Title: CALCIUM INGESTION SUPPRESSES APPETITE AND PRODUCES ACUTE OVERCOMPENSATION OF ENERGY INTAKE INDEPENDENT OF PROTEIN IN HEALTHY ADULTS[1,2,6]

Authors: Javier T. Gonzalez[3,4,5], Benjamin P. Green[3], Meghan A. Brown[3], Penny L.S. Rumbold[3], Louise A. Turner[3], Emma J. Stevenson[3].

[1]This study was funded by Northumbria University. [2]Supplemetal data and Supplemental Figures 1-3 are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at jn.nutrition.org.

[3]Department of Sport, Exercise and Rehabilitation, Northumbria University, Newcastle-upon-Tyne, United Kingdom. [4]Department for Health, University of Bath, Bath, United Kingdom.

[5]Address for correspondence:
Javier T. Gonzalez
Department for Health,
University of Bath,
Bath,
BA2 7AY.
Email: J.T.Gonzalez@bath.ac.uk
Tel: +44 (0)1225 385518

Word count: 4556 **Number of figures:** 4 **Number of tables:** 2

Author names for indexing: Gonzalez JT, Green, BP, Brown MA, Rumbold PLS, Turner LA, Stevenson EJ.

Running title: Acute Effects of Protein and Calcium on Appetite.

Conflict of interest and funding disclosure: PLRS and EJS have received funding from The Dairy Council for unrelated work. JTG and EJS have received funding from FrieslandCampina for unrelated work. BP Green, MA Brown, and LA Turner, no conflicts of interest. ARLA Foods Ingredients amba donated the calcium supplement but had no role in the study design.

[6]Abbreviations:

AUC, time-averaged area under the curve

CAL, high-calcium

CON, control

DPP-IV, dipeptidyl peptidase-IV

EI_{CON}, energy intake following control preload

EI_{EXP}, energy intake following experimental preload

GIP_{1-42}, glucose-dependent insulinotropic $polypeptide_{1-42}$

GLP-1_{7-36}, glucagon-like peptide-1_{7-36}

PRO, high-protein

PROCAL, high-protein and high-calcium

SEM, standard error of the mean

VAS, visual analogue scale

95% CI, 95% confidence interval

ΔCON, change from control

ΔEP, difference in energy content of the experimental and control preloads

ABSTRACT

Background: Prior evidence suggests high-calcium intake influences postprandial appetite and insulinemia, possibly due to elevated incretins. *In vitro* and *ex vivo* models demonstrate extracellular calcium and protein synergistically enhance secretion of incretins. This is yet to be shown in humans.

Objective: This study was designed to assess energy intake compensation in response to protein and calcium ingestion.

Design: Twenty healthy adults (13 men; 7 women) completed 4 trials in a randomized double-blind, crossover design, separated by $\geq$ 48 h. During trials, participants consumed preloads which were low in protein and calcium (CON; 4 g and 104 mg, respectively), high in protein (PRO; 29 g), high in calcium (CAL; 1170 mg) or high in both protein and calcium (PROCAL). Blood samples were collected at baseline, and 15, 30, 45 and 60 min following preload ingestion, to determine insulin and incretin hormone concentrations. Energy intake was assessed by a homogenous test-meal 60 min after the preload. Visual analogue scales were completed immediately before blood sampling to assess subjective appetite sensations.

Results: Relative to CON, PRO produced 100% (95% CI: 85, 115%) energy compensation, whereas CAL produced significant overcompensation 118% (95% CI: 104, 133%), which was significantly more positive than PRO ($P < 0.05$). PROCAL resulted in energy compensation of 109% (95% CI: 95, 123%), which tended to be greater than PRO ($P = 0.06$). The mean difference in appetite sensations relative to CON was not significantly different between PRO (-3; 95% CI: -8 to 3 mm), CAL (-5; 95% CI: -9 to 0) and PROCAL (-5; 95% CI: -10 to -1; $P > 0.05$).

Conclusions: The addition of protein to a preload results in almost perfect energy compensation, whereas addition of calcium, with or without protein suppresses appetite and produces over compensation of subsequent energy intake. The role of circulating insulin and

incretin concentrations in these responses however, remain unclear. Registered at clinicaltrials.gov: NCT01986036**.**

Keywords: females; food intake; fullness; glucagon-like peptide-1; hunger; insulin; males; protein.

INTRODUCTION

Habitual calcium intake is inversely associated with body fat percentage (1) and randomised controlled trials indicate that this may be a causal relationship, *ie*. calcium (plus vitamin D) supplementation augments fat loss under energy restriction (2). Whilst a decrease in dietary fat absorption is likely to partially account for this (3), fat excretion (typically increased by 2 g/d (3)) cannot account for the effect size typically reported in energy-restriction studies (equivalent to an additional ~5 g/d (2)). Thus, other mechanisms are likely to contribute. Some putative mechanisms include increased lipid utilization (4, 5) and reductions in ad libitum energy intake (6) and appetite sensations (7, 8).

Previous research has indicated that a single high-calcium (plus vitamin D) meal may decrease subsequent self-reported 24 h food intake (6). However in this study, energy intake did not differ during the controlled (non-self-report), laboratory period. This lack of an effect with non-self report measures has been shown by others (9). It was only when participants provided self-reported food diaries for the subsequent 24 h that energy intake was lower with a high-calcium (plus vitamin D) breakfast (6). Therefore it remains to be determined whether calcium intake can influence acute food intake in humans, with precise measurement of energy intake.

Notwithstanding this, we have previously reported that the addition of calcium to a mixed-macronutrient meal suppresses postprandial appetite sensations whilst concomitantly elevating insulinaemia (7, 8). These responses may be (in part) due to the gastrointestinal peptides, glucose-dependent insulinotropic polypeptide$_{1\text{-}42}$ (GIP$_{1\text{-}42}$; formerly known as gastric inhibitory peptide) and glucagon-like peptide-1$_{7\text{-}36}$ (GLP-1$_{7\text{-}36}$) (8). GIP$_{1\text{-}42}$ and GLP-1$_{7\text{-}36}$ are secreted by enteroendocrine cells in the gastrointestinal tract and are degraded by the enzyme dipeptidyl peptidase-IV (DPP-IV (10)). Evidence from both human embryonic kidney cells (11), and an isolated rodent intestinal model (12) suggest that the secretion of

these peptides is elevated by stimulation of the extracellular calcium sensing receptor [present in the human gastrointestinal tract (13)] by an elevated extracellular/luminal calcium concentration. Moreover, this effect is potentiated by the presence of amino acids (11, 12). Taken in concert with the observation that milk peptides display DPP-IV inhibitory activity (14), the presence of protein and calcium in a meal may act synergistically to enhance plasma glucose-dependent insulinotropic polypeptide and glucagon-like peptide-1 concentrations. This may in turn, make a contribution to a reduction in appetite and improve energy intake compensation.

Therefore, the primary aim of this study was to assess the effects of protein and calcium in a preload on subsequent compensation of energy intake. Secondary aims were to assess the subjective appetite, and plasma insulin, GIP_{1-42} and $GLP\text{-}1_{7-36}$ responses to the preloads.

