METABOLIC, ENDOCRINE AND APPETITE-RELATED RESPONSES TO ACUTE AND DAILY MILK SNACK CONSUMPTION IN HEALTHY, ADOLESCENT MALES

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This work was supported by the Dairy Council UK in the award of a PhD studentship to Benjamin. P. Green, but this organization had no role in the study design, analysis or manuscript preparation.

Abstract

Comprising of two experiments, this study assessed the metabolic, endocrine and appetite-related responses to acute and chronic milk consumption in adolescent males (15-18 y). Eleven adolescents [mean ± SD age: 16.5 ± 0.9 y; BMI: 23.3 ± 3.3 kg/m^2] participated in the acute experiment and completed two laboratory visits (milk vs. fruit-juice) in a randomized crossover design, separated by 7-d. Seventeen adolescents [age: 16.1 ± 0.9 y; BMI: 21.8 ± 3.7 kg/m^2] completed the chronic experiment. For the chronic experiment, a parallel design with two groups was used. Participants were randomly allocated and consumed milk (n = 9) or fruit-juice (n = 8) for 28-d, completing laboratory visits on the first (baseline, day-0) and last day (follow-up, day-28) of the intervention phase. On laboratory visits (for both experiments), measures of appetite, metabolism and endocrine responses were assessed at regular intervals. In addition, eating behavior was quantified by ad libitum assessment under laboratory conditions and in the free-living environment by weighed food record. Acute milk intake stimulated glucagon (P = .027 [16.8 pg·mL; 95% CI: 2.4, 31.3]) and reduced ad libitum energy intake relative to fruit-juice (P = .048 [-651.3 kJ; 95% CI: -1294.1, -8.6]), but was comparable in the free-living environment. Chronic milk intake reduced free-living energy intake at the follow-up visit compared to baseline (P = .013 [-1910.9 kJ; 95% CI: -554.6, -3267.2]), whereas the opposite was apparent for fruit-juice. Relative to baseline, chronic milk intake increased the insulin response to both breakfast (P = .031) and mid-morning milk consumption (P = .050) whilst attenuating blood glucose (P = .025). Together, these findings suggest milk consumption impacts favorably on eating behavior in adolescent males, potentially through integrated endocrine responses.

Keywords: Milk, Fruit-Juice, Appetite, Adolescents, Snack, Energy Intake

Introduction

Snacking has become commonplace and characterizes a major element of modern eating behavior, yet is often considered to contribute to the current obesity epidemic (Chapelot, 2011). Snacking is defined as an episode of food consumption occurring outside the context of typical main meals, including all food and beverage items (Chapelot, 2011). Snack foods are readily available in a variety of settings, including the school environment (Savige, Macfarlane, Ball, Worsley, & Crawford, 2007), and therefore snacking is highly prevalent, particularly among children and adolescents. For example, 98% of 12- to 17-y-old students in a recent UK study reported consuming one or more snacks daily, and this was greatest among male adolescents (Macdiarmid et al., 2009). Consequently, snacking contributes significantly to daily energy and nutritional intake in young people (Ovaskainen et al., 2006), which is potentially problematic and may lead to overconsumption of calories, free-sugars and nutrient-poor, energy-dense foods. Indeed, while the health effects associated with such dietary behaviours are well known (Chapelot, 2011) the promotion of more healthful snacks could benefit overall dietary intake, nutritional status and actually act as a marker for healthier eating habits.

From a child and adolescent perspective, fruit-juice drinks, sugar-sweetened beverages and milks are frequently reported as common beverage snack items consumed between main meals (Duffey et al., 2012). Despite fruit-juice providing vitamins, minerals and antioxidants, and sugar-sweetened beverages which hold a negligible nutritive value, high rates of consumption appear to promote weight gain in children and adolescents (Dennison, 1996; Dennison, Rockwell, & Baker, 1997; Woodward-Lopez, Kao, & Ritchie, 2011). Interestingly, the opposite may stand true for milk-based beverages (Dror, 2014). Indeed, emerging evidence suggests that milk-based beverages protect against adiposity in children and adolescents (Abreu et al., 2014; Barba, Troiano, Russo, Venezia, & Siani, 2005; Moore, Singer, Qureshi, & Bradlee, 2008), and the replacement of sugar-sweetened beverages with milk or water, but not fruit-juice, is inversely associated with body fatness throughout the transition from childhood to adolescence (Zheng et al., 2015). Relative to fruit-juice drinks and sugar-sweetened beverages, milk-based beverages are recognized as a nutrient-dense foodstuff and contain a host of constituents that improve the overall nutritional quality of the child and adolescent diet (Fiorito, Mitchell, Smiciklas-Wright, & Birch, 2006). Outside of the health-related benefits, recent evidence also indicates that high rates of milk

consumption are positively associated with academic performance and motivation for learning in adolescents compared to sugar-sweetened beverage intake (Kim et al., 2016).

Efforts to establish the relationship between milk and adiposity have identified several plausible mechanisms, all of which may be attributed to the nutritional composition of milk. Literature from cell and adult studies indicate that dairy calcium stimulates adipocyte lipolysis (Zemel, Shi, Greer, Dirienzo, & Zemel, 2000), increases energy expenditure (Zemel, et al., 2000), fat oxidation and faecal fat excretion (Melanson, Donahoo, Dong, Ida, & Zemel, 2005; van Loon, Saris, Verhagen, & Wagenmakers, 2000). Beyond calcium, milk proteins (whey and casein, and their products of digestion) may act to potentiate peptides from gastrointestinal, pancreatic and adipose tissue origin (Anderson & Moore, 2004; Bowen, Noakes, & Clifton, 2006; Schneeman, Burton-Freeman, & Davis, 2003), increasing perceptions of satiety (Dove et al., 2009; Gilbert et al., 2011) and thus reducing energy intake (Dove, et al., 2009). In addition, medium chain triglycerides, conjugated linoleic acid and lactose may also be implicated in the role of milk-based foods on reducing energy intake (Aziz & Anderson, 2007). Taken together, it appears that milk-based beverages have a unique potential to influence elements of energy balance. In this sense, milk contains a host of components and bioactive constituents that act individually, and probably synergistically, to impart beneficial effects on body mass regulation through actions related to appetite, eating behavior and metabolism. It is prudent to highlight, however, that the majority of this appetite and metabolic research has been conducted in adult populations, and at present there remains a dearth of mechanistic information in children and adolescents.

