association. 2013) and was approved by the University of Northumbria, Faculty of Health and Life Sciences Ethics Committee. Written informed consent was obtained from each child's parent or main carer and assent was given by the child prior to data collection.

2.2. Participants

Boys aged 8-11 y were recruited from a primary school located within the city of Newcastle upon Tyne (North East England, UK). To enable recruitment, consent was obtained from the Head Teacher

of the school they attended. A recruitment pack was distributed to all eligible boys who expressed an interest in participating and they were asked to take this home to their parent (or main carer). The pack contained a letter addressed to their parent/main carer with a full explanation of the study and consent forms for them and their child (if able) to sign and return to school. Signed consent was received from 24 boys, of which 23 participated in the study. Boys were excluded from participating if they were diabetic or took any form of medication known to affect taste, smell or appetite.

2.3. Study protocol

Prior to the first visit to the University laboratory, each boy was provided with a food diary. With the help of their parent (or main carer) they were requested to weigh and record all foods and fluids consumed from 1700 h the day before each visit until 2100 h, at which point they were required to begin a 12 h overnight fast. Following the first visit, they were provided with a copy of the food diary so that their food and fluid intake could be replicated prior to visit two. With the assistance of the parent (or main carer) they were asked to refrain from sport or physical activity from 1700 h until arrival at school on the morning of each visit.

The boys were required to attend the University laboratory on two different days, separated by 1 week. On the morning of each visit, following a 12 h overnight fast, the boys attended school at 0830 h. From waking, they were permitted to drink only water and with the assistance of their parent (or main carer) were asked to note this amount in the food diary to enable replication prior to the second visit. For logistical reasons, the boys were organised into testing groups of five to seven. At school (0830 h), the boys were met by two members of the research team and transported to the University for 0845 h so that they could each provide one fasted capillary blood sample.

During the first visit, the stature and seated height of each boy was measured to the nearest 0.01 m using a Harpenden Portable Stadiometer (Holtain Limited, Pembs, UK) to calculate age (y) from peak height velocity (APHV) [23]. Body mass was measured to the nearest 0.1 kg using portable SECA scales (SECA United Kingdom) whilst wearing light clothing. Waist circumference was measured to the nearest 0.01 m with a non-elastic flexible tape at each boy's natural waist whilst standing, as a measure of central adiposity [24]. In both visits, immediately following the collection of the blood sample, each boy was provided with breakfast, after which they were escorted back to school by two researchers.

2.4. Blood sampling

To obtain blood samples, the same fingertip capillary blood sampling and handling method utilised by Green and colleagues [20] was followed. Prior to blood collection, 33 µL per mL of aprotinin and 30 µL per mL of DPP-IV inhibitor were added to a microvette and pre-cooled, to prevent the cleavage of

GLP-1_{7-36} by proteases and thus aid in the preservation of this analyte. The fingertip was pierced with a sterile automated lancet (Accu-Check, Mannheim, Germany) and blood was collected (300 µL) into a pre-cooled EDTA microvette. Immediately following blood collection, the microvettes were placed on ice and then spun at 1500 *g* for 10 min in a multispeed microcentrifuge. Aliquots of the plasma supernatant were pipetted into labelled Eppendorfs and stored at −80 °C for quantification of GLP-1_{7-36}, glucagon, leptin and insulin at a later time-point. Together with the fingertip capillary blood sample, a further 20 µL of whole blood was collected from the same puncture site into sodium heparinized capillary tubes and transferred into Eppendorfs containing 1 mL haemolysis solution (EKF Diagnostics) to determine blood glucose. Samples were subsequently shaken to encourage haemolysis, placed on ice and processed immediately.