PARTICIPANTS AND METHODS

Study design

This study was a double-blind (both investigators and participants were blinded to the intervention), randomized crossover study consisting of 4 main trials, comprised of control (CON), high-calcium (CAL), high-protein (PRO) and high-protein and high-calcium (PROCAL) trials (registered on clinicaltrials.gov as NCT01986036). Each trial was separated by $\geq$ 2 d but $\leq$ 7 d. Trials were conducted in the nutrition and metabolism laboratories of Northumbria University (Newcastle-upon-Tyne, UK) in accordance with the Second Declaration of Helsinki, and following approval from the Northumbria University Faculty of Health and Life Sciences Ethics Committee. Random assignment (www.randomization.com), blinding, and the preparation of preload meals, was performed by PLS Rumbold, who had no further involvement in data acquisition.

132

133 **Participants**

134 A sample size estimation was conducted based on the reported 9.3% difference in *ad libitum*
135 energy intake following a single high-calcium meal vs. a low-calcium meal (6). Given that
136 the day-to-day variation in this measure is 8.9% (15), it was estimated that 16 participants
137 would provide more than an 80% chance of statistically detecting a difference with $P < 0.05$.
138 In order to account for potential dropouts, following informed written consent, 20 participants
139 (12 M, 8 F) were recruited from the Northumbria University student and staff population
140 (characteristics displayed in Table 1) between October 2013 and January 2014. Inclusion
141 criteria included a BMI between 18.5 and 29.9 kg/m^2 and aged 18-40 y. Participants were
142 excluded if they smoked, had any history of food allergies, metabolic disorders such as type 2
143 diabetes or displayed dietary restraint (defined as a score of >13 on the Three-Factor Eating
144 Questionnaire (16)). No direct male-female comparisons were made due to the difference in
145 group sizes, however, for information in the homogeneity of the participants, their
146 characteristics are provided as males alone, females alone, and the total group.

147

148 **Main trials**

149 Participants arrived in the laboratory at 0800 ± 1 h after an overnight fast (10-14 h) and 24 h
150 of physical activity standardization. Participants were asked to refrain from alcohol and
151 caffeine for 24 h, and to record and replicate their evening meal prior to trials. For all female
152 participants, all main trials were carried out during the early follicular phase of the menstrual
153 cycle (3-6 d following the first day of menses). An intravenous catheter was inserted into an
154 antecubital vein and, following a baseline blood sample and visual analogue scale (VAS),
155 participants consumed one of 4 preloads (CON, PRO, CAL, PROCAL). A timer was started
156 when participants consumed the first mouthful of the preload, following which, blood

157 samples and VAS were taken at 15, 30, 45 and 60 min post-preload. Food intake was then
158 assessed (60 min following preload ingestion) by providing participants with a homogenous
159 pasta meal (as previously described (17)), which they were asked to consume until
160 "comfortably full". The mass of food consumed was then converted into energy intake taking
161 into account water losses from reheating. The time frame following the preload was based on
162 our previous findings where appetite sensations following a high-calcium breakfast were
163 divergent within the first 60 min postprandial (7, 8). Participants were initially served a sub-
164 serving of the whole portion, which was augmented at regular intervals. This method
165 prevents the overwhelming sensation of the whole portion of pasta, whilst never allowing the
166 serving bowl to be empty and thus preventing the cessation of the eating occasion due to the
167 end of a "portion".
168
169 **Preloads**
170 All preloads contained instant porridge oats (Oatso Simple Golden Syrup, Quaker Oats UK,
171 Leicester, UK) and water to provide 0.5 g carbohydrate/kg body mass. These were cooked in
172 a microwave for 2 min at 1000 W, prior to a 5-min cooling period before serving. On CAL
173 trials, a milk-extracted calcium powder (Capolac®, Arla Foods Ingredients amba, Denmark;
174 from the same batch that has previously been independently validated (18)) was added to the
175 porridge to increase the calcium content by 15 mg/kg body mass. On PRO trials, milk protein
176 concentrate (MyProtein.co.uk, Northwich, UK) was added to increase the protein content of
177 the porridge by 0.35 g/kg body mass. To test the synergy of protein and calcium, the
178 PROCAL preload comprised of the addition of protein and calcium in identical absolute
179 quantities to PRO and CAL trials (Table 2). The calcium concentration of the drinking water
180 used to make the porridge was determined in duplicate using a photometric technique
181 (Modular P, Roche Diagnostics Ltd., West Sussex, UK). This was determined as 0.82 ± 0.01

mmol/L (given an atomic mass of 40.078 g/mol this equates to 3.27 ± 0.03 mg/dl), and was taken into account in the calcium content of the preloads (Table 2).

Anthropometric variables

Body mass was determined to the nearest 0.1 kg using balance scales (Seca, Birmingham, UK) upon arrival at the laboratory, where participants wore only light clothing. Stature was measured to the nearest 0.1 cm using a stadiometer (Seca, Birmingham, UK).

Subjective ratings

Subjective appetite ratings were assessed using previously validated, 100 mm VAS (19), upon arrival at the laboratory (in the fasted, resting state). Questions asked included: "how hungry do you feel?", "how full do you feel?", "how satisfied do you feel?" and "how much do you think you can eat?". These were also converted into a composite appetite score (which combines hunger, fullness, satisfaction and prospective consumption to provide a single value) as used previously (20).

Blood sampling and analysis

Blood samples were collected into EDTA tubes with 25 μL of aprotinin per mL of whole blood and were immediately centrifuged (10 min, 1509 g, 4°C). Aliquots of plasma were stored at -80°C before analysis. Plasma was analysed for insulin (IBL International GmbH, Hamburg, Germany), GIP_{1-42} (Immuno-Biological Laboratories Co., Ltd, Japan) and $GLP\text{-}1_{7-36}$ concentrations (MesoScale Discovery, Maryland, USA), using commercially-available kits. Samples from all trials for each individual participant were always included on the same plate to minimise variation. Intra-assay coefficients of variation were below 10%.

Statistical analysis

Due to difficulties with blood sampling from one participant, data for all blood variables are *n* = 19. Where data for a single timepoint during a individual's trial was missing [11 points were missing out of a total of 380 (< 3%) for each blood-based variable], the linear interpolation was used to complete the data set. For clarity and to account for the additional energy in the high protein trials (whilst the calcium contained negligible additional energy), energy intake is reported as both absolute values (intake at the test meal only kJ) and energy compensation (%) calculated as follows:

$$\text{Energy compensation} = (EI_{CON}/EI_{EXP}+\Delta EP)*100$$

Where EI represents *ad libitum* energy intake following the control (EI_{CON}) or experimental (EI_{EXP}) preloads and ΔEP represents the additional energy (above control) provided by the experimental preload. Energy compensation was calculated for PRO, CAL and PROCAL trials, with CON as the reference and data for energy compensation are reported at mean ± 95% confidence intervals (95% CI), thus if the 95% CI do not overlap with 100, then there was significant under- or over-compensation.

Plasma variables and subjective ratings were converted into time-averaged postprandial area under the curve (AUC) values. Data are expressed as mean ± standard error of the mean (SEM) for absolute data, whereas 95% confidence intervals (95% CI) are presented for mean differences relative to CON (i.e. PRO-CON, CAL-CON and PROCAL-CON) and were analysed using Prism v5 (GraphPad Software, Dan Diego, CA). Data were checked for normal distribution using the Shapiro-Wilk normality test and were log-transformed if appropriate, prior to statistical analysis. Male vs. female participant characteristics were compared by independent Student's *t* tests. Two-way (trial x time) repeated-measures ANOVA were used to detect differences between plasma and appetite variables over time. A one-way ANOVA was used to detect differences between all trials

(CON vs. PRO vs. CAL vs. PROCAL) in energy intake, energy compensation, AUC data and to compare the mean differences of each trial relative to the control trial (PRO-CON vs. CAL-CON vs. PROCAL-CON). After a significant effect, post-hoc tests, adjusted for multiple comparisons (Holm-Sidak) were used to determine the location of variance. Differences were considered significant at $P < 0.05$. Associations between variables [expressed as the change relative to the CON trial (ΔCON)] were assessed by Pearson product-moment correlation coefficients.