According to the acute literature (Birch, McPhee, Bryant, & Johnson, 1993; Mehrabani, Salehi-Abargouei, Asemi, Feizi, & Safavi, 2014; Zandstra, Mathey, Graaf, & van Staveren, 2000), mid-morning dairy snack consumption [ice-cream (Birch, et al., 1993), yogurt (Zandstra, et al., 2000) and milk (Mehrabani, et al., 2014)] reduces energy intake and increases energy expenditure (Apolzan et al., 2006) in children and adolescents (3- to 15-y-old). However, these studies are primarily limited to acute child investigations utilizing dissimilar preloads (differing according to volume and energetic content) and single energy intake assessment (laboratory based *ad libitum* assessment). Moreover, no quantitative measures of subjective appetite and/or appetite- and metabolism-related peptides were included which may have provided valuable insights concerning the mechanisms impacting on appetite and eating behaviour, and thus remains to be examined. Without a better understanding of the mechanisms impacting on appetite and eating behavior following dairy

consumption, it remains challenging to reconcile the potential effects of different dairy foods on energy regulation in children and adolescents. Consequently, this study investigated the effect of acute and chronic (28-d) mid-morning milk snack consumption on subsequent metabolic, endocrine and appetite-related responses.

Materials and Methods

Experimental Design

A randomized crossover design was implemented with two experimental conditions to investigate the acute effects of milk consumption on subsequent energy intake, circulating concentrations of glucagon-like peptide-1 ($GLP\text{-}1_{7\text{-}36}$), glucagon, insulin, leptin and blood glucose, energy expenditure and subjective appetite. Experimental visits consisted of mid-morning milk (< 2% fat) and an isoenergetic and isovolumetric serving of fruit-juice, each separated by 7-days. To investigate the effects of chronic milk consumption on the abovementioned metabolic, endocrine and appetite-related responses, a parallel design with two intervention groups was used. Participants were randomly allocated to groups, and received either daily mid-morning milk (<2% fat) or an isoenergetic and isovolumetric fruit-juice for 28 days. Participants made two experimental visits to the nutrition and metabolism laboratory, which were scheduled on the first (day-0, baseline) and last (day-28, follow up) days of the intervention phase. Participants were matched according to age (16.1 ± 1.1 vs. 16.4 ± 0.7 y), body mass (69.4 ± 18.3 vs. 68.2 ± 10.5 kg), body mass index ([BMI] 22.0 ± 5.0 vs. 21.6 ± 2.5 $kg \cdot m^2$) and habitual calcium intake (814.5 ± 118.4 vs. 836.0 ± 274.9 mg·d) intake. Habitual calcium intakes were estimated using a validated food frequency questionnaire for determining calcium and vitamin D intake in adolescents (Taylor et al., 2009). All testing was completed during school-term time.

Participants

Participants were recruited from a local secondary school in the North-East of England, after attendance at an initial information seminar. Adolescent males between 15 and 18 y of age were eligible to participate. Eleven male adolescents (mean ± $_{SD}$; age: 16.5 ± 0.8 y; body mass: 73.4 ± 11.5 kg; stature: 1.8 ± 0.1 m; BMI: 23.3 ± 3.3 kg/m^2) were recruited for the

acute experiment and another 19 different participants (mean ± SD; age: 16.1 ± 0.9 y; body mass: 68.8 ± 13.9 kg; stature: 1.8 ± 0.0 m; BMI: 21.8 ± 3.7 kg/m^2; habitual calcium intake: 790 ± 217 mg·d) for the daily experiment. All participants were free of milk-related allergies, diabetes or other metabolic disorders (and medication) known to affect taste, smell and appetite.

The Faculty of Health and Life Sciences Ethics Committee at Northumbria University reviewed the experimental procedures and approved the study. The study was conducted in accordance with the Declaration of Helsinki of 1975, as revised in 2013. All participants provided written informed parental consent and student assent before any study-related procedures were performed. This trial was registered at clincaltrials.gov: NCT02487342.

Pre-Trial Standardisation

Participants were instructed to record all food and fluid consumption 24 h preceding the first visit for both the acute and chronic experiments, using a self-report, weighed food diary. Participants were also advised to refrain from caffeine and alcohol consumption (≥ 24-h) and strenuous physical activity (≥ 24-h) before each experimental visit (days-0 and -28 of the daily experiment). Participants were requested to replicate these dietary and exercise behaviors for subsequent experimental visits. Between waking and arrival at the nutrition and metabolism laboratory, consumption of water only was permitted. Participants were requested to record, document and replicate morning water consumption for subsequent experimental visits.

Experimental Protocol

On experimental visits for both the acute and chronic experiments (days-0 and -28 of the daily experiment), participants arrived at the nutrition and metabolism laboratory at 0745 h. After 30 min rest, a baseline fingertip-capillary blood sample was drawn, expired gas sample (300 s resting sample) collected, and participants completed a series of baseline subjective appetite visual analogue scales (VAS). A standardized cereal and milk breakfast meal was provided at 0830 h. Participants were given 15 min to consume the entire contents of the breakfast meal.

Participants subsequently remained at rest in the laboratory for 180 min in an environment free from food cues. This period started upon the first mouthful of the breakfast meal. Further samples of fingertip-capillary blood and subjective appetite VAS were collected at 30 min intervals during the 180 min. Expired gas samples (300 sec) were collected at 25-30, 55-60, 85-90, 115-120, 145-150, and 175-180 min. Mid-morning snacks were administered at 90 min. At 180 min, a homogenous *ad libitum* pasta meal was provided. Participants were instructed to eat until comfortably full and satisfied. On completion of the *ad libitum* pasta meal, participants were free to leave the laboratory and returned to school via chaperon. They were asked to record any further food and fluid items consumed during the remainder of the day utilizing a combined weighed self-reported food record and 24 h dietary recall technique. The intervals between test meals were selected to be representative of a typical school day. During the intervention phase for the daily experiment, participants were instructed to maintain their usual feeding and physical activity practices.