2.5. Blood analysis

The concentrations of GLP-1_{7-36} (pg/mL), glucagon (pg/mL), leptin (pg/mL) and insulin (pmol/L) were determined by electrochemiluminescence, using a human hormone multiplexed sandwich immunoassay (Sector Imager 2400, MesoScale Discovery, Rockville, MD. USA). In preparation for the measurement of GLP-1_{7-36} (pg/mL), glucagon (pg/mL), leptin (pg/mL) and insulin (pmol/L), a stock calibrator was diluted (fourfold serial diutions) with a Metabolic Assay Working Solution (provided by the manufacturer), to create an eight point standard curve. As advised by the manufacturer, the calibrators and the fingertip capillary plasma samples were analysed in duplicate on one assay plate to eliminate inter-assay variation. Forty µL of plasma supernatant was extracted from each Eppendorf and pipetted into each well. The multiplex assay uses capture antibodies namely, anti-GLP-1 ($_{7-36}$) amide, anti-insulin, anti-glucagon and anti-leptin, in solution. The capture antibodies and the fingertip capillary plasma samples are added to an electroluminescent compound (MSD SULFO-TAG™ label). Over two incubation periods, GLP-1_{7-36} (pg/mL), glucagon (pg/mL), leptin (pg/mL) and insulin (pmol/L) along with the electroluminescent compound, bind to their specific capture antibody onto a working electrode surface located within each well. A read buffer solution is then added to provide the appropriate chemical environment for electrochemiluminescence and to enable a voltage to be applied to the plate electrodes which causes the labels on the electrode surface to emit light. The intensity of light emitted, is measured.

The lower limits of detection (sensitivity) for GLP$1_{7–36}$, glucagon, leptin and insulin were, 1.0 pg/mL, 20 pg/mL, 22 pg/mL and 9.0 pmol/L respectively, as specified by the manufacturer. Intra-assay coefficients of variation (CV) were established by the measurement of one, fasted fingertip capillary plasma sample, three times on the same assay plate. For GLP-1_{7-36}, glucagon, leptin and insulin, these were established as 11%, 9%, 19% and 11%, respectively.

Blood glucose was quantified by the glucose oxidase method using an automated point of care glucose analyser (BiosenC_line, EKF Diagnostics). The method electro-chemically measures β-D-glucose as it is converted to gluconic acid. Prior to use, the analyser was calibrated with a solution of known glucose concentration (12 mmol/L).

2.6. Statistical analysis

For all boys and when split according to lean and overweight/obese body composition, means ±SEM were calculated for GLP-$1_{7\text{-}36}$, glucagon, leptin, insulin and glucose. Within-subject reproducibility between samples for visits one and two was assessed by utilising a range of statistical methods. Deming regression tests for and provides a value for average systematic and proportional bias on a group level [25]. Mean difference, provides a value for typical error [26]. Bland-Altman limits of agreement (LOA) [27] was also used to indicate relative bias (mean difference) and random imprecision. Typical imprecision as a percentage coefficient of variation (CV %) was also calculated to quantify random imprecision. All values were checked for heteroscedasticity by the examination of box plots, scatter plots and related Pearson's correlation coefficients of the absolute differences (imprecision) and the means of measurements [27]. If heteroscedasticity was apparent with an r value of 0.4, the data was log transformed (natural) and stated as a geometric mean and ratio (x/÷) LOA.

To aid in the interpretation of the statistical analysis, clinically significant differences deemed to be meaningful were acquired for each analyte in advance of data collection. The clinically significant differences utilised were based on published adult research that had explored the effects of food intake, appetite or within subject reliability of fingertip sampling, in relation to GLP-$1_{7\text{-}36}$, glucagon, leptin, insulin or glucose [10, 21, 22, 28]. The use of clinically meaningful differences based on adult data was due to the lack of reproducibility literature to date, for healthy children. Due to the adult studies being largely conducted over set time periods, the clinically meaningful values obtained were time-averaged area under the curve [AUC]. The values were therefore determined to be 2.1 pg/mL, 7.4 pg/mL [10, 28], 222.0 pg/mL [21], 4.8 pmol/L and 0.5 mmol/L[22] respectively, for GLP-$1_{7\text{-}36}$, glucagon, leptin, insulin and glucose respectively.

