Body weight loss, effective satiation and absence of homeostatic neuropeptide compensation in male Sprague Dawley rats schedule fed a protein crosslinked diet.

Nikki Cassie, Richard L. Anderson, Dana Wilson, Anne Pawsey[#], Julian G. Mercer and Perry Barrett*

Rowett Institute, University of Aberdeen, Foresterhill, Aberdeen AB25 2ZD

[#]Dept of Physics and Astronomy, University of Edinburgh EH9 3FD

*Corresponding author

Dr Perry Barrett

Rowett Institute

University of Aberdeen

Foresterhill.

Aberdeen AB25 2ZD

Keywords: food reformulation, satiety, satiation, transglutaminase

Abstract

Food structure contributes to the induction of satiation and the maintenance of satiety following intake of a meal. There is evidence from human studies that protein-crosslinking of a milk-protein based meal may enhance satiety, but the mechanism underpinning this effect is unknown. We investigated whether a rat model would respond in a similar manner and might provide mechanistic insight into enhanced satiety by structural modification of a food source. Rats were schedule fed a modified AIN-93M based diet in a liquid form or protein-crosslinked to produce a soft-solid form. This was compared to a modified AIN-93M solid diet. Average daily caloric intake was in the order solid > liquid > crosslinked. Body composition was unaltered in the solid group, but there was a loss of fat in the liquid group and a loss of lean and fat tissue in the crosslinked group. Compared to rats fed a solid diet, acute responses in circulating GLP-1, leptin and insulin were eliminated or attenuated in rats fed a liquid or crosslinked diet. Quantification of homeostatic neuropeptide expression in the hypothalamus showed elevated levels of *Npy* and *Agrp* in rats fed the liquid diet. Measurement of food intake after a scheduled meal indicated that reduced energy intake of liquid and crosslinked diets is not due to enhancement of satiety. When continuously available ad-libitum, rats fed a liquid diet showed reduced weight gain despite greater 24h caloric intake. During the dark phase, caloric intake was reduced, but compensated for during the light phase. We conclude that structural modification from a liquid to a solidified state is beneficial for satiation, with less of a detrimental effect on metabolic parameters and homeostatic neuropeptides.

Introduction

Both human and rat studies show that satiation and satiety are a complex integration of the characteristics of food, including form, volume, caloric and macronutrient content (Gerstein 2004, Westerterp-Plantenga et al. 2004, Marmonier & Sylvestre 2000, Phillips & Powley 1996, Rolls et al. 1998). Evidence from human studies shows that caloric intake and subsequent satiety properties of a meal are directly influenced by the structural and textural characteristics of the food presented which can have effects on hormonal profiles, metabolic responses and appetite ratings (Martens et al. 2011, 2012, Mattes 2008, Mattes & Rothacker 2001, Moukarzel & Sabri 1996 Wilkinson et al. 2001).

There are various ways that food structure and texture can be altered for different requirements. Although the percentage content and amino acid composition of protein in a diet can be varied, dietary protein also provides an opportunity to manipulate the physical characteristics of food, for example following enzymatic crosslinking (Buchert et al. 2010). Several enzymes have been employed for protein crosslinking, including the naturally occurring enzyme transglutaminase (TG) (Simpson et al. 2012, Kuraishi et al. 2001). TG can alter the structure and texture of food high in protein by increasing viscosity and causing gelation (Buchert et al. 2010, Kuraishi et al. 2001). Although the functional characteristics of most foods that are high in protein can be altered by TG, casein is optimal for protein crosslinking due to its open structure (Bönisch et al. 2004, Lorenzen et al. 1998). TG is used in various forms in the food industry to alter the characteristics of food, for example in the processing of meat and fish products (Kuraishi et al. 2001, Motoki et al. 1998, Yokoyama et al. 2004), in baking (Autio et al. 2005), and in dairy produce (Jaros et al. 2006).

Recently emerging evidence has shown that crosslinking dietary protein to modify texture and structure without detriment to nutritional quality may have beneficial effects on human metabolic profile and appetite ratings (Juvonen et al. 2011, 2012). However, the mechanism underpinning this response is unknown, although there is some evidence to suggest that crosslinking proteins with TG increases the resistance of proteins to enzymatic breakdown (Lorenzen et al. 1998, Flanagan et al. 2003, Monogioudi et al. 2011) which could delay digestion and increases transit time. Other evidence suggests that TG crosslinked diet has an effect on the upper gastrointestinal (GI) tract, modulating the digestive process through physical means such as gelation and aggregation in the stomach (Kong et al. 2008, Juvonen 2009, Kristensen & Jensen 2011), which can then trigger a cascade of hormonal responses.

Currently there is no information on the effect that crosslinking has on the appetite regulatory regions of the brain. One key area is the arcuate nucleus (ARC) in the hypothalamus of the forebrain which harbours two important populations of neurons pertinent to the homeostatic

regulation of food intake. These are the orexigenic neurons that express neuropeptide Y (NPY) and agouti-related peptide (AGRP) and the anorexigenic neurons that express cocaine and amphetamine regulated transcript (CART) and the product of pro-opiomelanocortin (POMC) cleavage, α-melanocyte-stimulating hormone (α-MSH) (Coll, Farooqi & O'Rahilly 2007, Morton et al. 2006).

Protein crosslinking to enhance satiation or satiety without increasing energy density and macronutrient content could have numerous applications in assisting an overall objective of decreasing energy intake. The aim of this study was to utilise TG to crosslink protein in a casein supplemented rodent diet (AIN-93M) without the exclusion of water, providing a direct comparison of a solid and liquid form without changing volume or energy density. In using the Sprague Dawley rat model we allow for a mechanistic exploration of how the crosslinking of dietary protein affects food intake, body weight, hormonal profile and neuropeptide levels in the ARC. To achieve this we have modified a schedule feeding protocol (Johnstone et al 2006) which facilitates immediate food consumption on presentation of food, thus enabling gene expression and metabolic parameters to be determined relative to food intake.

Materials and Methods

Animals

Male Sprague Dawley rats of 7-8 weeks of age were purchased from Charles River, UK. On arrival, rats were housed on a 12 hour light: 12hour dark cycle, with lights on at 7 am (Zeitgeber (ZT) 0) and lights off at 7pm (ZT12). Rats were acclimatized to an *ad libitum* standard chow diet (Special Diet Services, UK, #871505 CRM (P); 69% carbohydrate, 22% protein, 9% fat by energy, 3.58 kcal/g) for 7 days. The rats were then housed individually with *ad libitum* access to pelleted AIN-93M (Special Diet Services, UK, 75.9% carbohydrate, 14.7% protein, 9.4% fat; 3.77 kcal/g) with water being freely available throughout the study. Average daily caloric intake on AIN-93M was calculated for each individual rat. This value was used to provide the daily (100%) caloric intake for each individual rat during scheduled feeding. On the day prior to the start of the scheduled feeding, the rats were scanned by EchoMRI™, (Echo Medical Systems, Houston, TX, USA) to determine body composition. Animal husbandry and experiments were carried out under a Project Licence approved by the Home Office in accordance with the Animals (Scientific Procedures) Act of 1986. The experiments also received ethical approval from the Rowett Institute Ethics Committee.