RESULTS

Energy intake

Repeated measures ANOVA detected a significant effect for energy intake at the test-meal ($P < 0.05$). Following adjustment for multiple comparisons, energy intake after PROCAL (3419 ± 345 kJ; $P < 0.05$) was significantly less than after CON (4126 ± 395 kJ), but not after PRO (3699 ± 304 kJ; $P > 0.05$) or CAL (3501 ± 253 kJ; $P > 0.05$).

Energy compensation was significantly greater (overcompensation) with CAL vs. PRO (Figure 1; $P < 0.01$) and tended to be greater with PROCAL vs PRO ($P = 0.06$). PRO produced almost perfect compensation (perfect compensation = 100%), whilst participants overcompensated following CAL (Figure 1).

Subjective appetite sensations

Two-way repeated measures ANOVA revealed a significant main effect of time for all subjective appetite variables (all $P < 0.001$). With regards to the composite appetite score, the main effect of trial was not significant ($P > 0.05$). There was however, a significant trial x time interaction effect ($P < 0.05$), whereby, following adjustment for multiple comparisons, PROCAL was lower than CON at 45 min post-preload (Figure 2A).

For all other appetite variables, there was no significant main effect of trial detected (all $P > 0.05$). Hunger, fullness, satisfaction and prospective consumption all displayed significant interaction (trial x time) effects (all $P < 0.05$; Supplemental Figure 1).

Repeated measures ANOVA revealed a significant effect for the composite appetite AUC ($P < 0.05$), whereby PROCAL was lower than CON (Figure 2B). The hunger AUC displayed a significant overall effect ($P < 0.05$), although following adjustment for multiple comparisons, there were no significant difference detected between specific trials (all $P > 0.05$). There was no overall effect for satisfaction or prospective consumption AUC (both $P > 0.05$), although the main effect for fullness AUC approached significance ($P = 0.06$).

When expressed as the change in appetite sensations relative to control (mean difference ± 95% CI; Figure 2C), PRO did not suppress appetite sensations (-3 mm, 95% CI: -8 to 3; $P > 0.05$), whereas the reduction with CAL vs. CON (-5 mm, 95% CI: -9 to 0; $P = 0.06$) approached significance, and PROCAL significantly reduced the composite appetite AUC relative to CON (-5 mm, 95% CI: -10 to -1; $P = 0.023$). However, no significant differences were observed between PRO-CON vs. CAL-CON vs. PROCAL-CON (main effect: $P > 0.05$).

Plasma variables

Plasma insulin concentrations displayed a main effect of trial ($P < 0.01$) and a main effect of time ($P < 0.001$), with no significant interaction (trial x time) effect ($P > 0.05$; Figure 3A). Plasma GIP_{1-42} concentrations also demonstrated a main effect of trial ($P < 0.01$) and a main effect of time ($P < 0.001$), with no significant interaction effect detected ($P > 0.05$; Figure 3B). Likewise, plasma $GLP\text{-}1_{7-36}$ concentrations displayed main effects of trial ($P < 0.001$) and time ($P < 0.001$) with no significant interaction effect ($P > 0.05$; Figure 3C).

Repeated measures ANOVA revealed a significant overall effect for insulin, GIP_{1-42} and GLP-1_{7-36} AUC ($P < 0.01$, $P < 0.05$ and $P < 0.001$, respectively). Following adjustment for multiple comparisons, the insulin AUC was higher with PROCAL vs. CON (Supplemental Figure 3A). The GIP_{1-42} AUC was not significantly different between each trial (Supplemental Figure 3B), whilst the GLP-1_{7-36} AUC was higher with PRO and PROCAL vs. CON (Supplemental Figure 3C).

There were no differences between PRO, CAL and PROCAL in the change in insulin AUC relative to CON (Figure 4A), however, PRO and PROCAL produced significantly more positive changes relative to CON, when compared to CAL (Figure 4B and 4C).

Associations between variables

The only correlations that were statistically significant were for the ΔCON composite appetite score AUC vs. ΔCON energy intake ($r = 0.37$, $P < 0.05$; Supplemental Figure 2A), ΔCON plasma GIP_{1-42} AUC vs. ΔCON plasma GLP-1_{7-36} AUC ($r = 0.46$, $P < 0.001$; Supplemental Figure 2B) and ΔCON composite appetite score AUC and ΔCON plasma GLP-1_{7-36} AUC ($r = -0.35$, $P < 0.05$; Supplemental Figure 2C). Estimated habitual calcium intake (range: 253-2700 mg/d; median: 973 mg/d) did not correlate with either ΔCON plasma GIP_{1-42} AUC or ΔCON plasma GLP-1_{7-36} AUC ($r = -0.04$, $P > 0.05$ and $r = -0.02$, $P > 0.05$, respectively).

DISCUSSION

Here we demonstrate that a high-protein preload produces almost perfect energy compensation, whilst a high-calcium preload (with and without protein) reduces appetite and results in overcompensation of subsequent energy intake (i.e. less energy intake relative to

the energy in the preload). This coincided with an elevation in insulinaemia, which could not be clearly attributed to responses of the incretin hormones GIP_{1-42} and $GLP\text{-}1_{7-36}$.

Previous evidence has suggested that dietary calcium may play a role in appetite control (6). However, the self-report nature of the measures used, combined with contradictory evidence (9, 21), make this somewhat equivocal. The data in the present study, acquired from a laboratory setting suggest that calcium, has the potential to acutely reduce postprandial appetite sensations and subsequent energy intake to a sufficient degree to offset any additional energy provided by the preload. Energy compensation was almost perfect (i.e. ~100%) in the PRO trial, whereas significant overcompensation occurred with CAL and tended to occur with PROCAL (Figure 1B). These data are consistent with the subjective appetite responses observed (Figure 2C), whereby PROCAL lowered appetite relative to control and CAL tended to lower appetite, relative to CON.

The lack of any detectable increase in incretin hormone concentrations with protein-calcium co-ingestion could be due to either the habitual calcium intake of the participants, or the blood-sampling site. A double-blind, placebo-controlled study has demonstrated that 3 weeks of calcium supplementation (1000 mg/d) results in a potentiation in postprandial plasma $GLP\text{-}1_{7-36}$ concentrations in response to a high-calcium meal, relative to a low-calcium control meal (22). This effect was not seen after 3 weeks of placebo supplementation. Therefore, a high-habitual calcium intake may be required to observe an acute effect of calcium intake on plasma incretin hormones. We attempted to explore this in the present study by examining the association between self-reported habitual calcium intake and the change in plasma incretin concentrations with PROCAL vs. CON. No significant correlation was observed between either GIP_{1-42} or $GLP\text{-}1_{7-36}$, and habitual calcium intake. Although the limitations associated with food frequency questionnaires make it difficult to draw firm conclusions from these observations.