3. Results

3.1. Participant characteristics

A total of 23 boys took part in the study however, two were excluded from statistical analysis due to non-standardisation of food intake prior to their second visit. In addition, owing to issues related to blood collection, results for GLP-$1_{7\text{-}36}$, leptin, insulin and glucose are provided for 20 boys and for glucagon, 17 boys. Participant characteristics are presented in Table 1. According to UK age and sex-specific BMI centiles [29], the majority of boys were classified as having a healthy body mass (76%) and 24% were classified as overweight/obese. Mean age (y) at peak height velocity (APHV) was

-3.4±0.2 y and -3.2±0.3 for the lean and overweight/obese boys, respectively. The APHV of the lean boys indicated they were an average of 3 y and 4.8 months and the overweight/obese boys were an average of 3 y 2.4 months, from reaching their peak height velocity.

Table 1. Participant characteristics.

Participant characteristic	Lean boys (n = 16)	Overweight/obese boys (n=5)
Age (y)	10.5±0.2	10.3±0.3
Body mass (kg)	34.9±1.0	47.6±3.6
Stature (m)	1.45±0.0	1.44±0.0
BMI (kg/m^2)	16.6±0.3	22.8±0.9
Waist circumference (cm)	60.4±1.0	74.1±3.0
Age at peak height velocity (y)	-3.4±0.2	-3.2±0.3

3.2. Reproducibility of GLP-1$_{7-36}$, glucagon, leptin, insulin and glucose

3.2.1. Deming regression

In relation to reproducibility for all boys between visits one and two, Deming regression analysis revealed no evidence of systematic [intercept (95% confidence interval (CI)] or proportional bias [slope (95% CI)] in fasted plasma concentrations of any of the analytes or glucose. For GLP-1$_{7-36}$, the intercept (95% CI) was -0.1 (-2.2 to 2.1) and slope (95% CI) was 0.9 (0.5 to 1.4) (Figure 5.1A), glucagon intercept (95% CI) was 1.4 (-45.9 to 48.8)] and slope (95% CI) was 1.1 (0.7 to 1.5) (Figure 5.1B), leptin intercept (95% CI) was -2549 (-7260 to 2162) and slope (95% CI) was 1.5 (0.9 to 1.9) (Figure 5.1C), insulin intercept (95% CI) was -204.6 (-23.1 to 315.5) and slope (95% CI) was 0.8 (0.3 to 1.1) (Figure 5.1D) and for glucose the intercept (95%) was 2.1 (-3.6 to 7.8) and slope (95% CI) was 0.6 (-0.6 to 1.8) (Figure 5.1E).

When split according to body composition, Deming regression analysis of fasted plasma concentrations for the lean boys revealed no evidence of systematic [intercept (95% (CI)] or proportional bias [slope (95% CI)] for GLP-1$_{7-36}$, glucagon and insulin (Table 2). For leptin, there was evidence of a proportional difference, whilst for glucose there was a significant difference between visits one and two (Table 2). For the overweight/obese boys, there was no evidence of systematic [intercept (95% (CI)] or proportional bias [slope (95% CI)] for GLP-1$_{7-36}$, glucagon, leptin and glucose (Table 2). For insulin, there was evidence of a proportional bias between visits one and two (Table 2).