Dietary manipulations

AIN-93M diet was also purchased in powdered form for use in the preparation of modified diets. The 'solid' diet consisted of 78% powdered AIN-93M, 18% H_2O, 2% additional casein, 0.8% emulsifier, and 1% inactivated transglutaminase (TG, Activa MP, Ajinomoto foods) and was moulded into a pellet with a caloric value of 2.98 kcal/g. 'Liquid' and 'crosslinked' diets were composed of (per 100g before solubilisation) 55.1% powdered AIN-93M, 43.1% casein, 0.8% emulsifier (Grinsted® SSL P 55 VEG, Danisco Nutrition & Health,UK) and 1% inactivated or active TG. The mixture (minus TG) was dissolved in water, then for gelation, the TG enzyme was added to the solubilized constituents and the mixture was allowed to crosslink at 4℃ for 16h. For the liquid diet, heat inactivated TG was added to maintain proportional content of constituents and protein. The caloric value of the diet was 1kcal/ml. After an overnight incubation with TG at 4 °C, the diet solidifies with little or no change in volume resulting in a semi-rigid gel. Evidence of crosslinking by active TG and the absence of crosslinking with inactivated TG in the liquid diet were assessed by testing the viscosity at 20°C with a stress controlled rotational rheometer (AR-G2, TA instruments, UK). The steady state viscosity was measured in duplicate with an increasing stress range, which resulted in Shear rates from 0.01 to 30 s^{-1} (Figure 1A). The test product crosslinked with the active TG showed a higher viscosity than the liquid with the inactivated TG and could therefore be considered to be solidified to a semi-rigid gel.

Study 1: Response to schedule fed experimental diets

The protocol is outlined in Figure 1B (study 1). The scheduled feeding program involved limiting access to food to two periods of food availability, both during the dark phase, such that the consumption of food is well co-ordinated between all rats once access is restored. In Study 1, groups received one of the 3 individual diets (solid, liquid or crosslinked). Each rat received 100% of ad-libitum intake. This was calculated for each individual rat from their daily intake over 12 days of feeding stock AIN-93M pellet, which showed little variation over the 12 day period for each group (between day 1 and 12 - 100±1.2%). All food for one day was presented from the start of scheduled feeding at two intervals - ZT13-15 for the first meal with food left over from this meal being made available at ZT20-22 for a second scheduled meal. This paradigm of scheduled feeding facilitated immediate intake on presentation of food whilst maintaining sufficient food intake to prevent weight loss after adaptation to scheduled feeding. Echo MRI scanning for body composition was performed on days 12 and 18. On the final day of the study, the rats were killed either at time 0 minutes (n=7), or had free access to the daily ration of 100% of *ad libitum* energy intake of the specified diet for 90 minutes and before being killed (n=7). Rats were anaesthetised with isoflurane, followed by decapitation. Trunk blood

(approximately 10ml) was collected into 15ml polypropylene tubes containing heparin and 8mM DPP-IV inhibitor (KR-62436 Hydrate, Sigma) and stored on ice until processing for plasma. Brains were removed and frozen on dry ice. Blood was centrifuged at 1000g for 15 minutes at 4°C and the plasma was removed to microfuge tubes and stored as aliquots at -80°C.

Study 2: Effect of diet on early and late phase satiety (Figure 1B)

The test for early phase satiety is based on the hypothesis that if rats are less satiated, the capacity to eat a diet presented will be greater in the period immediately after the test meal has been consumed. Similarly the test for late phase satiety is based on the capacity of the rats to eat a diet presented at the next scheduled meal with a greater degree of satiety resulting in less food consumed at the next scheduled meal. The protocol is outlined in Figure 1b (study 2). To assess the effect of the experimental diets on satiety, a scheduled feeding protocol was established as in study 1 with n=7 rats receiving solid, liquid or protein crosslinked diet. On the eighth day after the implementation of scheduled feeding, the effect of diet on early phase satiety was assessed. For this test, rats received their scheduled meal at ZT13 for 2h. After removal of uneaten food, rats were allowed free access to stock AIN-93M pellets for 1h (ZT15-16). Consumed food was measured by weigh back of uneaten food. At ZT20, rats were offered their uneaten experimental diet for 2h as before. On completion of the early phase satiety test, the following 2 days reverted to the 2 meal scheduled feeding protocol (ZT 13-15 and ZT20-22) with intake permitted to be no more than calculated daily caloric intake of each individual rat. On eleventh day after the implementation of scheduled feeding, the effect of diet on late phase satiety was assessed. For this test rats received their scheduled access to experimental diet at ZT13 for 2h. At the next scheduled meal at ZT20 rats were given free access to stock AIN-93M pellets for 1h with caloric consumption determined by weigh back of uneaten food. This was followed for a further 1 hour at ZT21 by the remainder of their experiment diet not eaten during the first scheduled feed.

Study 3: The effect of unrestricted *ad libitum* access to either solid, liquid or crosslinked experimental diets (Figure 1B)

The protocol is outlined in Figure 1B (study 3). After 7 days acclimatization, rats were fed AIN-93M for 12 days to determine average daily caloric intake. On day 12, body composition was determined by Echo MRI. The rats were then fed with continuous *ad libitum* access to

experimental solid, liquid or crosslinked modified AIN-93M diets (prepared as above) for a further 7 days (n=7 per group). During this time, food intake was determined twice per day for the period between ZT0 and ZT12 (light phase intake) and between ZT12 and ZT24 (dark phase intake). On the final day of the study, body composition was determined by Echo MRI.

Circulating hormones and metabolic parameters

The plasma analysis of glucose, non-esterified fatty acids (NEFAs) and triglycerides was performed using calorimetric assays, in the Konelab 30 instrument (Thermo Fisher Scientific, Basingstoke, UK). GLP-1 and leptin were measured by the Core Biochemical Assay Laboratory, Cambridge using the Meso Scale Discovery Total GLP-1 Kit (K150JVC-1, Gaithersburg, MD, USA) for the measurement of amidated GLP-1 (7-36), and a previously published leptin assay (Franks et al. 2005). Plasma insulin levels were determined using a Millipore rat insulin RIA (RI-3K, Darmstadt, Germany) according to manufacturer's instructions.

Brain gene expression

Brain tissue was cut on a cryostat at a thickness of 20µm, with sections collected on poly D-Lysine coated slides. Gene expression in the appetite regulatory areas of the brain was investigated using established *in situ* hybridization methods and ^{35}S labelled anti-sense riboprobes as described previously (Mercer et al. 1996). Sections containing the arcuate nucleus (ARC) were probed for *Npy, Pomc, Agrp* and *Cart* mRNA expression. Following hybridization, the slides were apposed to autoradiographic film for up to 7 days as appropriate. Quantification was achieved by image analysis using Image Proplus v7.0 software with reference to a ^{14}C microscale exposed to film at the same time as the brain sections.

Statistical analysis

Statistical analysis was performed using SIGMAPLOT 11.0 software (Systat software Chicago, IL, USA) to reveal effects of both time relative to meal and dietary formulation. Food intake and body weight were analysed by One-Way or Two-Way ANOVA as indicated in the text followed by *post-hoc* Holm-Sidak test. For data in Figure 2D, data were log transformed as they did not pass normality and equal variance. Equal variance testing also failed after log transformation and therefore significance/non-significance between groups was confirmed by a t-test allowing for unequal variance. Body composition comparisons were performed using

a paired t-test. Circulating hormones, metabolic parameters and brain neuropeptide gene expression were analysed using Two-Way ANOVA and *post-hoc* Holm-Sidak test. Results are expressed as mean ± SEM. In all analysis, P< 0.05 is considered statistically significant.