With regard to the sampling site, veins in the antecubital fossa may not provide a representation of the major site of action. As previously mentioned, GIP_{1-42} and $GLP\text{-}1_{7-36}$ are secreted by enteroendocrine cells in the gastrointestinal tract. DPP-IV in the endothelium acts immediately, reducing the quantity of $GLP\text{-}1_{7-36}$ entering the hepatic circulation by approximately 75% from that which is originally secreted (10). Upon passing through the liver, degredation leaves 10-15% to enter the systemic circulation (10), where further degredation by DPP-IV in plasma and secreted by adipose tissue can take place (23). It is postulated that $GLP\text{-}1_{7-36}$ may be able to activate neurons in the intestine and liver (10), which permits central effects (on appetite and insulin secretion) independent of the systemic circulating concentration. Thus, to what degree the concentration measured in an antecubital vein reflects that in the enterocyte and hepatoportal region, which may be the sites of most interest, is unclear.

In addition, it should also be acknowledged that numerous other putative mechanisms may also contribute to the appetite effects of protein and calcium intake, including delayed gastric emptying (24), plasma amino acid concentrations (25), and the concentrations of other other gastrointestinal hormones such as cholecystokinin (26), peptide YY (12) and gastrin (27). Notwithstanding this, we chose to concentrate on the incretin hormones given the insulin responses previously observed in humans (7, 8, 18) and *in vitro*/*ex vivo* [11, 12].

The design and timing of the preload prior to energy intake assessment (1 h), was chosen based on previous observations that calcium intake displays a time-dependent suppressant effect on appetite sensations in this period (7, 8) and also due to this time period typically producing close to 100% compensation with preload designs (28) and is validated somewhat by the almost 100% compensation seen in the PRO trial. This does however, constrain the applicability of the findings to this time period, and extrapolation to longer time periods are not recommended without further research. In addition, the quantity of calcium

and provided in preloads is equivalent to ~800 ml milk. Therefore the practical application of these findings currently lies in fortification, rather than with normal milk composition. Nonetheless, this does provide a proof-of-principle and may be used to augment the satiety effects of pre-meal high-protein snacks (29, 30) and a dose-response study would be a logical progression. The primary outcome was determined as energy intake at the test-meal, however, PRO and PROCAL preloads also contained additional energy (Table 2), which means that any subsequent reduction in energy intake should be interpreted as appropriate energy compensation rather than a reduction *per se*.

In conclusion, the consumption on a preload containing additional protein results in almost perfect energy compensation, whilst the addition of calcium, with or without protein, suppresses appetite and energy intake such that overcompensation ensues with no apparent protein-calcium synergy. It remains unclear whether these responses are attributable to changes in plasma insulin, $GIP_{1\text{-}42}$ or $GLP\text{-}1_{7\text{-}36}$ concentrations.

ACKNOWLEDGEMENTS

The authors' responsibilities were as follows - JTG, BPG, PLRS and LAT designed the research; JTG, BPG, MB, LAT and PLRS conducted the research; EJS provided essential materials; JTG analysed the data and wrote the paper; JTG had primary responsibility for final content. All authors have read and approved the final manuscript.

REFERENCES

1. Tidwell DK, Valliant MW. Higher amounts of body fat are associated with inadequate intakes of calcium and vitamin D in African American women. Nutr Res 2011;31(7):527-36.
2. Onakpoya IJ, Perry R, Zhang J, Ernst E. Efficacy of calcium supplementation for management of overweight and obesity: systematic review of randomized clinical trials. Nutr Rev 2011;69(6):335-43.
3. Christensen R, Lorenzen JK, Svith CR, Bartels EM, Melanson EL, Saris WH, Tremblay A, Astrup A. Effect of calcium from dairy and dietary supplements on faecal fat excretion: a meta-analysis of randomized controlled trials. Obes Rev 2009;10(4):475-86.
4. Gonzalez JT, Rumbold PL, Stevenson EJ. Effect of calcium intake on fat oxidation in adults: a meta-analysis of randomized, controlled trials. Obes Rev 2012;13(10):848-57.
5. Gonzalez JT, Stevenson EJ. New perspectives on nutritional interventions to augment lipid utilisation during exercise. Br J Nutr 2012;107(3):339-49.
6. Ping-Delfos WC, Soares M. Diet induced thermogenesis, fat oxidation and food intake following sequential meals: influence of calcium and vitamin D. Clin Nutr 2011;30(3):376-83.
7. Gonzalez JT, Rumbold PLS, Stevenson EJ. Appetite sensations at rest, during exercise and recovery: impact of a high-calcium meal. Appl Physiol Nutr Metab 2013;38(12):1260-7.
8. Gonzalez JT, Stevenson EJ. Calcium co-ingestion augments postprandial glucose-dependent insulinotropic peptide1-42, glucagon-like peptide-1 and insulin concentrations in humans. Eur J Nutr 2014;53(2):375-85.

9. Lorenzen JK, Nielsen S, Holst JJ, Tetens I, Rehfeld JF, Astrup A. Effect of dairy calcium or supplementary calcium intake on postprandial fat metabolism, appetite, and subsequent energy intake. Am J Clin Nutr 2007;85(3):678-87.

10. Holst JJ. The physiology of glucagon-like peptide 1. Physiol Rev 2007;87(4):1409-39.

11. Conigrave AD, Quinn SJ, Brown EM. L-amino acid sensing by the extracellular Ca2+-sensing receptor. Proceedings of the National Academy of Sciences of the United States of America 2000;97(9):4814-9.

12. Mace OJ, Schindler M, Patel S. The regulation of K- and L-cell activity by GLUT2 and the calcium-sensing receptor CasR in rat small intestine. J Physiol 2012;590(Pt 12):2917-36.

13. Sheinin Y, Kallay E, Wrba F, Kriwanek S, Peterlik M, Cross HS. Immunocytochemical localization of the extracellular calcium-sensing receptor in normal and malignant human large intestinal mucosa. The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society 2000;48(5):595-602.

14. Nongonierma AB, FitzGerald RJ. Susceptibility of milk protein-derived peptides to dipeptidyl peptidase IV (DPP-IV) hydrolysis. Food chemistry 2014;145:845-52.

15. Gregersen NT, Flint A, Bitz C, Blundell JE, Raben A, Astrup A. Reproducibility and power of ad libitum energy intake assessed by repeated single meals. Am J Clin Nutr 2008;87(5):1277-81.

16. Stunkard AJ, Messick S. The three-factor eating questionnaire to measure dietary restraint, disinhibition and hunger. J Psychosom Res 1985;29(1):71-83.

17. Gonzalez JT, Veasey RC, Rumbold PL, Stevenson EJ. Breakfast and exercise contingently affect postprandial metabolism and energy balance in physically active males. Br J Nutr 2013;110(4):721-32.

18. Gonzalez JT, Green BP, Campbell MC, Rumbold PL, Stevenson EJ. The influence of calcium supplementation on substrate metabolism during exercise in humans: a randomized controlled trial. Eur J Clin Nutr 2014;68(6):712-8.

19. Flint A, Raben A, Blundell JE, Astrup A. Reproducibility, power and validity of visual analogue scales in assessment of appetite sensations in single test meal studies. International Journal of Obesity and Related Metabolic Disorders 2000;24(1):38-48.

20. Anderson GH, Catherine NL, Woodend DM, Wolever TM. Inverse association between the effect of carbohydrates on blood glucose and subsequent short-term food intake in young men. Am J Clin Nutr 2002;76(5):1023-30.

21. Soerensen KV, Thorning TK, Astrup A, Kristensen M, Lorenzen JK. Effect of dairy calcium from cheese and milk on fecal fat excretion, blood lipids, and appetite in young men. Am J Clin Nutr 2014;99(5):984-91.