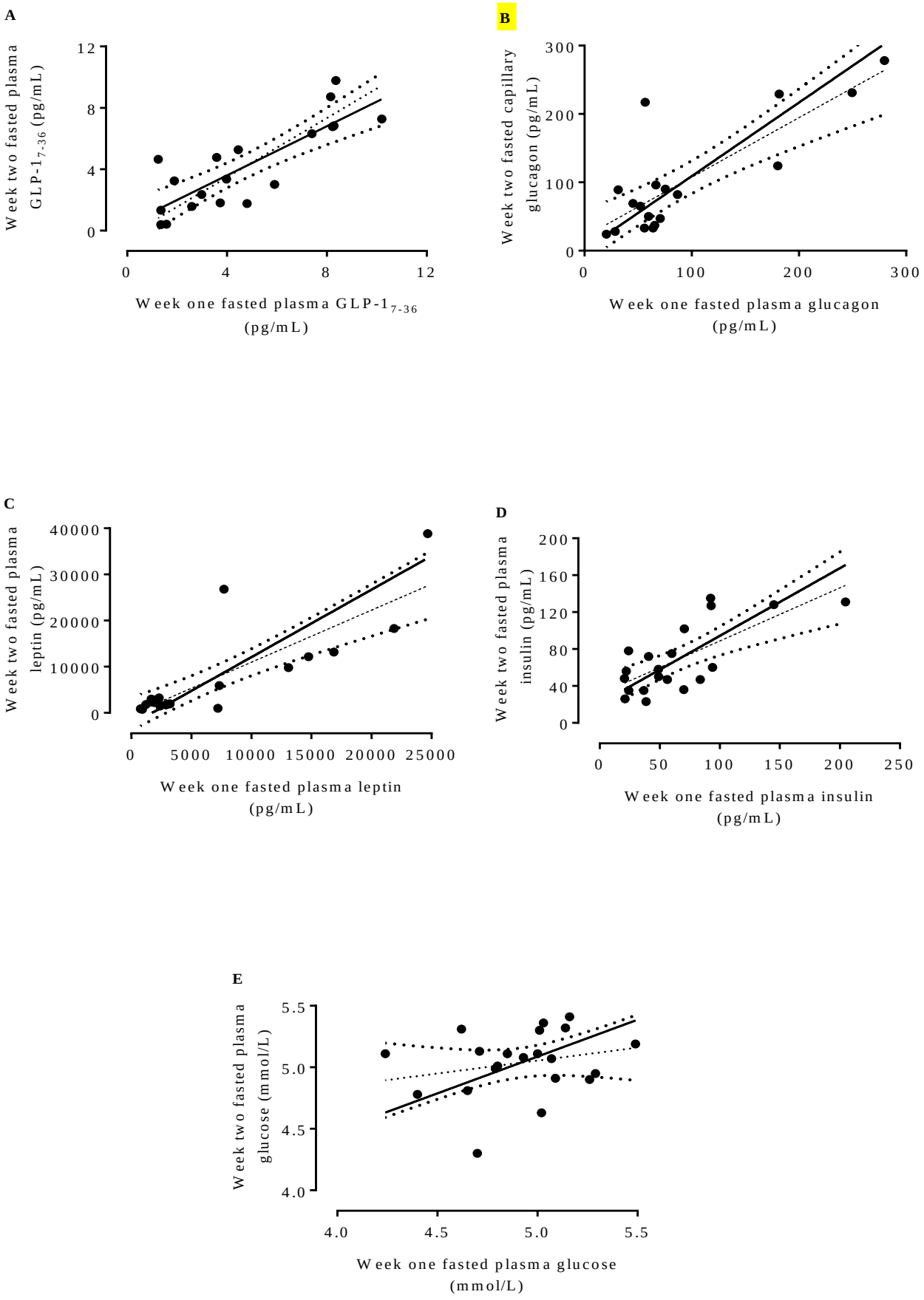

Figure 2. Deming regression scatter-plots of fasted plasma GLP-$1_{7\text{-}36}$ (pg/mL, panel A), glucagon (pg/mL, panel B), leptin (pg/mL, panel C), insulin (pmol/L, panel D) and glucose (mmol/L, panel E) for visit one versus visit two. The solid black line indicates the line of equality. The dashed line represents the regression line with the corresponding 95% CI falling in between the black dotted lines. Individual data points denote means of visit one versus visit two for fasted plasma GLP-$1_{7\text{-}36}$, glucagon, leptin, insulin and glucose. For conversion of GLP-$1_{7\text{-}36}$ (pg/mL) and insulin (pg/mL) to SI units, multiply by 0.298 and 0.172, respectively.