Test Meals

Breakfast

Breakfast consisted of semi-skimmed milk (Tesco, UK) and Kellogg's Rice Krispies (Kelloggs, Manchester, UK), in a cereal to milk ratio of 30 g: 125 mL. The quantity issued was designed to provide 10% of the participants estimated daily energy requirement for protein, fat and carbohydrate (14%, 14% and 72%, respectively) as used previously (Astbury, Taylor, French, & Macdonald, 2014).

Mid-Morning Snacks

For both experiments, mid-morning snack items consisted of milk (< 2% fat, Tesco, UK), and an orange fruit-juice (Tesco, UK). All items were isovolumetric (217 mL) and isoenergetic (427 kJ), yet differed according to macro-nutrient composition (**Table 1**). Milk was selected as the dairy preload as this represents the most commonly consumed dairy food for this sex and age group (Green, Turner, Stevenson, & Rumbold, 2015). The volume selected was based on nationally representative consumption patterns (Bates, Lennox, & Swan, 2010), and is similar to other snack-based studies (Almiron-Roig, Grathwohl, Green, & Erkner, 2009; Mehrabani, et al., 2014). All packaging labels were removed and snack items were served in appropriate opaque serving containers.

For the acute experiment, participants completed two experimental conditions and were issued with one of the two snacks in a counterbalanced randomized manner. For the chronic experiment, a parallel design with two intervention groups was employed whereby participants were randomly allocated to groups and received either daily milk or fruit-juice for the 28-d intervention phase. During the intervention phase, the lead investigator visited participants on the school campus on a daily basis. A temporary nutrition laboratory was setup within the school where participants arrived between 1100-1115 h daily to consume their mid-morning snack. Participants were registered on a daily basis to monitor compliance, and snacks were consumed in the presence of the researcher (during weekdays only). On Fridays, participants were provided with the snacks for weekend periods. Participants were requested to consume the snacks once daily between 1100-1115 h, and return empty containers the following Monday (as a measure of compliance).

Pasta Meal

Lunchtime food intake (180 min) was evaluated by means of a homogenous pasta meal and comprised of pasta (Tesco, UK), tomato sauce (Tesco, UK), cheddar cheese (Tesco, UK) and olive oil (Tesco, UK) and provided 859 kJ of energy per 100 g portion (205 kcal; energy contributions 14% protein, 52% carbohydrate and 34% fat). The test lunch was offered *ad libitum*. Participants were initially provided with 400 g of the pasta meal. Research staff continuously refilled participant's bowls (before the dish became empty) until participants indicated they were comfortably full. Detailed information concerning the nutrient composition of the pasta meal and the method of cooking has been reported in previous studies (Gonzalez et al., 2015). In addition, similar pasta meals have been used successfully in previous adolescent research (Rumbold et al., 2013). Energy intake from the pasta meal was calculated based on the amount consumed and nutritional composition as indicated by the manufacturer. To facilitate this, research staff covertly weighed the meal prior to serving, and immediately following meal termination.

Subjective Appetite

Subjective measures of appetite were assessed using validated 100 mm, paper based VAS (Flint, Raben, Blundell & Astrup, 2000). Scales were anchored with diametrically opposed feelings of extremity, and addressed hunger ('how hungry do you feel?'), gut fullness ('how

full do you feel?'), prospective food consumption ('how much do you think you can eat?'), and satisfaction ('how satisfied do you feel?'). Subjective measures of appetite were reported immediately prior to each fingertip-capillary blood sample.

Gas Analysis

To collect gas samples, a mouthpiece attached to a two-way, non-rebreathing valve (model 2730, Hans Rudolph, Kansas City, Missouri) was used. Gas samples, collected in Douglas Bags, were analyzed for concentrations of oxygen and carbon dioxide using a paramagnetic and infrared transducers, respectively (Servomex 5200S, Crowborough, East Sussex, UK). In addition, bag volume and temperature of expired gas samples were determined using a dry gas meter (Harvard Apparatus, Edenbridge, Kent, UK) and thermistor (model 810-080, ETI, Worthing, UK), respectively. Participants inserted the mouthpiece and rested for approximately 2 min before each expired gas sample (300 sec) was collected. Samples were collected at 25-30, 55-60, 85-90, 115-120, 145-150, and 175-180 min. Rates of energy expenditure (kJ), and substrate oxidation were estimated based on caloric equivalents of carbohydrate utilization and lipid oxidation, using stoichiometric equations as described (Frayn, 1983) with the assumption that protein oxidation was negligible.

Free-Living Energy Intake

Participants recorded all food and drink items consumed for the remainder of each experimental day. This was completed utilizing a combined weighed self-reported food record and dietary recall, used previously with adolescent populations (Rumbold, St Clair Gibson, Stevenson, & Dodd-Reynolds, 2011). Participants were requested to give full comprehensive recordings of all food and drink items consumed, weighing all items prior to and following consumption (if leftovers were present). Following each study day, the lead investigator visited the participants on a one-to-one basis on school campus grounds and completed 24 h recall interviews. Interviews used a two-pass approach (Ashley, Bovee, & Andersen, 2003), and lasted approximately 15 min per participant. All interviews took place at the same time each day and were conducted by the same researcher. One trained member (the lead investigator) of the research team examined all food records utilizing the nutritional software package Nutritics (Nutritics Professional v3.09, Nutritics, Ireland).

Blood Sampling and Analysis

At seven separate intervals, during laboratory visits (for both the acute and chronic experiment), fingertip-capillary (0.3 mL) blood samples were drawn into pre-cooled EDTA-treated microvettes. Samples were collected whilst participants lay in a semi-supine position at pre-breakfast (t = 0 min) and at 30, 60, 90, 120, 150 and 180 min following breakfast consumption for the determination of plasma GLP-1_{7-36}, glucagon, insulin, and leptin. Research from our laboratory has previously provided affirmation that fingertip-capillary blood sampling offers an appropriate methodological and reproducible approach for the quantification of appetite-related peptides in a resting state (Green, Gonzalez, Thomas, Stevenson, & Rumbold, 2014; Allsop, Rumbold & Green, 2016). Consequently, pre-analytical (e.g. sample treatment) and analytical (e.g. sample handling) procedures were followed as previously described (Green, et al., 2014). Briefly, microvettes contained aprotinin (33 μL·mL whole blood) and a di-peptidyl peptidase IV inhibitor (30 μL·mL whole blood) for the preservation of GLP-1_{7-36} and glucagon.