Results

Effect of changing food structure by protein crosslinking on food intake and body mass.

Upon introduction of scheduled feeding, food intake adaptation to scheduled meals was evident by a stable intake at both ZT13-15 and ZT20-22 from day 3 onwards (Figure 2A-C). At the end of the 6th day of the scheduled feeding, caloric intake, averaged between day 3-6, at ZT13-15 was significantly different between groups and there was an interaction between time and diet (Two-Way ANOVA P<0.001). Rats fed the liquid diet consumed more calories than rats fed the solid (P<0.018) or the crosslinked diet (P<0.001), and rats fed the solid diet consumed more calories than rats fed the crosslinked diet (P<0.001). Similarly, caloric intake at ZT20-22 was significantly different between groups with rats fed the solid diet consuming more calories than rats fed either the liquid (P<0.001) or crosslinked diets (P<0.001), and rats fed the liquid diet consumed more than rats fed the crosslinked diet (P<0.001). Within diet there was a greater caloric intake of solid diet at ZT20-22 compared to ZT13-15 (63% and 37% respectively; P<0.001), but there was no significant difference between food intake at ZT13-15 and ZT20-22 for either the liquid or crosslinked diets; with both diets, approximately 50% of daily intake was consumed at each scheduled meal. After 6 days on the scheduled feeding programme, the average caloric intake, calculated between days 3 to 6 was solid diet - 95.2±2.1 kcal; liquid diet - 77.2±0.6 kcal; crosslinked - 59.9±0.4 kcal (One-Way ANOVA; P<0.001). The rats that had been fed the solid diet consumed significantly more calories over 2 meals than those consuming either the liquid (P<0.001) or crosslinked diets (P<0.001, Figure 3A), but this level was not significantly different to the daily ad-libitum caloric intake of stock diet, measured over a 12-day period prior to the start of scheduled feeding (97.1±2.6 kcal). The rats receiving the liquid diet consumed more calories than the crosslinked group (P<0.001, Figure 3A) and for both groups this was significantly less than the daily ad-libitum caloric intake of stock diet (P<0.01)

Daily bodyweight measurements were taken prior to and during the scheduled feeding period. On the day prior to implementation of scheduled feeding, there were no differences in body weight between the three groups (Figure 3B). During the scheduled feeding period the body weights of rats in all three groups showed an initial decrease during the period of adaptation, but when adapted to scheduled meals, both solid and liquid diet fed rats increased body

weight. Average daily weight gain over the last 4 days of scheduled feeding, when adaptation to scheduled meals was evident, was not different to the last 4 days before scheduled feeding in rats receiving a solid diet (6.7±0.11g/day vs 7.36±0.48g/day respectively, P=0.23). In rats receiving the liquid diet, although growth was evident, weight gain was lower during scheduled feeding than ad-libitum feeding (3.62±0.26g/day vs 7.46±0.31g/day respectively, P<0.001). However, body weight of rats receiving the crosslinked diet did not increase after the introduction of scheduled feeding (ad-libitum fed 6.98±0.3g/day, scheduled fed -0.31±0.65g/day). At the end of the scheduled feeding period, rats consuming the liquid diet had significantly lower body weight than rats that had been fed the solid diet (liquid: 379.4 ± 6.0 g vs. solid: 404.8 ± 3.6g, P<0.001, Figure 3B). Rats consuming the crosslinked diet had significantly lower body weight than rats that had been fed either the solid diet or the liquid diet (crosslinked: 363.2 ± 3.8g vs. solid: 404.8 ± 6.0g, P<0.001; vs. liquid: 379.4 ± 6.0g, P<0.001, Figure 3B).

At the end of the scheduled feeding period, analysis of body composition revealed changes in fat (Figure 3C) and lean mass (Figure 3D), compared to prior to scheduled feeding. There was a significant decrease in fat mass in rats fed the liquid diet (pre: 39.1 ± 1.9g vs. post: 22.6 ± 1.2g, P<0.001) or crosslinked diet (pre: 44.8 ± 2.75g vs. post: 24.0 ± 2.0g, P<0.001), but no difference in the rats fed a solid diet (Figure 3C). There was also a significant decrease in lean mass in rats consuming the crosslinked diet (pre: 316.7 ± 4.4g vs. post: 274.9 ± 2.3g, P=0.043, Figure 3D), but no change in lean mass in rats fed either the solid or liquid diets (solid: pre: 313.9 ± 5.8g vs. post: 313.5 ± 5.1g, P=0.86; liquid: pre: 308.7 ± 4.4g vs. post: 307.3 ± 4.4g, P=0.72, Figure 3D). Visual inspection of the stomach 90min after food presentation showed that rats fed the crosslinked and liquid diets had enlarged stomachs (supplementary Figure 1).

Protein crosslinking has an effect on circulating hormones and metabolic parameters.

GLP-1, insulin and leptin response after an *ad libitum* caloric intake.

The circulating level of the hormones GLP-1, insulin and leptin were analysed from terminal blood samples at 0 and 90 minutes post-food ingestion. Two-Way ANOVA revealed that there was a significant effect of time (P<0.001) and form (P=0.032), with interaction between time and form (P=0.025) on GLP-1 levels (Figure 4A). A *post-hoc* Holm-Sidak test revealed GLP-1 levels were significantly raised from 0 to 90 minutes in rats that received solid (P<0.001) or crosslinked diet (P=0.004), but not in rats consuming the liquid diet (P=0.091).

Two-Way ANOVA revealed that there was no effect of time (P=0.96) on plasma leptin levels, but there was an effect of diet form (P<0.001; Figure 4B). Circulating leptin levels were higher at baseline in rats fed the solid diet, compared to the liquid and crosslinked diets (both P<0.001). This difference was still present at 90 minutes (both P<0.001). The group consuming the crosslinked diet had higher leptin levels compared to the liquid diet group at both time points (0 min; P=0.035, 90 min; P=0.029).

Form and time significantly affected plasma insulin levels (P<0.001 for both; Figure 4C) and there was a significant interaction between these two factors (P<0.001). There was a significant increase in circulating insulin on the solid diet at 90 minutes compared to the 0 minute controls (P<0.001), but this effect was not observed for the other two forms of diet. Additionally, fasting insulin levels were lower in the liquid diet fed animals than in the rats receiving the solid diet (P<0.001). At 90 minutes, solid diet fed animals had higher plasma insulin in response to feeding compared to liquid (P<0.001) and crosslinked (P<0.001) diets. Rats fed a crosslinked diet exhibited a small but significant increase in insulin levels at 90 minutes in comparison to the liquid diet group (P<0.05).

Glucose, NEFA and triglyceride profile after an *ad libitum* caloric intake

The circulating levels of glucose, non-esterified fatty acids (NEFAs) and triglycerides were analysed from terminal blood samples at baseline (0 min) or 90 minutes post-food ingestion (Figure 5).