Table 2. Deming regression analysis between visits one and two of the lean (n=16) and (n=5) overweight/obese

Analyte	Lean only		Overweight/obese only	
	Intercept (95% CI)	**Slope (95% CI)**	**Intercept (95% CI)**	**Slope (95% CI)**
GLP-1_{7-36} (pg/mL)	-1.0 (-2.7 to 0.8)	1.0 (0.7 to 1.4)	4.3 (-9130 to 9298)	-18.0 (-1865 to 1829)
Glucagon (pg/mL)	3.3 (-60.5 to 54.0)	1.1 (0.6 to 1.5)	7.8 (-141.3 to 157.0)	1.0 (-0.0 to 2.0)
Insulin (pmol/L)	-24.5 (-8.3 to 39.4)	1.5 (0.5 to 2.6).	55.2 (-9.2 to 119.7)	0.4 (-0.1 to 0.9)
Leptin (pg/mL)	416.6 (-782.2 to 1615.0)	0.7 (0.4 to 0.9)	36654.0 (-47208.0 to 120517.0)	-1.2 (-6.4 to 4.0)
Glucose (mmol/L)	4.7 (1.3 to 8.2)	0.7 (-0.6 to -0.8).	-3.0 (-18.4 to 12.4)	1.6 (-1.5 to 4.8)

3.2.2. Bland-Altman limits of agreement (LOA, mean difference and typical imprecision (CV %)

Table 3 displays the mean $_{\pm SEM}$ of all analytes for visits one and two, as well as mean differences, typical imprecision expressed as a percentage coefficient of variation (CV %) and Bland Altman LOA. Between visits one and two, the CV % for plasma GLP-1_{7-36}, glucagon, leptin and insulin were high, and low for plasma glucose. Bland-Altman LOA enabled the calculation between visits of relative bias (mean difference) ± random imprecision (1.96 standard deviations (SD) of the difference). As such LOA showed good agreement between visits one and two for plasma GLP-1_{7-36}, although there was large random imprecision. Limits of agreement for plasma glucagon, leptin and insulin exceeded the aforementioned predetermined clinical values and showed large random imprecision. For glucose, LOA were good between visits and random imprecision was low.

The means $_{\pm SEM}$ for visits one and two, mean differences, typical imprecision (CV %) and Bland Altman LOA for all analytes when split according to lean and overweight/obese body composition, are displayed in Table 4. When compared with the predetermined clinical values, the LOA for the lean boys of GLP-1_{7-36}, glucagon, leptin and glucose showed good reproducibility, whilst insulin showed poor reproducibility between visits one and two. For the overweight/obese boys, the LOA showed good reproducibility for GLP-1_{7-36} and glucose, whilst for glucagon, insulin and leptin reproducibility remained poor, between visits.

Table 3. Means ±SEM, mean differences ±SEM and CV % between visit one and visit two of fasting plasma GLP-1_{7-36} (pg/mL), glucagon (pg/mL), leptin (pg/mL), insulin (pmol/L) and glucose (mmol/L) for all boys (n=21).

	GLP-1_{7-36} (pg/mL)	**Glucagon (pg/mL)**	**Leptin (pg/mL)**	**Insulin (pmol/L)**	**Glucose (mmol/L)**
Visit one mean ±SEM	4.7±0.6	92.8±18.0	6679.8±1582.9	63.0±10.0	4.9±0.1
Visit two mean ±SEM	4.6±0.7	101.5±19.1	7218.4±2169.4	70.2±8.0	5.0±0.1
Mean difference ±SEM	0.1±0.1	8.7±1.1	746.9±586.5	7.2±2.0	0.1±0.0
CV %	68.8	45.0	57.0	48.7	5.3
Bland-Altman LOA	-0.5±3.3	8.5±93.1	538.3±11209.2	7.2±67.3	0.1±0.7

SEM standard error mean; CV % percentage coefficient of variation.