22. Trautvetter U, Jahreis G. Effect of supplementary calcium phosphate on plasma gastrointestinal hormones in a double-blind, placebo-controlled, cross-over human study. Br J Nutr 2013:1-7.

23. Lamers D, Famulla S, Wronkowitz N, Hartwig S, Lehr S, Ouwens DM, Eckardt K, Kaufman JM, Ryden M, Muller S, et al. Dipeptidyl peptidase 4 is a novel adipokine potentially linking obesity to the metabolic syndrome. Diabetes 2011;60(7):1917-25.

24. Shafer RB, Levine AS, Marlette JM, Morley JE. Do calories, osmolality, or calcium determine gastric emptying? The American journal of physiology 1985;248(4 Pt 2):R479-83.

25. Jordi J, Herzog B, Camargo SM, Boyle CN, Lutz TA, Verrey F. Specific amino acids inhibit food intake via the area postrema or vagal afferents. J Physiol 2013;591(Pt 22):5611-21.

26. Nakajima S, Hira T, Hara H. Calcium-sensing receptor mediates dietary peptide-induced CCK secretion in enteroendocrine STC-1 cells. Molecular nutrition & food research 2012;56(5):753-60.

27. Behar J, Hitchings M, Smyth RD. Calcium stimulation of gastrin and gastric acid secretion: effect of small doses of calcium carbonate. Gut 1977;18(6):442-8.

28. Almiron-Roig E, Palla L, Guest K, Ricchiuti C, Vint N, Jebb SA, Drewnowski A. Factors that determine energy compensation: a systematic review of preload studies. Nutr Rev 2013;71(7):458-73.

29. Astbury NM, Taylor MA, French SJ, Macdonald IA. Snacks containing whey protein and polydextrose induce a sustained reduction in daily energy intake over 2 wk under free-living conditions. Am J Clin Nutr 2014;99(5):1131-40.

30. Akhavan T, Luhovyy BL, Brown PH, Cho CE, Anderson GH. Effect of premeal consumption of whey protein and its hydrolysate on food intake and postmeal glycemia and insulin responses in young adults. Am J Clin Nutr 2010;91(4):966-75.

Table 1 Participant characteristics and fasting plasma variables[1]

	Total (n = 20)	Males (n = 13)	Females (n = 7)	P value[2]
Characteristics				
Age (y)	23 ± 1	24 ± 1	22 ± 1	0.15
Body mass (kg)	71.0 ± 2.4	77.4 ± 1.7	59.0 ± 2.4	< 0.001
Height (cm)	175 ± 2	180 ± 2	164 ± 2	< 0.001
BMI (kg/m^2)	23.2 ± 0.6	23.9 ± 0.7	21.9 ± 1.1	0.11
Habitual calcium intake (mg/d)	1000 ± 126	1080 ± 169	855 ± 180	0.41
Fasting plasma variables[3,4]				
Insulin (pmol/L)	91 ± 8	79 ± 9	112 ± 14	0.049
GIP_{1-42} (pmol/L)[5]	2.1 ± 0.3	2.4 ± 0.4	1.7 ± 0.3	0.25
GLP-1_{7-36} (pmol/L)[5]	0.41 ± 0.08	1.58 ± 0.40	0.99 ± 0.28	0.32

[1]All values are means ± SEM.

[2]Male vs female, compared by independent Student's t test.

[3]Mean of 4 visits.

[4]For blood variables n = 12 for males, and 7 for females.

[5]GIP_{1-42}, glucose-dependent insulinotropic polypeptide$_{1-42}$; GLP-1_{7-36}, glucagon-like peptide-1_{7-36}.

Table 2 Nutritional composition of the preloads[1,2]

	CON	PRO	CAL	PROCAL
Energy (kJ)	773 ± 27	1244 ± 43	783 ± 27	1253 ± 43
Energy (kcal)	185 ± 6	297 ± 10	187 ± 6	299 ± 10
Carbohydrate (g)	36 ± 1	37 ± 1	36 ± 1	38 ± 1
Fat (g)	3 ± 0	4 ± 0	4 ± 0	4 ± 0
Protein (g)	4 ± 0	29 ± 1	5 ± 0	29 ± 1
Calcium (mg)	104 ± 4	104 ± 4	1170 ± 40	1170 ± 40
Energy Density (kJ/g)	2.1 ± 0.0	3.1 ± 0.0	2.1 ± 0.0	3.1 ± 0.0

[1]All values are means ± SEM.

[2]CAL, high-calcium; CON, control; PRO, high-protein;; PROCAL, high-protein and high-calcium.

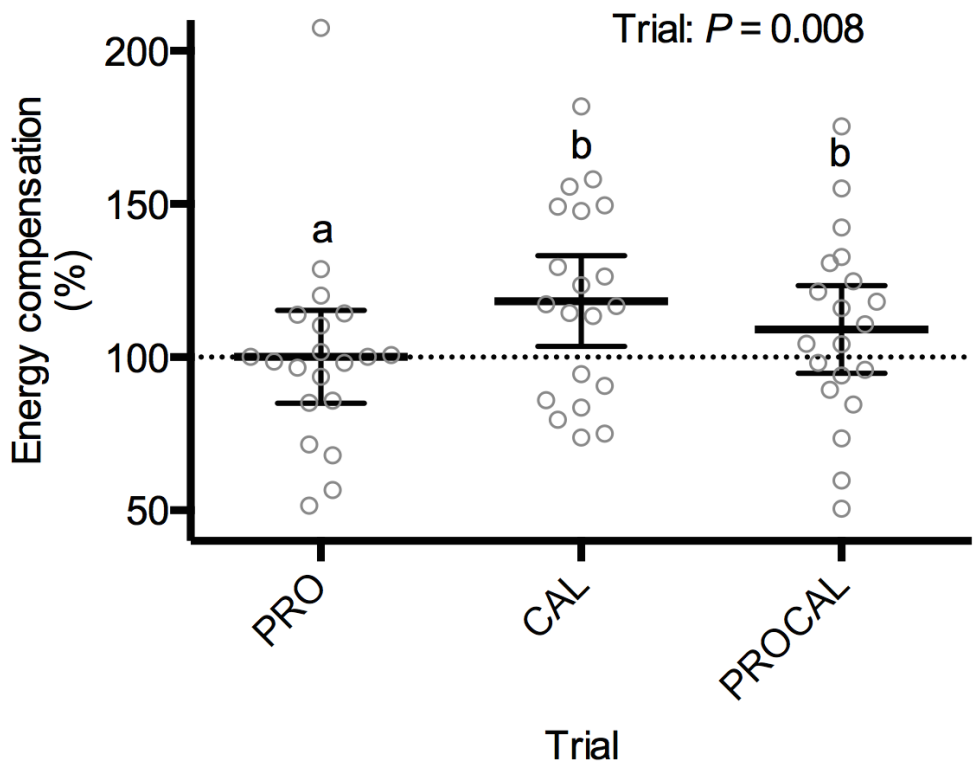


FIGURE 1. Energy compensation (%) during an *ad libitum* test meal 1 h following CON, PRO, CAL, or PROCAL preloads, in humans. Values are individual differences (circles) and means ± 95% confidence intervals (horizontal lines); $n = 20$. Labelled means without a common letter differ ($P < 0.05$). CAL, high-calcium; CON, control; PRO, high-protein; PROCAL, high-protein and high-calcium.