Two-Way ANOVA revealed that there was a significant effect of time (P=0.003) and form (P=0.048) on glucose levels (Figure 5A), and a significant interaction between the two (P<0.001). Baseline levels of glucose in the liquid group were lower than in the solid (P=0.029) or crosslinked groups (P=0.003). There was no change in circulating glucose concentrations 90 minutes after food presentation in the solid (P=0.19) or crosslinked groups (P=0.15). However, there was a significant effect of time for rats receiving the liquid form of the diet, with glucose levels rising after 90 minutes (P<0.001).

Two-Way ANOVA revealed that there was a significant effect of time (P<0.001) and form (P<0.001) on NEFA levels (Figure 5B) and an interaction between time and form (P<0.001). There was no difference in baseline levels of NEFAs between the 3 groups. NEFA levels significantly decreased 90 minutes post food ingestion in the solid (P=0.048) and crosslinked groups (P=0.041). There was no change in NEFA levels in the liquid group at the 90 minute time point, with NEFA levels being higher in the liquid group than in either the solid or crosslinked groups (both P<0.001).

Two-Way ANOVA revealed a significant effect of time (P<0.001) and form (P<0.001) on circulating triglyceride levels (Figure 5C), and an interaction between time and form (P<0.001). There was no difference in baseline levels of triglycerides between the three groups. Triglyceride levels increased over the 90 minutes post-food ingestion in all three diet groups (all groups P<0.001). At both time points, triglyceride levels were lower in the liquid group compared to rats fed the solid or crosslinked diets (all P<0.001).

Agrp, Npy, Cart and Pomc hypothalamic neuropeptide expression

Expression of appetite regulatory neuropeptide mRNA (*Npy, Agrp, Cart* and *Pomc)* in the ARC of the hypothalamus (Figure 6) was compared between the three diet forms at the two time points (0 or 90 min) by Two-Way ANOVA and *post-hoc* Holm-Sidak testing.

Analysis of the expression of *Npy* and *Agrp* in the ARC revealed a significant effect of form (*Npy*: P=0.03; *Agrp*: P=0.02), with rats that were fed a liquid diet having elevated expression compared to those that were fed solid or crosslinked diets. However, there was no effect of time (*Npy:* P=0.57; *Agrp:* P=0.24), and no interaction between form and time (*Npy:* P=0.69; *Agrp:* P=0.73, Figures 6 A,B). There was no effect of time or form on *Cart* or *Pomc* expression (time; *Cart*: P=0.45; *Pomc*: P=0.29, form; *Cart*: P=0.59; *Pomc*: P=0.82, Figures 6 C,D), and no interaction (*Cart* P=0.69, *Pomc* P=0.78).

The effect of diet on subsequent satiety.

The impact of diet on early- and late-phase satiety was assessed in a separate cohort of rats (study 2) receiving the same three diets on the feeding schedule already described (Figure 7).

Prior to test 1 (early-phase satiety), average daily caloric intake over a 5-day period when food intake was adapted to scheduled meals, was higher in the solid diet fed rats (86.25±2.04kcal) compared to both the liquid (78.17±1.01kcal, P<0.001) and crosslinked diet fed rats (59.33±0.63kcal, P<0.001), and greater in the liquid group compared to the crosslinked group (P<0.001, Figure 7A). However, on the day of the test, at the first scheduled meal, there was no difference in caloric intake between the groups on the three diets (ZT13-15; solid: 39.0 ± 2.0 kcal; vs. liquid: 39.1 ± 0.9 kcal P=0.95; vs. crosslinked: 34.6 ± 1.7 kcal, P=0.75; liquid: 39.1 ± 0.9 kcal; vs. crosslinked: 34.6 ± 1.7 kcal, P=0.73, Figure 7B). However, during the test meal (stock AIN-93M pellet), the rats that had previously consumed the solid diet ate significantly less during the 1 hour access immediately after the scheduled feed when compared to the groups previously fed the liquid or crosslinked diets (ZT15-16; solid: 3.9 ± 1.0 kcal; vs. liquid:

17.4 ± 2.2 kcal, P<0.001; crosslinked: 18.5 ± 2.2 kcal, P <0.001, Figure 7B). There was no difference in test meal intake between rats fed the liquid and crosslinked diet. However, at ZT20-22, with access to the remainder of their respective diets, the solid group consumed significantly more calories than the liquid and crosslinked groups (ZT20-22; solid: 45.7 ± 2.2 kcal; vs. liquid: 35.0 ± 2.3 kcal, P<0.001; crosslinked: 33.6 ± 1.3 kcal, P<0.001), but there was no difference in caloric intake between liquid and crosslinked fed groups (Figure 7B). Total caloric intake (scheduled plus test caloric intake) was similar in all three groups to the daily average on an ad-libitum intake (Figure 7C).

On test day 2 (late-phase satiety test), at the first scheduled meal (ZT13-15), caloric intake in rats that were fed a solid diet (43.3 ± 2.5 kcal) was higher than that of rats fed a liquid diet (liquid: 35.4 ± 1.6 kcal, P<0.05, Figure 7D). The rats that received the crosslinked diet ingested fewer calories (32.3± 0.8 kcal) than the group receiving the solid diet (P<0.01), but their intake was not significantly different to that of the liquid group (P=0.23, Figure 7D). At the test meal (stock AIN-93M pellet between ZT20-21), rats that had previously consumed the solid diet ate significantly less (55.3 ± 3.6 kcal) of the test meal than the liquid group (74.0 ± 4.3 kcal, P= 0.036) and trended toward a lower intake when compared to the crosslinked group (70.3± 6.1 kcal, P= 0.079). At ZT21-22 during the final hour of a scheduled feed, rats were given access to the leftover portion of their schedule fed diet. The solid group consumed significantly fewer calories (4.8 ± 0.6 kcal) than the liquid group (9.1 ± 1.2 kcal, P= 0.024), and trended toward a lower intake compared to the crosslinked group (8.6 ± 1.3 kcal, P= 0.076, Figure 7D). Total caloric intake (scheduled plus test caloric intake) was similar between all three groups and approximately 20% greater than the average daily caloric intake on an ad libitum diet (Figure 7~~6~~E).

Food intake, body weight and body composition on 24 hour *ad libitum* access to solid, liquid or protein crosslinked diets.

Rats had continuous *ad-libitum* access to the solid, liquid or crosslinked diets for 7 days. On the day prior to the introduction of *ad libitum* access to one of the three experimental diets there were no differences in body mass between the three groups (solid: 359.7 ± 8.2 g vs. liquid: 365.3 ± 5.1g vs. crosslinked 369.5 ± 4.7g; all comparisons P>0.05, Figure 8A). On the final day, after 7 days on experimental diets, the rats consuming the liquid diet had significantly lower body weight than rats fed the solid or crosslinked diets (One-Way ANOVA Day 21; liquid: 380.6 ± 3.8 g vs. solid: 411.0 ± 7.4 g, P<0.001; crosslinked: 407.3± 4.4 g, P=<0.001, Figure 8A). Body composition by Echo MRI at the end of the study week found an increase in fat mass in rats fed the solid diet (solid; pre: 34.2 ± 1.5g vs. post: 40.1 ± 1.5g, P=0.007), a

decrease in rats fed the liquid diet (liquid pre: 31.7 ± 1.5g vs. post: 26.6 ± 1.4g, P=0.0012), but no change in fat mass in rats fed the crosslinked diet (crosslinked; pre: 31.9 ± 2.1g vs. post: 34.1 ± 1.5g, P=0.094; Figure 8B. There was no change in lean mass within or between groups (Figure 8B).