Following blood collection, samples were centrifuged immediately. Microvettes were spun at 1509 *g* (3000 rpm) for 10 min in a multispeed micro-centrifuge. Quantitative assessments of GLP-1_{7-36} (pg·mL), glucagon (pg·mL), leptin (pg·mL) and insulin (pmol·L) were simultaneously determined in 40 μL of plasma by electrochemiluminescence using a human hormone multiplex assay kit (Sector Imager 2400, MesoScale Discovery, Maryland, USA). Of note, the addition of protease inhibitors to samples for the preservation of GLP-1_{7-36} and glucagon does not influence measured concentrations of plasma leptin and insulin (Bielohuby, Popp, & Bidlingmaier, 2012). Samples from each participant were analyzed within the same run to eliminate inter-assay variation. Intra-assay coefficients of variation were determined by the repeated measurement of a single baseline fingertip-capillary blood sample three times. For the acute experiment, average inter-assay coefficients of variation were 11%, 10%, 12% and 12% for GLP-1_{7-36}, glucagon, leptin and insulin, respectively. For the daily experiment, average inter-assay coefficients of variation were 10%, 8%, 6% and 8% for GLP-1_{7-36}, glucagon, leptin and insulin, respectively.

Additional fingertip-capillary (0.02 mL) blood samples for the determination of blood glucose were drawn into sodium heparinized capillary tubes and transferred into eppendorfs containing 1 mL haemolysis solution (EKF Diagnostics). Samples were subsequently shaken to encourage haemolysis, placed on ice and processed immediately. Concentrations of blood

glucose were quantified instantaneously by glucose oxidase method (BiosenC_line, EKF Diagnostics). Concentrations of blood glucose were quantified instantaneously by the glucose oxidase method using an automated glucose analyzer (BiosenC_line, EKF Diagnostics), based on an electro-chemical measuring principle following the conversion of β-D-glucose to gluconic acid. Prior to use, the analyzer was calibrated with a solution of known concentration (12 mmol·L), provided by the manufacturer.

Sample-Size Estimate and Power

An a priori sample-size estimate was conducted on the basis of the ability to detect a difference in postprandial plasma glucagon, an appetite-related peptide responsible for actions including increased satiety and reduced food intake (anorexigenic behaviors). For the acute experiment, sample-size estimation was conducted based on a pre-determined clinically significant time-averaged AUC difference for plasma glucagon. Given that the reported typical percentage error of between-day fingertip-capillary plasma glucagon is 8.2% (Green et al., 2014), it was estimated that 10 participants would provide > 80% chance of statistically detecting a difference with $P < 0.05$. For the purpose of the chronic study, sample-size estimation was conducted based on observations from the acute experiment that mid-morning milk consumption elicits a 16.8 pg·mL greater plasma glucagon response relative to a serving of fruit-juice, alongside the previously mentioned between-day typical error. Consequently, it was estimated that 18 participants (nine per group) would provide > 80% chance of statistically detecting a difference with $P < 0.05$.

Statistical Analysis

Computer software package JMP®, version 12.2.0 (SAS Institute Inc., Cary, NC) was used to perform all statistical analysis. Data were checked for normal distribution with the use of the Kolmogorov-Smirnov normality test and were log-transformed if appropriate before statistical analysis. Statistical significance was accepted at an α level of $P \leq .05$. All data are presented as mean ± $_{SEM}$ unless otherwise stated, with effects expressed as mean difference ± 95% confidence intervals (CI).

For the acute experiment, comparisons between experimental visits for baseline fingertip-capillary variables, baseline subjective appetite, energy expenditure, *ad libitum* and free-living energy intake were assessed using paired samples t-tests. Area under the curve (AUC) values were computed for fingertip-capillary variables and subjective appetite using the trapezoidal rule for the post-breakfast (0-90 min) and post-snack (90-180 min) periods, and these values were subsequently time averaged. The postprandial period was split into 0-90 min and 90-180 min as the time points after the breakfast meal and mid-morning snack may influence the effect of particular appetite-related components (e.g. hormonal, metabolic, physical or cognitive) (Blundell et al., 2010). Data concerning postprandial time-averaged AUC estimates of fingertip-capillary variables and subjective appetite were assessed using paired samples t-tests with 95% confidence intervals (CI) comparing between experimental visits.

To investigate the effect of daily milk or fruit-juice on appetite and eating behavior, values obtained during the baseline experimental visit (day-0) were compared with intervention follow up experimental visits (day-28). As the methodological approach of experimental visits was identical between the acute and daily experiment, statistical procedures were conducted in the same manner as stated for the acute experiment. This comprised paired samples t-test analysis to determine differences for fingertip-capillary variables, subjective appetite, energy expenditure, *ad libitum* and free-living energy intake, with effects expressed as mean difference ± 95% CI relative to the baseline experimental visit (day 0). Reflecting the results at the level of data measurement, the use of CI indicates the direction of the effect studied (du Prel, Hommel, Rohrig, & Blettner, 2009). If the CI does not include the value of zero, it can be assumed a directional effect exists (Shakespeare, Gebski, Thiagarajan, & Jay Lu, 2006). Differences between baseline (day-0) and intervention follow up experimental visits (day-28) for the milk and fruit-juice group were analyzed using independent t-tests to determine contrasts between groups for fingertip-capillary variables, subjective appetite, energy expenditure, *ad libitum* and free-living energy intake, with effects expressed as mean difference ± 95% CI.

Results

In total, all 11 participants completed the acute experiment. Due to difficulties associated with attendance at the intervention follow up experimental visit (day-28), data for the chronic experiment are presented for 17 participants [milk group (n = 9) fruit-juice group (n = 8)].

Hormonal Variables

In the acute experiment, fasted plasma concentrations of GLP-$1_{7\text{-}36}$, glucagon, insulin, leptin and blood glucose concentrations were comparable between conditions. This remained during the post-breakfast period (time-averaged $AUC_{0\text{-}90\ min}$), suggesting that the postprandial concentrations of these peptides were comparable following breakfast. Following the acute mid-morning milk snack, however, time-averaged $AUC_{90\text{-}180\ min}$ estimates of glucagon were greater relative to the fruit-juice snack (95.5 ± 6.4 pg·mL vs. 76.7 ± 7.2 pg·mL, respectively; P = .027 [16.8 pg·mL; 95% CI: 2.4, 31.3]).