Table 4. Means ±SEM, mean differences ±SEM, CV % and Bland-Altman limits of agreement between visit one and visit two of fasting plasma GLP-1_{7-36} (pg/mL), glucagon (pg/mL), leptin (pg/mL), insulin (pmol/L) and glucose (mmol/L) for lean (n=16) and overweight/obese boys (n=5).

		GLP-1_{7-36} (pg/mL)	**Glucagon (pg/mL)**	**Leptin (pg/mL)**	**Insulin (pmol/L)**	**Glucose (mmol/L)**
Lean	**Visit one mean ±SEM**	4.7±0.7	89.1±19.3	3397.8±802.3	56.3±6.4	5.0±0.1
	Visit two mean ±SEM	3.9±0.8	98.8±21.7	2647.4±565.8	62.0±8.4	5.1±0.1
	Mean difference ±SEM	0.9±0.1	9.7±2.4	750.4±236.5	3.8±2.0	0.1±0.0
	CV %	41.5	45.8	53.3	32.8	5.2
	Bland-Altman LOA	0.8±1.4	6.4±50.5	32.6±150.8	5.2±26.0	-0.1±0.4
Overweight/obese	**Visit one mean ±SEM**	4.3±1.5	109.1±57.4	15307.2±2940.9	98.6±45.7	4.8±0.2
	Visit two mean ±SEM	7.5±1.9	113.8±55.8	17596.3±3348.2	96.8±20.3	4.9±0.4
	Mean difference ±SEM	3.2±0.4	4.7±37.3	2289.1±407.3	1.7±25.4	0.1±0.2
	CV %	78.5	59.6	67.3	72.7	4.7
	Bland-Altman LOA	3.2±0.5	4.7±37.3	-10.15±323.8	-1.7±55.8	0.1±0.3

SEM standard error mean; CV % percentage coefficient of variation.

4. Discussion

To the authors' knowledge, this is the first study to examine between-day reproducibility of fasted plasma satiety-related analytes, namely GLP-1_{7-36}, glucagon, leptin, insulin and glucose, in 8 to 11 y-old boys. The results provide initial data for between-day reproducibility in the above mentioned analytes and also for glucose when obtained by fingertip capillary sampling, from children. The main findings showed that for all boys, glucose and GLP-1_{7-36} are reproducible between-days, when obtained by fingertip capillary sampling from 8-11 y boys. When analysed according to body composition, between-day reproducibility was maintained for GLP-1_{7-36} in the lean boys and for glucose, in those classified as overweight/obese. Comparison of blood glucose between lean and overweight/obese children is therefore possible when obtained by fingertip capillary sampling. For fingertip derived GLP-1_{7-36} however, comparison is not advised according to body composition due to greater imprecision established between-days in overweight/obese boys. The reproducibility data obtained presently could provide important information with regards to appetite-related research with children. Fingertip capillary sampling of glucose and GLP-1_{7-36} might also be a feasible alternative to the more invasive methods of blood draw, in future paediatric appetite-related research.

For all boys, fasting glucose was found to be the most reproducible between-days, illustrating that the analyte can be reliably measured by fingertip capillary sampling, in paediatric appetite-related intervention studies. When examined according to body composition, the strong reproducibility of glucose was maintained, but only in the overweight/obese boys. In the lean boys, there was evidence of systematic and proportional bias between-days, although all other statistical tests supported good reproducibility. As individual values for both visits for the lean boys were within the normal range for fasting blood glucose [30], this suggests that comparison of the analyte can be made between lean and overweight/obese groups, when utilising fingertip capillary sampling. This is important due to the associations of glucose with satiety, as according to the 'glucostatic theory'[14], the presence of this analyte instigates insulin release so that the sugar may be absorbed [13]. Along with insulin, other hormones are simultaneously released such as GLP-1_{7-36}, the actions of which restrict food intake [31]. The results of the present study thus demonstrate the necessity for inclusion and measurement of glucose in acute appetite-related clinical studies with children.