Food intake was determined over each 24h period of the 7 day duration of experimental diet feeding and recorded as dark phase (between ZT12 and ZT0) and light phase (between ZT0 and ZT12) intake. On average over a 24 hour period, the caloric intake of rats on the liquid diet was higher than rats on the solid or crosslinked diets (24 hour intake; liquid: 112.5 ± 1.1 kcal; vs. solid: 92.5 ± 1.0 kcal, P<0.001; vs crosslinked: 101.8 ± 1.9 kcal, P= 0.022). The rats consuming the crosslinked diet also had a higher caloric intake than the solid group (24 hour intake; P=0.002, Figure 9A). During the dark phase, the solid group consumed more calories than the liquid or crosslinked groups (dark phase solid: 83.0 ± 1.3 kcal; vs. liquid: 61.0 ± 0.6 kcal, P<0.001; vs. crosslinked: 55.4 ± 1.6 kcal P<0.001). The crosslinked group consumed less than the liquid group (dark phase; P= 0.005, Figure9B). In the light phase, rats fed the solid diet consumed fewer calories than the rats receiving the liquid or crosslinked diets (light phase; solid: 9.5 ± 0.6 kcal; vs. liquid: 51.5 ± 1.0 kcal, P<0.001; crosslinked: 45.8 ± 1.5 kcal P<0.001, Figure 9C), and as in the dark phase, intake of rats fed the crosslinked diet was less than rats fed the liquid diet (P=0.002).

Discussion

Human studies have provided evidence that the physical form or structure of food presentation produces differential effects on both subjective and behavioural measures of satiation and satiety (Moukarzel & Sabri 1996, DiMeglio & Mattes 2000, Bertenshaw 2008, Martens et al. 2012, Martens et al. 2011). These studies indicate that food presented in a solid form suppresses hunger more effectively than food in a liquid form. Such studies are often acute interventions where a test meal is fed followed by an assessment of hunger ratings or subsequent intake of food. Similar comparisons in animal models are difficult to perform because experimental animals may not voluntarily consume unfamiliar diets to the required quantity or in a timely fashion, due to intrinsic neophobia. The current study was designed to overcome variability in the consumption of an experimental diet by using a scheduled feeding protocol, which limits access to food to two, 2h periods of availability, and caloric content per day to a level that was individually assessed for each animal to 100% of ad-libitum daily intake. This feeding schedule corresponds to the principal periods during the light-dark cycle when the Sprague-Dawley rat normally consumes food (Bake et al 2014). Observation showed that this protocol ensured that the rats were sufficiently hungry when presented with food that

consumption started soon thereafter. At the first presentation each day (at the beginning of the dark phase), the total food for the day is presented allowing the rat to eat to satiety, with the remaining food presented in the latter half of the dark phase.

A limitation of structural changes to diet form in an experimental context, is that in most cases a change in structure is accompanied by a change in caloric density. We were able to resolve this challenge by solubilizing a defined diet with a high enough protein content such that when the protein was crosslinked by transglutaminase the structure of the diet changed from a liquid to a soft solid state. Using this approach we were, uniquely, able to compare diets of exactly the same caloric density in solid and liquid forms. To make the diet a solid from a liquid form by protein crosslinking, the minimum protein content required was 13% by weight, which increased the percentage protein by energy of the liquid and crosslinked diets to 52%. The third diet used for reference had a more rigid structure (solid diet), but with a similar percentage protein by weight (approximately 14% by energy).

Using our scheduled feeding protocol, rats that were fed the solid form of the diet usually consumed all the available food by the end of the second meal. This equated to an average of 98% of caloric intake of ad-libitum of stock diet, resulting in a near normal growth rate. In contrast, rats receiving the liquid or crosslinked diets did not consume all the food presented over the course of the two meals, leaving on average 25% and 35% of available calories, respectively. Within scheduled meals there was an effect of time of day on food intake. The intake of the crosslinked diet was significantly less compared to the solid and liquid diets at both scheduled meals. However, intake of the solid meal at ZT20-22 was 70% higher compared to ZT13-15. This is consistent with a spike of food intake prior to the start of the light phase (Bake et al 2014). However, intake of the liquid or crosslinked diet was similar at both time points, comprising approximately 50% of intake at each meal. Thus the effect of diet was more evident at the latter half of the dark phase.

At 52% protein by energy the reconstituted liquid and crosslinked diets could be considered high protein diets, and thus to be potentially appetite suppressive (Batterham et al 2006, Stengel 2013). However, we show that rats fed either of these two diets were able to consume more food in the first hour after the first scheduled feed than rats fed the solid diet (study 2, early phase satiety trial), implying that satiety may be short lived and that the percentage energy from protein may not have contributed to the initial reduced intakes. With a caloric density 3-fold lower than that of the solid diet, one possible explanation for this reduction in intake is that rats may have achieved early stage satiety due to the volume of the liquid or crosslinked diet consumed. Observation of the stomach found that it was distended at 90min after intake of a liquid or crosslinked diet indicative of the accumulation of the diet

(supplementary Figure 1). This would be consistent with evidence from experiments varying gastric volume by drainage or use of pyloric cuffs that shows the rate of gastric emptying affects subsequent food intake (Davis and Smith 1990, Davis et al 1998).

Over 7 days, following initial body weight loss upon the introduction of scheduled feeding, differences in caloric intake were translated into changes in body weight, with the solid and liquid diet fed rats sustaining a body weight increase, albeit at a lower rate for rats fed liquid diet. For rats on the solid diet there was no change in body fat and lean mass indicating that the available caloric intake was sufficient to maintain growth. In rats fed the liquid diet, lean mass was unchanged but fat mass was lower than the pre-diet fat mass. This could reflect insufficient caloric consumption over the scheduled feeding periods to maintain fat mass, but another possible contributory factor is processing of a liquid diet, with the calories and nutrients consumed assimilated differently in comparison to the processing of a solid diet. Rats fed the crosslinked diet, consuming fewer calories than the other groups, maintained a stable body weight after an initial drop experienced by all rats when the scheduled feeding paradigm was introduced. This was manifest in a lower lean tissue mass in addition to a lower fat mass, but indicates after this initial drop, intake was sufficient to maintain the acquired body mass.

Evidence from studies of humans reveals that changing the form of food presentation can change the postprandial hormonal response (Moukarzel & Sabri 1996, Raben et al. 2003, Mattes 1996, DiMeglio & Mattes 2000, Anderson & Woodend 2003, Mattes 2001, Stull et al. 2008, Panahi et al. 2013). There are, however, insufficient comparable animal studies to explain the changes found in the current study at a mechanistic level. The plasma hormone measures reported here represent a first attempt to establish such an evidence base.

In Study 1, both the solid and crosslinked diets caused a postprandial increase in GLP-1 at 90 minutes, with the liquid diet showing no change in level between the two time points. This is in contrast to a human study where a liquid diet tended to raise GLP-1 levels and a crosslinked diet did not (Juvonen et al 2011), However, it should be noted that the human study was an acute response to a single meal, whereas in the current study the response was measured on the seventh day of diet manipulation during which period more chronic physiological adaptations may have taken place.