Following both chronic fruit-juice and milk snack consumption, fasted plasma concentrations of GLP-$1_{7\text{-}36}$, glucagon, insulin, leptin and blood glucose concentrations did not differ at the intervention follow up (day-28) compared to baseline (day-0) (**Figures 1 & 2**). No differences were observed for GLP-$1_{7\text{-}36}$, glucagon, insulin, leptin and blood glucose post-breakfast ($AUC_{0\text{-}90\ min}$) and post-snack ($AUC_{90\text{-}180\ min}$) between the intervention follow up (day-28) and baseline (day-0) following chronic fruit-juice (**Figures 1 & 2**). This finding remained following chronic milk consumption for GLP-$1_{7\text{-}36}$, glucagon, and leptin (**Figures 1 & 2**). Time-averaged $AUC_{0\text{-}90\ min}$ insulin, was elevated post-breakfast at the intervention follow up (day-28) compared to baseline (day-0) after chronic milk consumption (356 ± 54.6 pmol·L vs. 277.5 ± 48.8 pmol·L, respectively; P = .031 [79.4 pmol·L; 95% CI: 149.3, 9.4], **Figure 2, Panel A**). This remained evident for time-averaged $AUC_{90\text{-}180min}$ (198.4 ± 38.2 pmol·L vs. 163.5 ± 29.1 pmol·L, respectively; P = .050 [34.9 pmol·L; 95% CI: 66.0, 3.8], **Figure 2, Panel A**). Consistent with the insulinotropic effect of chronic milk, measures of time-averaged $AUC_{90\text{-}180\ min}$ glucose concentrations were attenuated at the intervention follow up (day-28) compared to baseline (day-0) (4.2 ± 0.1 mmol·L vs. 4.5 ± 0.1 mmol·L, respectively; P = .025 [-0.3 mmol·L; 95% CI: -0.0, -0.4], **Figure 2, Panel C**).

Energy Intake

Energy intake at the *ad libitum* pasta meal (**Figure 3 Panel A**) was lower following acute mid-morning milk consumption compared to fruit-juice (5459.7 ± 503.2 kJ vs. 6111.0 ± 461.2 kJ, respectively; P = .048 [-651.3 kJ; 95% CI: -1294.1, -8.6]), but was no different in the free-living environment (2936.0 ± 630.5 kJ vs. 3751.2 ± 802.6 kJ, respectively; P = .476

[815.1 kJ; 95% CI: 3268.2, -1638.0]. No statistical differences were found for total daily energy intake following acute mid-morning milk consumption compared to fruit-juice (10,289 ± 519.0 kJ vs. 11,755.9 ± 885.2 kJ, respectively; *P* = .213 [1466.4 kJ; 95% CI: 3923.0, -990.3]).

Following chronic fruit-juice consumption, energy intake at the intervention follow up (day-28) *ad libitum* pasta meal (**Figure 4**) was greater and approached statistical significance compared to baseline (day-0) (6272.0 ± 756.3 kJ vs. 5384.2 ± 413.6 kJ, respectively; *P* = .056 [887.8 kJ; 95% CI: 1813.7, -37.5]), but was no different in the free-living environment (*P* = .822 [-195.3 kJ; 95% CI: 1926.8, -2317.3]. Chronic milk consumption appeared to have the opposite effect. In this sense, energy intake at the intervention follow up (day-28) *ad libitum* pasta meal (**Figure 4**) remained comparable to baseline (day-0) (4994.4 ± 192.3 kJ vs. 4792.5 ± 308.3 kJ, respectively; *P* = .326 [201.8 kJ; 95% CI: 646.4, -242.7]), yet was lower in the free-living environment (3460.7 ± 317.6 kJ vs. 4960.7 ± 781.9 kJ, respectively; *P* = .013 [-1910.9 kJ; 95% CI: -554.6, -3267.2]). No statistical differences were found for total daily energy intake in either group.

Subjective Appetite

Subjective appetite data is illustrated in **Table 2**. In the acute experiment, fasted hunger, fullness, prospective food consumption, and satisfaction was comparable between conditions. This remained true during the post-breakfast period (time-averaged $AUC_{0\text{-}90\ min}$). Following the acute mid-morning milk snack, time-averaged $AUC_{90\text{-}180\ min}$ fullness was lower relative to the fruit-juice snack (20.8 ± 2.6 mm vs. 26.8 ± 4.0 mm, respectively; *P* = .038 [-6.0 mm; 95% CI: -11.6, -0.4]). Consistent with a reduction in subjective fullness, time-averaged $AUC_{90\text{-}180\ min}$ prospective food consumption was greater for milk relative to the fruit-juice snack (76.9 ± 3.5 mm vs. 72.7 ± 3.2 mm, respectively; *P* = .005 [4.9 mm; 95% CI: 1.8, 7.9]).

Following chronic fruit-juice consumption, fasted hunger, fullness, prospective food consumption, and satisfaction were all similar at the intervention follow up (day-28) compared to baseline (day-0). A similar pattern emerged following chronic milk consumption, however, measures of fasted prospective food consumption were elevated at the intervention follow up (day-28) compared to baseline (day-0) (75.1 ± 6.9 mm vs. 58.8 ± 3.6 mm, respectively; *P* = .041 [16.2 mm; 95% CI: 31.6, 0.8]). Post-breakfast time-averaged $AUC_{0\text{-}90\ min}$ hunger, fullness, prospective food consumption, and satisfaction were comparable at the intervention follow up (day-28) compared to baseline (day-0) for both groups. This

finding was also observed during the post-snack period (time-averaged $AUC_{90\text{-}180\ min}$) for both groups.

Metabolic Responses

In the acute experiment, fasted energy expenditure, carbohydrate and fat oxidation were comparable between conditions. Total post-breakfast (0-90 min) and post-snack (90-180 min) energy expenditure (kJ), carbohydrate (g) and fat (g) oxidation were not different between conditions. Following both chronic fruit-juice and milk snack consumption, fasted energy expenditure, carbohydrate and fat oxidation did not differ at the intervention follow up (day-28) compared to baseline (day-0). Similarly, no differences were observed for total post-breakfast (0-90 $_{min}$) and post-snack (90-180 $_{min}$) energy expenditure (kJ), carbohydrate (g) or fat (g) oxidation between the intervention follow up (day-28) and baseline (day-0) following both chronic fruit-juice and milk consumption.