Fasting plasma GLP-1_{7-36} also showed good between-day reproducibility, apart from displaying a high CV %. The CV % (68.8%) showed typical imprecision to be threefold greater than found previously in adults (22.7%) when utilising fingertip capillary sampling [20]. When split by body composition, the reproducibility of GLP-1_{7-36} was noted to improve for the lean boys, although typical imprecision (CV 41.5%) was still almost double than established in adults (CV 22.7% and 19.0 %) [20, 32]. Presently however, only one fingertip capillary sample was collected from the boys in each visit. In our most

recent study with adults, we collected five samples over 120 min [20] which may explain the reduced variation in values [26]. For the overweight/obese boys, imprecision between-days was further increased, as greater values were obtained for mean difference (3.2±0.4 pg/mL), CV % (78.5%) and Bland-Altman (LOA) (3.2±0.5 pg/mL). Previous adult reproducibility testing of GLP-1_{7-36} has shown that a difference of 2.1 pg/mL [33] would be deemed clinically meaningful [10]. The plasma concentrations of GLP-1_{7-36} determined presently, were within the range of those found previously for fasted 8-12 y boys, during resting conditions (median 9.9, interquartile range 4.3 to 24.7 pg/mL) [34]. Therefore, these results would suggest that plasma GLP-1_{7-36} can be measured by fingertip capillary sampling in lean 8-11 y boys. In view of the previous adult reproducibility findings and the greater imprecision established in the present study for overweight/obese boys, the comparison of fasting plasma GLP-1_{7-36} in children when split according to body composition, is not advised when obtained by this less invasive method.

In contrast to glucose and GLP-1_{7-36}, the reproducibility of insulin, glucagon and leptin was poor for all boys. Despite Deming regression analysis displaying no systematic or proportional bias, typical and random imprecision between-days on a group level, was high. The findings thus demonstrate that fasting values of insulin, glucagon and leptin are likely to alter research interpretation if fingertip capillary sampling is employed over venous methods. When the boys were spilt by body composition, there was an increase both in mean values and in overall imprecision in the overweight/obese boys for glucagon, leptin and insulin. Furthermore, Deming regression showed proportional bias between-days for insulin in those classified as overweight/obese and for leptin in the lean boys. These results suggest that as concentrations of leptin and insulin rise, imprecision also increases and thus illustrates that fingertip capillary values of fasting leptin and insulin cannot be compared between lean and overweight/obese children. Reasons for the high typical imprecision, could be that both leptin and insulin are tonic peptides which indicate long term nutritional status. The actions of leptin in particular, are to signal the brain as to the status of adiposity and so levels of this analyte increase according to body mass [35]. Fasting levels of insulin alongside glucose are known to increase with pubertal change [36] and earlier pubertal transition can be triggered by increased adiposity [37]. However, it should be noted, that as sampling was only 7 days apart, it is unlikely that extensive body mass or pubertal dependent changes in insulin and glucose would have occurred in the present population, on an intra-individual basis.

The between-day reproducibility of glucagon remained poor for the lean boys when split according to body composition. For those classified as overweight/obese, there was a slight improvement in reproducibility as shown by a decrease in mean difference and Bland-Altman LOA. For this analyte however, there was a lower number of participants in the lean and overweight/obese groups and this

may have contributed to the poor reproducibility [26]. Further reproducibility testing of fingertip capillary sampled glucagon in paediatric populations is therefore, warranted.