Lower circulating leptin levels in liquid and crosslinked diet rats compared to the rats fed the solid diet are likely to reflect a reduction in fat mass on these diets. However, there was a slightly higher basal (t=0) value in rats fed the crosslinked diet compared to the liquid fed group. Within diets, there was no effect of diet form on postprandial circulating leptin concentrations at 90 minutes after the start of feeding. In Study 1, it was ascertained that changing the form of the diet has an effect on circulating levels of glucose, triglycerides and

NEFAs. All three diets resulted in a rise in triglyceride levels, indicating that food had passaged beyond the stomach and that absorption of fat had occurred. However, the rats fed the liquid diet had an overall lower level, which could be indicative of the fat from the diet not being efficiently absorbed.

The effects of the different diet forms on circulating insulin levels appear to be complex and may indicate that there are additional effects of the diets. The significantly lower fasting insulin levels seen in the liquid diet fed rats, compared to those fed a solid diet, could be a direct link to lower fat mass and/or could be attributed to a positive change in insulin sensitivity (Askari et al. 2010, Zheng et al. 2016). However, a similar reduction in fat mass was also observed for the crosslinked diet but basal insulin was not found to be significantly different with respect to the solid diet. This reduction in fasting insulin in liquid fed rats may also be influenced by lower triglyceride levels which have been shown to positively correlate with circulating insulin levels (Godsland et al. 1992).

In accord with previous studies (Dimitriadis et al. 2011), an elevated plasma insulin concentration can be expected in rats at 90 minutes after feeding a solid diet, despite no evidence of elevated plasma glucose, i.e. after basal glycaemia has been restored. This appears to be corroborated by the reduction in circulating levels of NEFAs at 90 minutes which may be a result of insulin-dependent inhibition of lipolysis (Carpentier et al. 2005). Scheduled feeding on the liquid diet appears to have stimulated neither GLP-1 nor insulin secretion, but this may be due to poor nutrient absorption (in comparison to the solid diet), resulting in these hormones returning to basal levels within 90 minutes. This may explain the raised NEFA levels which would be expected to eventually increase after the animals have re-entered a fasting state (Dimitriadis et al. 2011). Nevertheless plasma glucose concentrations are raised, without a concurrent increase in circulating insulin suggesting a possible dysregulation of the glucose homeostatic machinery (given the levels of glycaemia already observed in these rats). However, crosslinking the diet appears to have a beneficial effect to enable a solid diet-like state with respect to GLP-1 and metabolite concentrations.

Evidence suggests that dietary protein may increase hindbrain and hypothalamic neuronal activation (Darcel et al. 2005, Schwarz et al. 2010, Phifer et al. 1998, Faipoux et al. 2007, Journel et al. 2012). There is, however, little information on how different diet forms impact on the appetite regulatory regions of the hypothalamus. Using anti-sense probes for the appetite regulatory peptides *Agrp, Npy, Cart* and *Pomc* mRNA, we observed a higher expression of *Agrp* and *Npy* in rats schedule fed the liquid diet which is not explained by these rats eating fewest calories since caloric intake was the same or greater than in the crosslinked group. Furthermore, elevated gene expression was evident at an intermediate body weight in the

liquid diet cohort (vs rats fed the solid or crosslinked diets). Levels of *Agrp and Npy* mRNA expression were not acutely affected by food intake, being elevated both before and 90 minutes after feeding. The findings suggest that the rats on the liquid diet have registered a caloric deficit over the course of the scheduled feeding period and are in negative energy balance, as raised levels of these neuropeptides are often seen in fasting or food restricted states (Hahn et al., 1998; Hambly et al., 2007). Significantly, this effect was suppressed in rats fed the crosslinked diet, despite their lower body weight and energy intake. As *Agrp* and *Npy* expression are known to be inhibited by GLP-1, it may be possible that sustained lower GLP-1 secretion in response to liquid caloric intake (Seo et al. 2008), may have contributed to this increase in expression of these neuropeptides. A high protein diet in mice has been shown to suppress *Npy* and *Agrp* expression in the hypothalamus (Batterham et al., 2006). The differential expression of *Agrp* and *Npy* between the crosslinked and liquid study and no suppression relative to the solid diet would not implicate the high protein content by energy of these two diets as contributing to a reduction in food intake in the schedule feeding paradigm.

The effect of diet on early- and late-phase satiety (study 2) was assessed in a separate study. We found that rats fed either the liquid or crosslinked diets consumed a similar number of calories from the stock diet during 60 minutes following either the first scheduled meal or at the start of the second scheduled meal and more calories compared to rats that consumed the solid diet. This finding is in line with rat and human studies previously undertaken which report excess consumption of calories after a liquid meal (Ramirez 1987, DiMeglio & Mattes 2000, Davidson & Swithers 2005). Therefore although satiation with a liquid or crosslinked diet reduces overall intake, the effect is short-lived making little difference to early- or late-phase satiety.

An elevated hypothalamic level of *Agrp* and *Npy* may contribute to the higher level of intake in the case of rats fed a liquid diet, but this does not explain a higher intake in rats that were fed the crosslinked diet. However, with both the liquid and crosslinked diet, the textural property of the pelleted stock diet may be perceived to be more palatable (or novel) than the experimental diet. This could lead to stimulation of food reward pathways that promote food intake (Menzies et al. 2012, Zhang et al, 1998, 2003, Kelley et al. 2003).

When rats were given constant ad-libitum access to diet (study 3), food intake measured during the light and dark phase revealed distinct diet-specific patterns of feeding behaviour. Rats fed the solid diet, as expected, consumed 90% of their daily food intake in the dark phase. In contrast, rats fed the liquid or crosslinked diets consumed up to 60% of their total daily caloric intake during the dark phase, but consumed the remainder of their intake in the light phase, when they are normally not active or consuming a significant amount of food. One

explanation for this observation could be that the volume of liquid or crosslinked diet limits food intake in late dark phase when there is a transient peak of food intake prior to light phase onset. Therefore, as a result of insufficient caloric intake, rats on these diets are unable to maintain satiety and as a result consume food during the light phase. This is consistent with longer term body weight defence mechanisms involving the hypothalamus and other brain regions (Saper et al 2002).

Behavioural and physiological processes from food intake to metabolic rate are regulated by the circadian clock and timed for optimum utilization of nutrients in a natural environment (Tahara and Shibata et al 2013). Perturbation of circadian regulated metabolic processes by diet, timing of food intake or mutations in clock components can be profound and may be an underlying factor in the development of obesity (Salgado-Delgado et al. 2010, 2013, Kohsaka A et al. 2007, Turek et al 2005, Chaix et al 2014). Thus, dysregulation of a circadian regulated feeding patterns may be a contributory factor leading to the consumption of more calories by rats fed liquid or crosslinked diets during the light phase compared to rats fed the solid diet. However, despite disruption of normal circadian intake of food, rats fed the crosslinked diet had a similar body weight trajectory to rats fed the solid diet, and rats fed the liquid diet showed a slower rate of growth that continues to diverge from the body weight trajectory of rats fed either the solid or crosslinked diets. Interestingly, despite higher caloric intake this was not detrimental for adiposity as fat mass did not increase (crosslinked group) or was decreased (liquid group). However, the underlying reason for this remains to be explored.