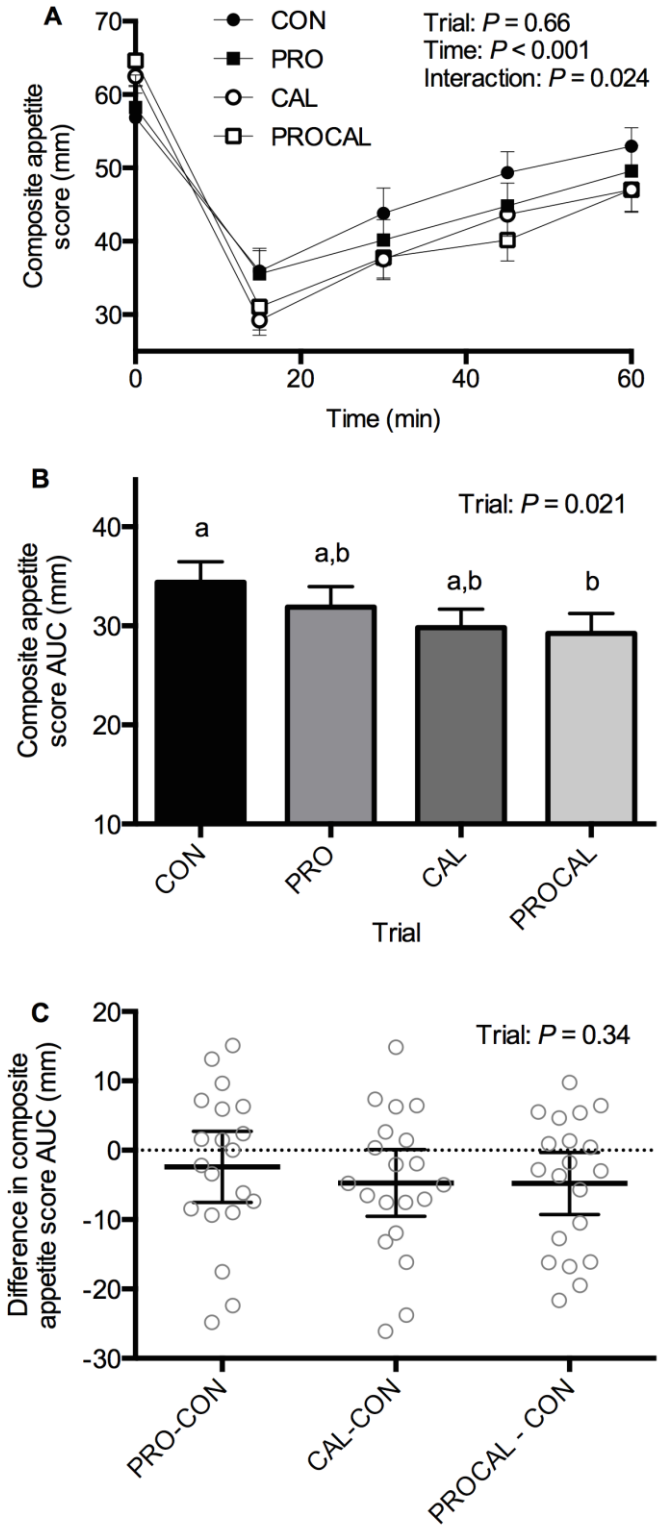


FIGURE 2. The composite appetite score following CON), PRO, CAL, or PROCAL preloads, in humans expressed over time (A), as a postprandial time-averaged (60 min) area under the curve (AUC; B) or as the mean difference ± 95% confidence intervals (horizontal lines; circles are individual data) between PRO, CAL and PROCAL, relative to CON (C); n = 20. Values in A and B are means ± SEM. CON, control; PRO, high-protein; CAL, high-calcium; PROCAL, high-protein and high-calcium; Labelled means without a common letter differ ($P < 0.05$). CAL, high-calcium; CON, control; PRO, high-protein; PROCAL, high-protein and high-calcium.

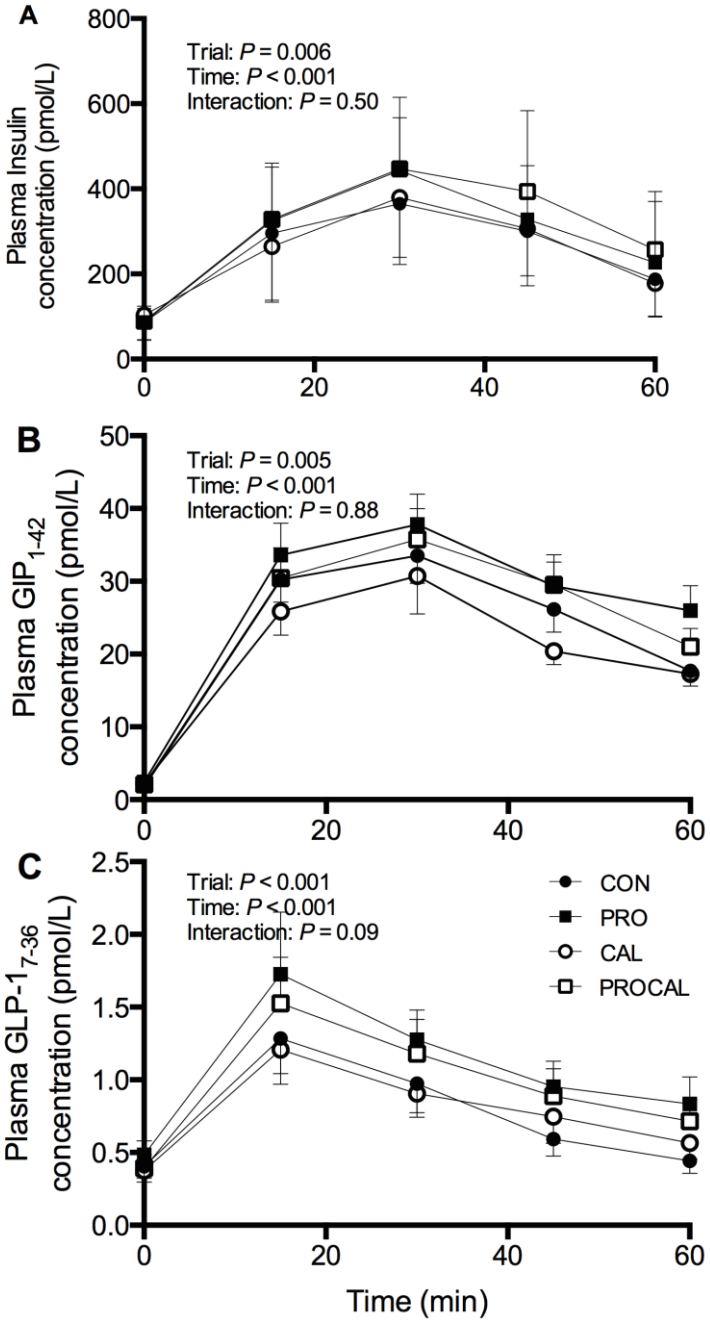


FIGURE 3. Plasma insulin (A), GIP_{1-42}, and $GLP\text{-}1_{7-36}$ concentrations following CON, PRO, CAL, or PROCAL preloads, in humans. Values are means ± SEM; *n* = 19. CAL, high-calcium; CON, control; GIP_{1-42}, glucose-dependent insulinotropic polypeptide$_{1-42}$; $GLP\text{-}1_{7-36}$, glucagon-like peptide-1_{7-36}; PRO, high-protein; PROCAL, high-protein and high-calcium.