Between Group Comparisons

The baseline (day-0) to intervention follow up (day-28) change in fingertip-capillary variables, subjective appetite, metabolic responses, *ad libitum* and free-living energy intake were not significantly different ($P \geq .05$ for all variables) between milk and fruit-juice as assessed by independent t-test (data not shown).

Discussion

Accumulating evidence suggests that milk-based dairy foods elicit anti-obesity properties through actions on eating behaviour and metabolism (Aziz & Anderson, 2007), yet research concerning the physiological mechanisms impacting on energy regulation among children and adolescents is sparse. To the best of our knowledge, the present study is therefore the first to assess the differential effects of acute and chronic (28-d) mid-morning milk consumption on subsequent metabolic, endocrine and appetite-related responses in adolescent males. The major findings of this study were that acute mid-morning milk consumption increased postprandial glucagon secretion and impacts on eating behaviour, reducing subsequent voluntary energy intake at the next meal (acute experiment), compared with fruit-juice. Alongside increased postprandial insulin secretion and attenuated blood glucose concentrations, reductions in eating behaviour were replicated under free-living conditions when milk was consumed chronically (28-d), whereas the opposite was apparent for fruit-

juice consumption. Findings arising from this study begin to provide some mechanistic insight that may have contributed to these observations.

The observation that acute and chronic mid-morning milk consumption reduces subsequent energy intake supports previous observations in children and adolescents (Birch, et al., 1993; Mehrabani, et al., 2014; Zandstra, et al., 2000), contributing further to the understanding that milk-based dairy foods exert the potential to impact favorably on eating behavior. In this study, energy intake at the *ad libitum* pasta meal following acute mid-morning milk consumption was 651.3 kJ (155.7 kcal) lower than when consuming fruit-juice, with no significant differences between conditions thereafter. Following chronic milk consumption, energy intake at the *ad libitum* pasta meal was comparable between baseline (day-0) and follow up (day-28) experimental visits, yet free-living energy intake was reduced by 1910.9 kJ (456.7 kcal), while the opposite was apparent following daily fruit-juice. It has been suggested that sustained reductions in daily energy intake averaging 460 to 690 kJ (110 to 165 kcal) may offer an effective approach for preventing excess weight accumulation in children and adolescents (Wang, Gortmaker, Sobol, & Kuntz, 2006). On the basis of our observations, it would be prudent for future investigations to assess whether milk-based dairy foods provide application to overweight and obese metabolically diseased pediatric populations utilizing longer observation periods.

The mechanism by which milk consumption affects eating behavior is not properly understood, however, there are several plausible explanations and constituents of milk that may act synergistically to elucidate its actions. It is widely recognized that dietary proteins are more satiating than energetic equivalents of carbohydrate and fat under most conditions, and suppress eating behavior at the next available opportunity (Anderson & Moore, 2004; Astrup, 2005; Rolls, Hetherington, & Burley, 1988). In this study, experimental preloads were isoenergetic (427 kJ) and isovolumetric (217 mL), yet differed according to macronutrient composition whereby milk contained considerably more protein than the fruit-juice drink (7.5 g vs. 1.1 g). From a physiological perspective, milk consumption reportedly lowers the postprandial secretion of blood glucose (Panahi et al., 2013), and may be brought about due to an insulinotropic effect (stimulating the production and activity of insulin) (Nilsson, Stenberg, Frid, Holst, & Bjorck, 2004; Ostman, Liljeberg Elmstahl, & Bjorck, 2001). The insulinotropic response may involve milk proteins, digestion and the release of plasma amino acids, and appetite-related hormone secretion, all of which are known to mediate insulin secretion (Nilsson, et al., 2004; Schmid, Schusdziarra, Schulte-Frohlinde,

Maier, & Classen, 1989; van Loon, et al., 2000). In addition, the postprandial response of the appetite- and metabolism-related peptides quantified in this study are profoundly influenced according to ingested macro- (and micro-) nutrient composition, and also energy content. Insulin and glucagon for example rise in response to protein feeding (Acheson et al., 2011). Considering the higher protein content of milk, a heightened insulin and glucagon and attenuated glucose response may have been expected. Indeed, greater circulating concentrations of insulin and glucagon have been related to anorexigenic behaviors in the adult literature, including increased satiety and acutely reduced food intake (Flint et al., 2007; Parker et al., 2013; Penick & Hinkle, 1961; Woods, Lutz, Geary, & Langhans, 2006).

Despite the consistent observation that acute and chronic (28-d) milk consumption reduced energy intake at the *ad libitum* pasta meal (acute experiment) and in free-living environment (chronic experiment), these observations were not reflected by differences in subjective appetite. In fact, our subjective appetite data infers contrasting observations, whereby milk consumption reduced subjective fullness and increased subjective prospective food consumption. This finding was unexpected, especially considering evidence from adult studies suggest acute and daily milk consumption attenuates subjective appetite (Dove, et al., 2009; Gilbert, et al., 2011). Nonetheless, the uncoupling relationship of VAS to reflect and forecast subsequent eating behaviour is common in some (Thivel et al., 2012; Thivel et al., 2011) but not all adolescent studies (Leidy, Ortinau, Douglas & Hoertel, 2013; Leidy et al., 2015). Consequently, this highlights the necessity to include measures of appetite-related peptides in younger populations. It could be reasonable to suggest that alterations in appetite-related peptides may therefore supersede subjective perceptions of food-related emotions to influence subsequent appetite and eating behavior in adolescents.