These studies explored the role and potential satiating mechanism of protein crosslinking, using TG as a possible avenue for beneficial food reformulation for calorie reduction. To our knowledge, these studies are the first to highlight that when using diets of a similar macronutrient content, the manipulation of physical form can affect feeding behaviour, physiology, postprandial response and the expression of hypothalamic neuropeptides involved in appetite regulation, although as previous study by Davidson and Swithers (Davidson and Swithers, 2005) tested the effect of increasing viscosity of an Ensure solution on caloric intake by adding a soluble fibre. They found that a more viscous Ensure pre-meal reduced intake of a subsequent test meal compared to a normal Ensure solution; however a confounding issue with this study was that supplementary soluble fibre used to increase viscosity of Ensure may reduce food intake (Adam et al., 2015).

In summary, schedule feeding a crosslinked dietary protein with TG can lead to lower levels of calorie intake and a reduction in the negative metabolic and homeostatic outcomes generated by consumption of a liquid diet. A lower caloric intake may be due to promotion of

satiation, but any satiety effect was short-lived. Together with beneficial effects of protein crosslinking on lower orexigenic drive in the hypothalamus, our research may provide the beginnings of an understanding of a mechanism involved in an enhanced satiation by a protein crosslinked diet. In the current obesogenic environment although protein crosslinking may not improve satiety, an enhanced satiation by protein crosslinking may be beneficial as part of a strategy for calorie restriction and weight loss in the human population.

Acknowledgements

This work was funded by the European Union Seventh Framework programme (Full4Health - grant number 266408) and the Scottish Government Rural and Environment Science and Analytical Services Division. We thank Danisco, Kettering, UK, for the gift of emulsifier. We thanks Dr Claus-Dieter Mayer of Biomathematics & Statistics Scotland for help with statistical analysis.

Declaration of interest

The authors have nothing to declare

References

Adam, C., Williams, P.A., Garden, K.E., Thomson, L.M., Ross, A.W. Does-dependent effects of a soluble dietary fibre (pectin) on food intake, adiposity, gut hypertrophy and gut satiety hormone secretion in rats. *PLoS ONE,* 10 (2015), e0115438.

Anderson, G.H., Woodend, D. Consumption of sugars and the regulation of short-term satiety and food intake. *The American Journal of Clinical Nutrition*, 78 (2003), 843S-849S.

Askari, H. Tykodi, G., Liu, J., Dagogo-Jack, S. Fasting plasma leptin level is a surrogate measure of insulin sensitivity. *The Journal of Clinical Endocrinology & Metabolism* 95 (2010), 3836-3843.

Autio, K., Kruus, K., Knaapila, A., Gerber, N., Flander, L., Buchert, J. Kinetics of transglutaminase-induced crosslinking of wheat proteins in dough. *Journal of Agricultural and Food Chemistry* 53 (2005), 1039-1045.

Bake, T., Morgan, D.G.A., Mercer J.G. Feeding and metabolic consequences of scheduled consumption of large, binge-type meals of high fat diet in the Sprague-Dawley rat. Physiology and Behaviour 128 (2014), 70-79.

Batterham, R.L., Heffron, H., Kapoor, S., Chivers, J.E., Chandarana, K., Herzog, H., Le Roux, C.W., Thomas, E.L., Bell, J.D., Withers, D.J. Critical role for peptide YY in protein-mediated satiation and body-weight regulation. *Cell Metabolism* 4 (2006), 223-233.

Bertenshaw, E.J., Lluch, A., Yeomans, M.R. Satiating effects of protein but not carbohydrate consumed in a between-meal beverage context. Physiology & Behavior, 93 (2008), 427-436.

Bönisch, M., Lauber, S., Kulozik, U. Effect of Ultra-high Temperature Treatment of the Enzymatic Cross-linking of Micellar Casein and Sodium Caseinate by Transglutaminase. *Journal of Food Science,* 69 (2004), E398-E404.

Brynes, A.E., Frost, G.S., Edwards, C.M.B., Ghatei, M.A., Bloom, S.R. Plasma glucagon-like peptide-1 (7-36) amide (GLP-1) response to liquid phase, solid phase, and meals of differing lipid composition. *Nutrition*, 14 (1998), 433-436.

Buchert, J., Ercili Cura, D., Ma, H., Gasparetti, C., Monogioudi, E., Faccio, G., Mattinen, M., Boer, H., Partanen, R., Selinheimo, E. Crosslinking food proteins for improved functionality. *Annual review of food science and technology,* 1 (2010),113-138.

Carpentier, A.C., Frisch F., Cyr, D., Genereux, P., Patterson, B.W., Giguere, R., Baillargeon J.-P. "On the suppression of plasma nonesterified fatty acids by insulin during enhanced intravascular lipolysis in humans." *American Journal of Physiology-Endocrinology and Metabolism* 289.5 (2005): E849-E856.

Chaix, A., Zarrinpar, A., Miu, P., Satachidananda, P., Time-restricted feeding is a preventative and therapeutic intervention against diverse nutritional challenges. Cell Metabolism, 20 (2014), 991-1005.

Clegg, M.E., Pratt, M., Markey, O., Shafat, A., Henry, C.J.K. Addition of different fats to a carbohydrate food: Impact on gastric emptying, glycaemic and satiety responses and comparison with in vitro digestion. *Food Research International*, 48 (2012), 91-97.

Coll, A.P., Farooqi, I.S., O'Rahilly, S. The hormonal control of food intake, *Cell,* 129 (2007), 251-262.

Collins, P.J., Houghton, L.A., Read, N.W., Horowitz, M., Chatterton, B.E., Heddle, R., Dent, J. Role of the proximal and distal stomach in mixed solid and liquid meal emptying. *Gut*, 32 (1991), 615-619.

Darcel, N., Fromentin, G., Raybould, H.E., Gougis, S., Gietzen, D.W., Tome, D. Fos-positive neurons are increased in the nucleus of the solitary tract and decreased in the ventromedial hypothalamus and amygdala by a high-protein diet in rats. *The Journal of nutrition*, 135 (2005),1486-1490.

Davidson, T.L., Swithers S.E. Food viscosity influences caloric intake compensation and body weight in rats. Obesity Research 13, (2005) 537-544

Davis, J.D., Smith, G.P. Learing to sham feed: behavioural adjustments to loss of physiological postingestional stimuli. Amercian Journal of Physiology, 259 (1990), R1228-1235

Davis, J.D., Smith, G.P., Sayer J.L. Closing th pylorus decreases the size of the large meals in the rat. Physiology and Behaviour, 63 (1998), 191-196

DiMeglio, D.P. & Mattes, R.D. 2000, "Liquid versus solid carbohydrate: effects on food intake and body weight. *International Journal of Obesity*, 24 (2000), 794-800.

Dimitriadis, G., Mitron, P., Lambadiari, V., Maratou, E., Raptis, S.A. Insulin effects in muscle and adipose tissue." *Diabetes Research and Clinical Practice* 93 (2011): S52-S59.

Faipoux, R., Tome, D., Gougis, S., Darcel, N., Fromentin, G. Proteins activate satiety-related neuronal pathways in the brainstem and hypothalamus of rats. *The Journal of Nutrition*, 138 (2008), 1172-1178.

Fisher, R.S., Malmud, L.S., Bandini, P., Rock, E. Gastric emptying of a physiologic mixed solid-liquid meal. *Clinical Nuclear Medicine*, 7 (1982), 215-221.