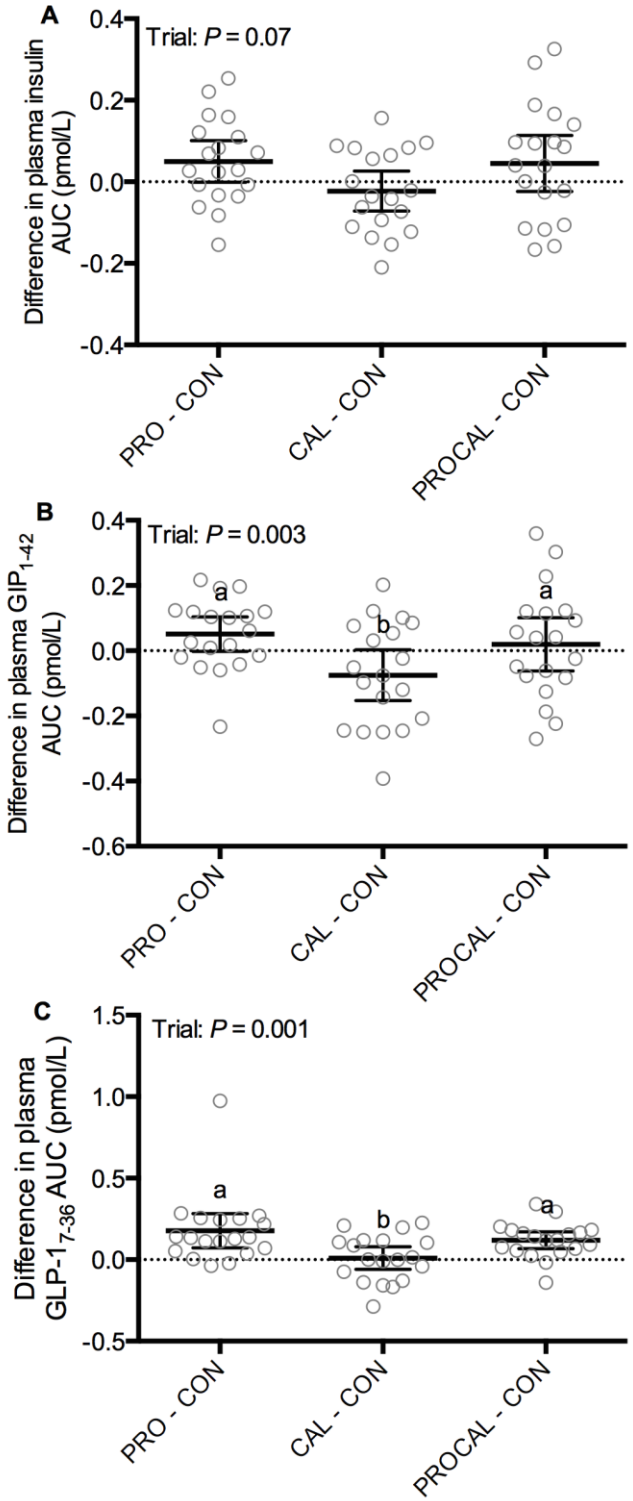
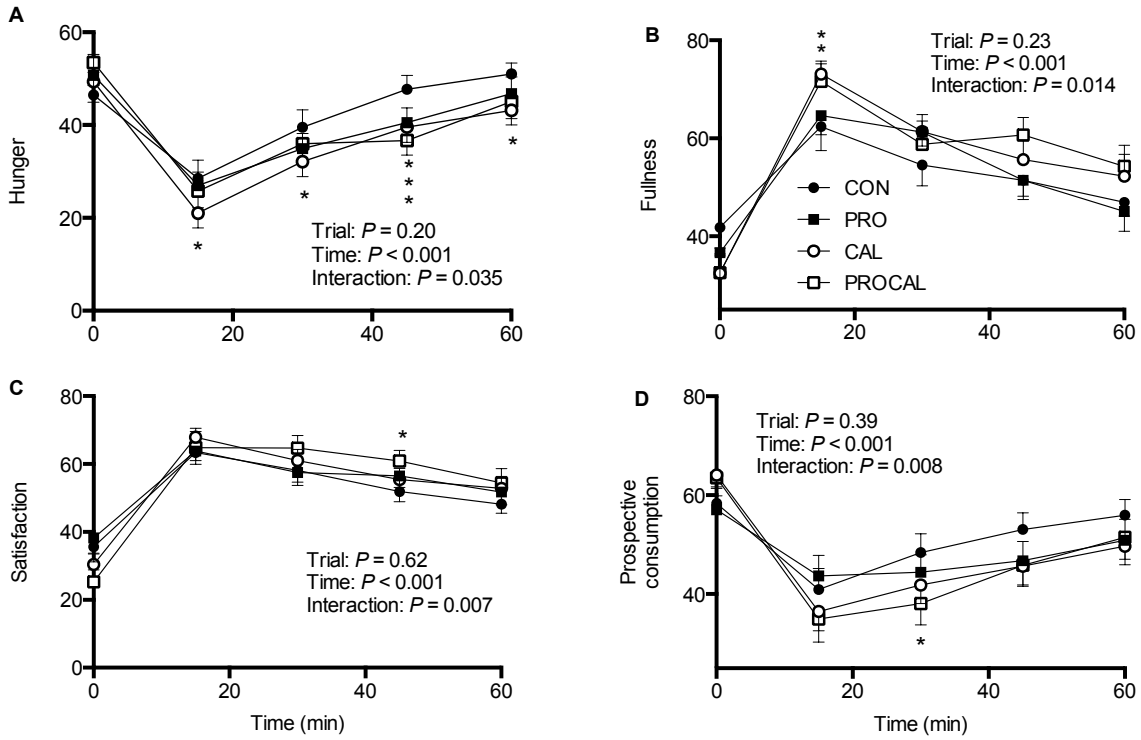