In the present study, we did not observe any capacity for acute or chronic mid-morning milk or fruit-juice consumption to impact on measures of energy expenditure or substrate metabolism. Reasons for this may involve the volume of product that was distributed in this study. There is evidence in the adolescent literature that single milk constituents or whole dairy food consumption exerts the ability to affect postprandial metabolism (Apolzan, et al., 2006). Although only available in abstract from, Apolzan et al. (2006) evaluated energy expenditure (kcal·min) for 240 min after consumption of a high energy (40% of participants energy requirement) low calcium non-dairy control (45 ± 2 mg Ca), supplemental calcium (670 ± 4 mg Ca) or a dairy-based product (687 ± 5 mg Ca) in overweight adolescent male and females. They observed a greater rate of energy expenditure

following the consumption of the dairy-based product compared with the low calcium non-dairy control, but only in overweight adolescent males. No differences were recorded following supplemental calcium ingestion, which may suggest additional constituents found within milk-based dairy foods act to impact on metabolism. To expand, it is well established, for example, that protein elicits a greater effect on diet induced thermogenesis (20-35% of energy consumed) compared to calorie matched intakes of carbohydrate (5-15% of energy consumed) or fat (0-3% of energy consumed) (Panahi, et al., 2013), albeit it in adults. Nonetheless, despite the higher protein content of the milk (compared to fruit-juice), it may be that the energy and calcium content of milk used in the present study (~272 mg Ca) was insufficient to elicit a significant effect on postprandial metabolism and may therefore demonstrate a dose response, yet the volume selected was based on nationally representative consumption patterns, and is similar to other snack-based studies. Furthermore, the lack of any effect may be attributed to the fact our participants were of normal weight.

The present study was conducted in a manner that reflected a typical school day, and thus practical application in a free-living environment. In this sense, the snack items provided, timing of the mid-morning snack, and volume provided are common for the studied age and sex. Additionally, the authors' believe various aspects of the methodological approach were extremely robust (energy and volume matched snacks, rigorous assessment of subjective appetite, appetite- and metabolism-related peptides, energy expenditure and eating behavior). The results presented from this study may provide some important practical implications. Firstly, the findings of this study provide a strong foundation for further appetite- and metabolism-related research in children and adolescent populations who are metabolically challenged. Based on our findings, it seems milk is an attractive alternative to fruit-juice and sugar-sweetened beverages and may begin to provide initial evidence for stakeholders to help shape the development of future nutrition provision for children and adolescents. Indeed, the inclusion of milk-based foodstuff as a component of a healthy balanced diet is recognized extensively, providing a significant contribution of several essential nutrients (Fiorito, et al., 2006). Thus, encouraging the consumption of milk at mid-morning, over fruit-juice or other energy-dense food snacks that offer little nutritional benefit may positively influence young people's eating habits, especially within the school environment.

Caution should be observed when extrapolating the results of this study as the findings are constrained to adolescent males over a relatively modest duration. Moreover, there are a few limitations that warrant mention. Firstly, while our study design provided

robust experimental control, the provision of a limited highly palatable meal (cheese and tomato pasta) may have induced an element of overconsumption or progressive dislike for that matter that (if faced with) may have influenced subsequent eating behaviour and thus satiety. In children, the amount of food consumed at a laboratory test meal has been shown to vary in children according to palatability (Keller, Kirzner, Pietrobelli, St-Onge & Faith, 2009), whereby the presentation of highly palatable foods promoted overconsumption at ad libitum assessments (Fearnbach, Thivel, Meyermann & Keller, 2015; Deighton, Frampton & Gonzalez 2016). Indeed, this has recently been shown for the meal used in this study (Deighton et al., 2016). However, the cited study presents evidence that eating behaviour at pasta meal better represents preceding appetite (Deighton et al., 2016). While it could be argued that presenting a highly palatable single course ad libitum meal may not reflect true eating practices it is important to note that the pasta meal utilised in this study is typical of what is commonly offered in a school setting, and similar meals have been used successfully in previous within-group adolescent research studies (Rumbold et al., 2013). Secondly, we did not control for factors such as usual breakfast and snacking habits in our sample of adolescent males. Although it could be argued this introduces a number of potentially confounding variables, retrospectively revisiting 24-hour food diaries for pre-trial standardisation few participants skipped breakfast or did not include snacks among their habitual dietary behaviors. Nonetheless, future investigation is certainly warranted to help further establish the longer-term health benefits of milk-based dairy food consumption in children and adolescents, particularly in those who are metabolically compromised. It would also be advantageous to follow on from this body of work by investigating female adolescents and younger populations.

To summarize, in an acute setting the consumption of milk influences short-term energy intake, reducing energy intake at an *ad libitum* pasta meal relative to an isoenergetic and isovolumetric serving of fruit-juice in adolescent males. This was replicated under free-living conditions whereby energy intake was reduced following chronic (28-d) mid-morning milk consumption, whereas the opposite was apparent for daily fruit-juice consumption. Acute mid-morning milk consumption increased postprandial glucagon secretion. In addition, chronic (28-d) mid-morning milk consumption elicited an increased postprandial insulin secretion and attenuates blood glucose concentrations. The findings presented throughout this study therefore indicate that acute and chronic mid-morning milk consumption can influence short-term eating behavior in male adolescents, and begin to illustrate that this may be facilitated through integrated endocrine responses.

Acknowledgements, financial support and conflict of interest

The authors' would like to thank teachers and staff, and in particular the participating students, from Cardinal Hume Catholic High School for their generous commitment, effort and understanding for the conduct of this research. The authors' also extend their gratitude to Kayleigh Cousins for her assistance with data collection.

This work was supported by the Dairy Council UK in the award of a PhD studentship to B. P. G., but this organization had no role in the study design, analysis or manuscript. The authors' declare no conflicts of interest.

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Figure Legends

Figure 1. Mean ± $_{SEM}$ concentrations of plasma GLP-$1_{7\text{-}36}$ (pg·mL; panel A) and plasma glucagon (pg·mL; panel B), obtained from fingertip-capillary blood samples. Graphs depicted on the left are from the chronic milk snack condition (n = 9) whereas graphs depicted on the right are from the fruit-juice condition (n = 8). For the left sided graphs, grey shaded boxes represent values obtained on the first experimental visit (day-0, baseline), whereas white shaded boxes represent values obtained on the last day (day-28, follow up) of each intervention phase. For the right sided graphs, grey shaded circles represent values obtained on the first experimental visit (day-0, baseline), whereas white shaded circles represent values obtained on the last day (day-28, follow up) of each intervention phase. To convert GLP-$1_{7\text{-}36}$ (pg·mL) and plasma glucagon (pg·mL) to their corresponding SI units multiply values by 0.298 and 0.287, respectively. Mid-morning snack items were distributed at 90 min post-breakfast, as represented by the grey shaded area.