The finding that fasting concentrations of glucose can be compared between lean and overweight/obese 8-11 y boys, demonstrates the practical application of the measurement of this analyte by fingertip capillary sampling in future appetite-related intervention research. Most notable however, is that fasting concentrations of GLP-1_{7-36} can also be measured in lean 8-11 y boys, by this less invasive method. Although research suggests that fasting concentrations of GLP-1_{7-36} are similar in lean, overweight and obese children [34, 38, 39], there is a dearth of evidence in relation to post-prandial responses [40]. Adult studies suggest that the post-prandial response of GLP-1_{7-36} is blunted in obesity [9]. To date, it is unclear whether the post-prandial response of GLP-1_{7-36} is blunted in obese children also [9, 38, 39]. Investigation into the reproducibility of post-prandial satiety-related responses, which include GLP-1_{7-36} when obtained by fingertip capillary sampling is therefore, warranted in children. In doing so, fingertip capillary sampling of satiety-related analytes alongside subjective measurement with visual analogue scales (VAS) could enable more rigorous investigations of paediatric appetite. A consequence of this might be a better understanding of the mechanisms which affect paediatric appetite and subsequent feeding behaviour which may have a role in obesity.

As this was the first study to measure between-day reproducibility of GLP-1_{7-36}, glucagon, leptin and insulin in a paediatric population, it is not without limitations. Firstly, this meant that findings had to be directly compared with those of adults, as this was the only available data. Concentrations of various hormones including insulin and leptin differ with age and sex and so a proportion of the imprecision noted in this study, could be due to biological events linked to pubertal status [41]. Such a dynamic could successively affect the comparison of GLP-1_{7-36} and glucagon. In addition, some analytical imprecision may have occurred. To prevent this, the pre-treatment, sample handling and analysis were all rigorously controlled, as demonstrated by the low intra-assay coefficients of variation (CV) obtained for GLP-1_{7-36} (11%), glucagon (9%) and insulin (11%). In the present study, it is not known whether the levels of any of the fasted analytes could have been affected by stress, as this psychological marker was not measured. However, to counteract any stress the boys may have felt prior to blood draw, the University laboratory was arranged to resemble a kitchen in the home. The blood sampling area was hidden from view and only those children who were entirely comfortable with the procedure participated. Future reproducibility investigations utilising fingertip capillary sampling, might therefore benefit from the additional measurement of cortisol. Nevertheless, at the time of writing, there are no paediatric studies that have measured between day reproducibility of GLP-1_{7-36}, glucagon, leptin and insulin, when utilising fingertip capillary sampling. Indeed the use of fingertip capillary sampling in this study is a strength and is a technique which reduces ethical concerns surrounding blood sampling in paediatric populations, particularly in comparison to the

more invasive venous sampling [20]. Consequently, the study findings enable the opportunity for future investigations to utilise fingertip capillary blood sampling to quantify, GLP-1_{7-36} and glucose, particularly with vulnerable populations such as paediatrics. The study also employed several statistical tests to provide a more thorough examination which included Deming regression analysis, a preferred statistical test employed in clinical research, as opposed to a t-test and Pearson's correlation.

To conclude, for all boys, fingertip derived glucose and GLP-1_{7-36} were reproducible between-days. The good reproducibility of glucose enables the comparison of this analyte between lean and overweight/obese 8-11 y boys. Decreased reproducibility of GLP-1_{7-36} in lean boys, in comparison to those classified as overweight/obese however, suggests that this analyte should not be compared between these two groups. The present findings offer the opportunity for researchers to utilise less invasive fingertip derived concentrations of glucose and GLP-1_{7-36} in future paediatric satiety-related investigations. Measurement of both glucose and GLP-1_{7-36} by fingertip capillary sampling could not only provide more rigorous investigation but also increase the practical application of paediatric appetite findings. Moreover, a better understanding of the mechanisms that have an affect on appetite and feeding behaviour in children, a population that is currently at increasing risk of obesity.

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