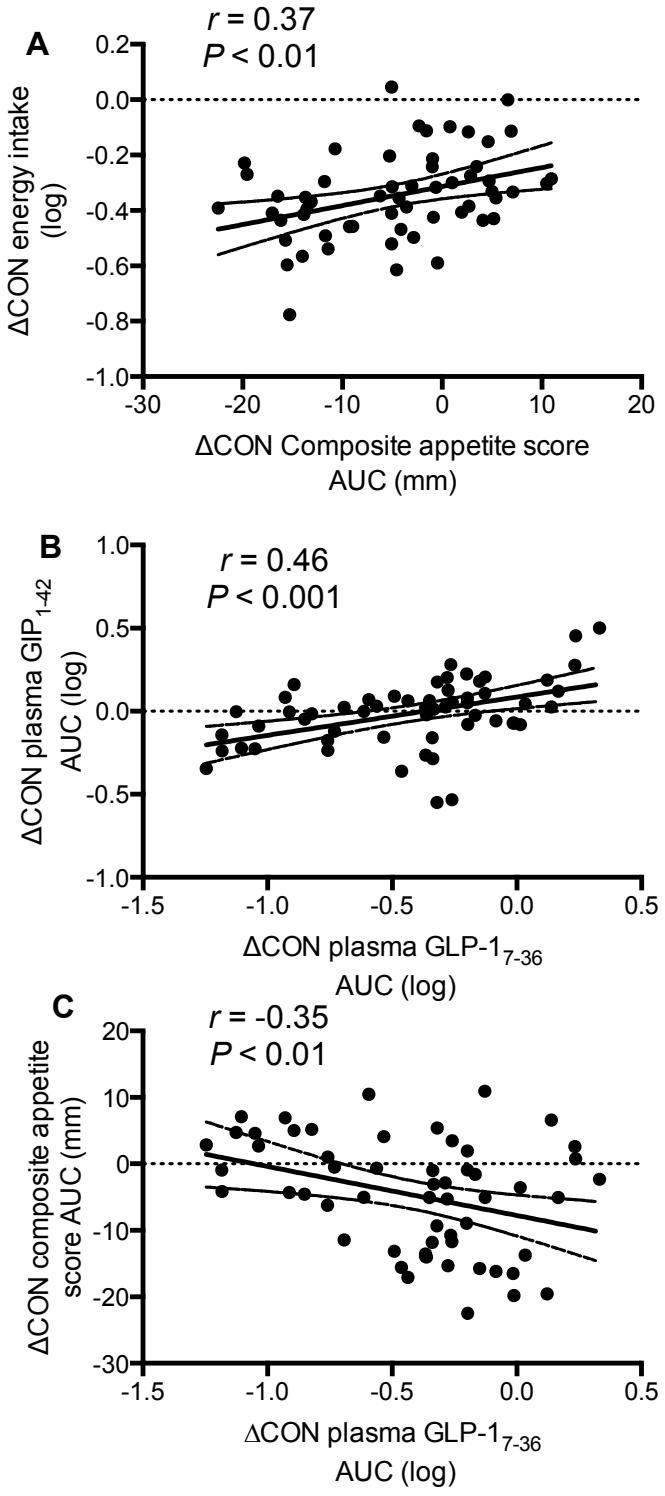
FIGURE 4. Plasma insulin (A), $GIP_{1\text{-}42}$, and $GLP\text{-}1_{7\text{-}36}$ postprandial time-averaged (60 min) area under the curve (AUC) following CON, PRO, CAL, or PROCAL preloads, in humans. Values are individual differences (circles) and mean difference ± 95% confidence intervals (horizontal lines) between PRO, CAL and PROCAL, relative to CON; $n = 19$. Labelled means without a common letter differ ($P < 0.05$). CAL, high-calcium; CON, control; $GIP_{1\text{-}42}$, glucose-dependent insulinotropic polypeptide$_{1\text{-}42}$; $GLP\text{-}1_{7\text{-}36}$, glucagon-like peptide-$1_{7\text{-}36}$; PRO, high-protein; PROCAL, high-protein and high-calcium.

ONLINE SUPPORTING MATERIAL



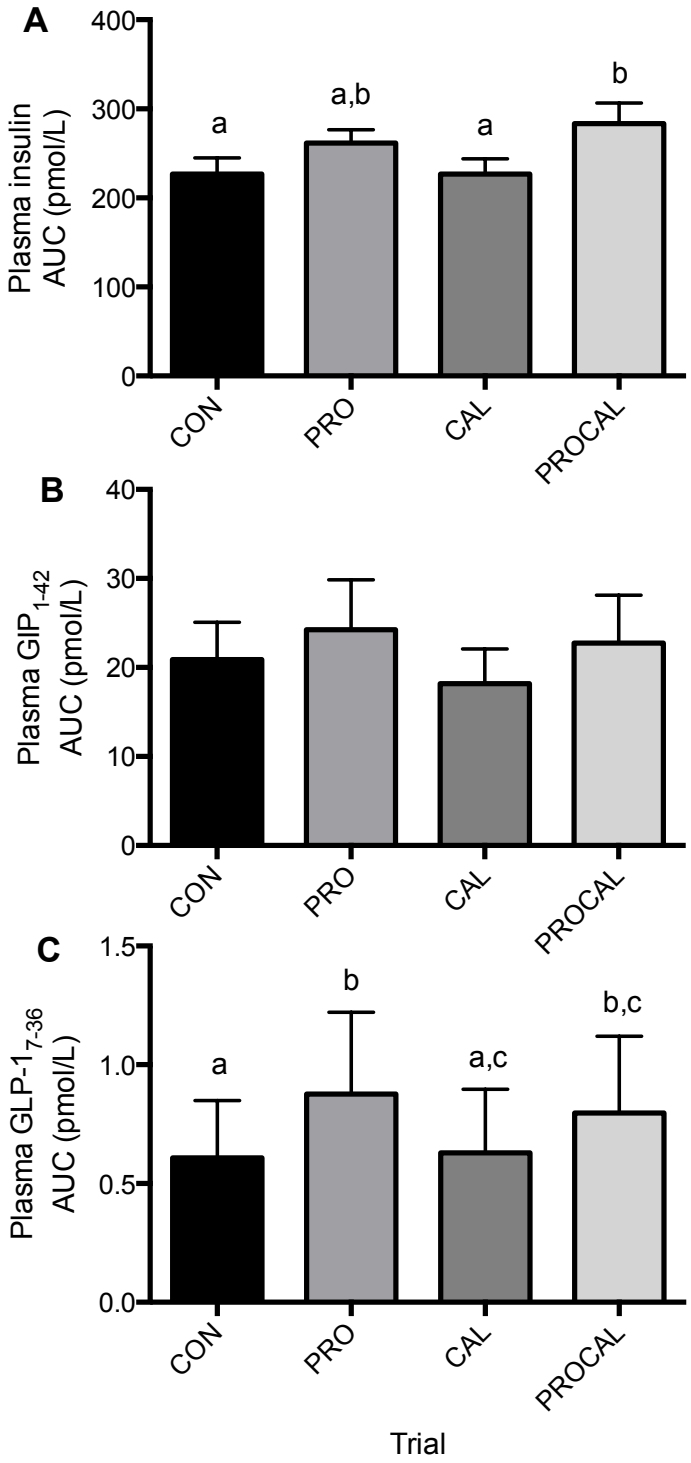
SUPPLEMENTAL FIGURE 1. Hunger (A), fullness (B), satisfaction (C) and prospective consumption (D) ratings following control (CON), high-protein (PRO), high-calcium (CAL), or high-protein and high-calcium (PROCAL) preloads, in humans. Values are means ± SEM; *n* = 20. *Different from CON (*P* < 0.05).

ONLINE SUPPORTING MATERIAL



SUPPLEMENTAL FIGURE 2. Correlations between the composite appetite score postprandial area under the curve (AUC) and energy intake (A), plasma GLP-17-36 AUC and GIP1-42 AUC (B), and GLP-17-36 AUC and the composite appetite score AUC. Data are expressed as the difference from the control trial (ΔCON); *n* = 20 for A, *n* = 19 for B and C. GIP_{1-42}, glucose-dependent insulinotropic polypeptide$_{1-42}$; GLP-1_{7-36}, glucagon-like peptide-1_{7-36}.

ONLINE SUPPORTING MATERIAL



SUPPLEMENTAL FIGURE 3. Plasma insulin (A), $GIP_{1\text{-}42}$, and $GLP\text{-}1_{7\text{-}36}$ postprandial time-averaged (60 min) area under the curve (AUC following control (CON), high-protein (PRO), high-calcium (CAL), or high-protein and high-calcium (PROCAL) preloads, in humans. Values are means ± SEM; n = 19. GIP_{1}-42, glucose-dependent insulinotropic $polypeptide_{1\text{-}42}$; $GLP\text{-}1_{7\text{-}36}$, glucagon-like peptide-$1_{7\text{-}36}$; Labelled means ($P < 0.05$).