Figure 2. Mean ± SEM concentrations of plasma insulin (pmol·L; Panel A), plasma leptin (pg·mL; Panel B) and capillary blood glucose (mmol·L; Panel C), obtained from fingertip-capillary blood samples. Graphs depicted on the left are from the chronic milk snack condition (n = 9) whereas graphs depicted on the right are from the fruit-juice condition (n = 8). For the left sided graphs, grey shaded boxes represent values obtained on the first experimental visit (day-0, baseline), whereas white shaded boxes represent values obtained on the last day (day-28, follow up) of each intervention phase. For the right sided graphs, grey shaded circles represent values obtained on the first experimental visit (day-0, baseline), whereas white shaded circles represent values obtained on the last day (day-28, follow up) of each intervention phase. Mid-morning snack items were distributed at 90 min post-breakfast, as represented by the grey shaded area. Please note: significant observations relate to time-averaged AUC fingertip-capillary variables.

Figure 3. Mean ± $_{SEM}$ *ad libitum* pasta meal (Panel A, n = 11) and free-living (Panel B, n = 11) energy intake (kJ) following acute mid-morning milk snack consumption, relative to an isoenergetic and isovolumetric serving of fruit-juice. Please note: free-living energy intake was considered as calories consumed/reported for the remainder of the study as calculated by weighed food record.

Figure 4. Mean ± $_{SEM}$ *ad libitum* pasta meal (Panel A, n = 11) and free-living (Panel B, n = 11) energy intake (kJ) on the first (day-0, baseline) and last (day-28, endpoint) day of each intervention phase. Grey shaded boxes represent values obtained during the daily milk snack condition (n = 9), whereas grey shaded circles represent values obtained during the daily fruit-juice condition (n = 8).

Table 1. Nutritional composition of the snack foods for both the acute and daily experiment.

	Milk[1]	Fruit-Juice[2]
Serving Size (+ mL water)	207 (10)	217
Energy content (kJ)	427	427
Carbohydrate (g)	10.2	32.0
Fat (g)	3.7	0.0
Protein (g)	7.5	1.1

Note: [1]milk (< 2% fat, Tesco, UK), and [2]fruit-juice (Pure orange juice smooth; Tesco, UK).

Table 2. (A) Time-averaged AUC subjective appetite data (n = 11) following acute (**A**) mid-morning milk snack consumption, relative to an isoenergetic and isovolumetric serving of fruit-juice. (**B**) Time-averaged AUC subjective appetite values obtained during the daily experiment on the first experimental visit (day 0, baseline), and last (day 28, endpoint) days of the intervention phase. Data following daily mid-morning milk snack consumption (n = 9) is expressed on the left side and data following daily fruit-juice (n = 8) is expressed on the right. All time-averaged AUC values are dichotomised according to post-breakfast ($_{0\text{-}90\ min}$) and post-snack ($_{90\text{-}180\ min}$) postprandial periods, with Values are expressed as mean ($_{SEM}$), alongside their corresponding 95% CI. * indicates a difference at the same time point relative to the fruit-juice, whereas ¥ indicates a difference from baseline observation at the same time during the endpoint visit.

A. Acute Experiment	Post-breakfast period (time-averaged $AUC_{0\text{-}90\ min}$)			Post-snack period (time-averaged $AUC_{90\text{-}180\ min}$)		
	Milk	Fruit-juice		Milk	Fruit-juice	
	Mean ($_{SEM}$)	Mean ($_{SEM}$)	95% CI	Mean ($_{SEM}$)	Mean ($_{SEM}$)	95% CI
Hunger (mm)	54.8 (4.3)	57.4 (4.8)	16-7, -11.6	69.5 (4.2)	67.4 (3.1)	10.8, -15.0
Fullness (mm)	35.0 (5.1)	31.7 (4.3)	9.8, -16.5	20.8 (2.6)	26.8 (4.0)	-11.6, -0.4*
Prospective Food Consumption (mm)	66.0 (3.3)	63.4 (4.6)	3.7, -8.9	76.9 (3.5)	72.1 (3.2)	1.8, 7.9 *
Satisfaction (mm)	37.4 (4.1)	32.3 (3.8)	9.2, -3.5	25.3 (3.5)	28.2 (3.4)	9.2, -3.5
B. Daily Experiment	Post-breakfast period (time-averaged $AUC_{0\text{-}90\ min}$) Milk			Post-breakfast period (time-averaged $AUC_{0\text{-}90\ min}$) Fruit-Juice		
	Day 0	Day 28		Day 0	Day 28	
	Mean ($_{SEM}$)	Mean ($_{SEM}$)	95% CI	Mean ($_{SEM}$)	Mean ($_{SEM}$)	95% CI
Hunger (mm)	50.8 (3.3)	59.3 (4.9)	21.6, -4.5	46.9 (6.0)	46.9 (6.0)	31.3, -14.2
Fullness (mm)	34.0 (5.1)	29.7 (5.2)	6.0, -14.7	43.6 (5.9)	36.6 (7.8)	9.8, -23.8
Prospective Food Consumption (mm)	68.2 (2.6)	73.3 (4.3)	15.1, -4.9	60.4 (6.1)	60.9 (6.0)	13.2, -13.3
Satisfaction (mm)	35.7 (1.8)	34.5 (4.2)	9.5, -11.9	44.2 (6.1)	37.1 (7.3)	8.0, -22.2
	Post-snack period (time-averaged $AUC_{90\text{-}180\ min}$) Milk			Post-snack period (time-averaged $AUC_{90\text{-}180\ min}$) Fruit-Juice		
	Day 0	Day 28		Day 0	Day 28	
	Mean ($_{SEM}$)	Mean ($_{SEM}$)	95% CI	Mean ($_{SEM}$)	Mean ($_{SEM}$)	95% CI
Hunger (mm)	75.8 (2.3)	78.4 (2.8)	10.1, -4.8	64.9 (4.3)	75.1 (4.2)	25.9, -5.3
Fullness (mm)	17.1 (2.6)	18.9 (3.8)	7.9, -4.3	30.5 (5.3)	21.9 (4.9)	5.1, -22.3
Prospective Food Consumption (mm)	81.1 (1.8)	85.3 (1.8)	9.5, -1.2	71.5 (3.7)	78.1 (4.4)	17.1, -3.8
Satisfaction (mm)	23.0 (3.8)	18.0 (2.1)	3.6, -13.6	32.1 (4.9)	23.5 (5.4)	2.3, -19.6