Flanagan, J., Gunning, Y. FitzGerald, R. Effect of cross-linking with transglutaminase on the heat stability and some functional characteristics of sodium caseinate. Food Research International, 36 (2003), 267-274.

Franks, P.W., Brage, S., Luan, J., Ekelund, U., Rahman, M., Farooqi, I.S., Halsall, I., O'rahilly, S., Wareham, N.J. Leptin predicts a worsening of the features of the metabolic syndrome independently of obesity. Obesity research, 13 (2005),1476-1484.

Fujise, T., Yoshimatsu, H., Kurokawa, M., Oohara, A., Kang, M., Nakata, M., Sakata, T. Satiation and masticatory function modulated by brain histamine in rats. *Proceedings of the Society for Experimental Biology and Medicine.Society for Experimental Biology and Medicine* 217 (1998), 228-234.

Gerstein, D.E., Woodward-Lopez, G., Evans, A.E., Kelsey, K., Drewnowski, A. Clarifying concepts about macronutrients' effects on satiation and satiety. *Journal of the American Dietetic Association,* 104 (2004), 1151-1153.

Godsland, I. F., Crook, D., Walton, C., Wynn, V., Oliver M.F. Influence of insulin resistance, secretion, and clearance on serum cholesterol, triglycerides, lipoprotein cholesterol, and blood pressure in healthy men. *Arteriosclerosis, Thrombosis, and Vascular Biology* 12 (1992), 1030-1035.

Hahn, T.M., Breininger, J.F., Baskin, D.G., Schwartz, M.W. Coexpression of Agrp and NPY in fasting-activated hypothalamic neurons. *Nature Neuroscience*, 1 (1998), 271-272.

Hambly, C., Mercer, J.G. Speakman J.R. Hunger does not diminish over time in mice under protracted caloric restriction. *Rejuvenation Research* 10 (2007), 533-542.

Hand, K. V., Bruen C.M., O'Halloran, F., Panwar, H., Calderwood, D., Giblin, L., Green, B.D. Examining acute and chronic effects of short-and long-chain fatty acids on peptide YY (PYY) gene expression, cellular storage and secretion in STC-1 cells. *European Journal of Nutrition* 52 (2013), 1303-1313.

Jaros, D., Partschefeld, C., Henle, T., Rohm, H. Transglutaminase in dairy products: chemistry, physics, applications. *Journal of Texture Studies*, 37 (2006), 113-155.

Johnstone, L.E, Fong, T.M. Leng, G. Neuronal activation in the hypothalamus and brainstem during feeding in rats. Cell Metabolism 4 (2006), 313-321.

Journel, M., Chaumontet, C., Darcel, N., Fromentin, G., Tome, D. Brain responses to high-protein diets. *Advances in Nutrition*, 3 (2012), 322-329.

Juvonen, K.R., Purhonen, A.K., Salmenkallio-Marttila, M., Lahteenmaki, L., Laaksonen, D.E., Herzig, K.H., Uusitupa, M.I., Poutanen, K.S., Karhunen, L.J. Viscosity of oat bran-enriched beverages influences gastrointestinal hormonal responses in healthy humans. *The Journal of nutrition,* 139 (2009), 461-466.

Juvonen, K.R., Karhunen, L.J., Vuori, E., Lille, M.E., Karhu, T., Jurado-Acosta, A., Laaksonen, D.E., Mykkänen, H.M., Niskanen, L.K., Poutanen, K.S. Structure modification of a milk protein-based model food affects postprandial intestinal peptide release and fullness in healthy young men. *British Journal of Nutrition,* 106 (2011), 1890-1898.

Juvonen, K.R., Lille, M.E., Laaksonen, D.E., Mykkänen, H.M., Niskanen, L.K., Herzig, K., Poutanen, K.S., Karhunen, L.J. Crosslinking with transglutaminase does not change metabolic effects of sodium caseinate in model beverage in healthy young individuals. *Nutrition journal.* 11 (2012), art no. 35

Kelley, A., Will, M., Steininger, T., Zhang, M., Haber, S. Restricted daily consumption of a highly palatable food (chocolate Ensure®) alters striatal enkephalin gene expression. *European Journal of Neuroscience*, 18 (2003), 2592-2598.

Keogh, J.B., Wooster, T.J., Golding, M., Day, L., Otto, B., Clifton, P.M. Slowly and Rapidly Digested Fat Emulsions Are Equally Satiating but Their Triglycerides Are Differentially Absorbed and Metabolized in Humans. *The Journal of Nutrition*, 141 (2011), 809-815.

Kohsaka, A., Laposky, A.D., Ramsey, K.M, Estrada, C., Joshu, C., Kobayashi, Y., Turek, F.W., Bass, J. High fat diet disrupts behavioural and molecular circadian rhythms in mice. Cell Metabolism, 6 (2007), 414-421.

Kong, F., Singh, R. Disintegration of solid foods in human stomach. *Journal of Food Science*, 73 (2008), R67-R80.

Kristensen, M., Jensen, M.G. Dietary fibres in the regulation of appetite and food intake. Importance of viscosity. *Appetite*, 56 (2011), 65-70.

Kuraishi, C., Yamazaki, K., Susa, Y. Transglutaminase: its utilization in the food industry. *Food Reviews International,* 17 (2001), 221-246.

Lorenzen, P.C., Schlimme, E., Roos, N. Crosslinking of sodium caseinate by a microbial transglutaminase. Food/Nahrung, 42 (1998), 151-154.

Marciani, L., Cox, E., Pritchard, S., Major, G., Hoad, C., Mellows, M., Hussein, M., Costigan, C., Fox, M., Gowland, P. Additive effects of gastric volumes and macronutrient composition on the sensation of postprandial fullness in humans. *European Journal of Clinical Nutrition*, 69 (2015), 380-384.

Marmonier, C., Chapelot, D., Louis-Sylvestre, J. Effects of macronutrient content and energy density of snacks consumed in a satiety state on the onset of the next meal. *Appetite,* 34 (2000),161-168.

Martens, M.J., Lemmens, S.G., Born, J.M., Westerterp-Plantenga, M.S. A solid high-protein meal evokes stronger hunger suppression than a liquefied high-protein meal. *Obesity*, (2011), 522-527.

Martens, M.J., Lemmens, S.G., Born, J.M., Westerterp-Plantenga, M.S. Satiating capacity and post-prandial relationships between appetite parameters and gut-peptide concentrations with solid and liquefied carbohydrate. *PLoS ONE*, 7 (2012), e42110.

Mattes, R.D. Dietary Compensation by Humans for Supplemental Energy Provided as Ethanol or Carbohydrate in Fluids. *Physiology & Behavior*, 59 (1996), 179-187.

Mattes, R.D. & Rothacker, D. 2001, "Beverage viscosity is inversely related to postprandial hunger in humans", *Physiology & Behavior,* vol. 74, no. 4, pp. 551-557.

Mattes, R.D. Food palatability, rheology, and meal patterning. *Journal of Parenteral and Enteral Nutrition,* 32 (2008), 572-574.

McClements, D.J., Decker, E.A., Park, Y., Weiss, J. Designing food structure to control stability, digestion, release and absorption of lipophilic food components. *Food Biophysics,* 3 (2008), 219-228.

Menzies, J.R., Skibicka, K.P., Egecioglu, E., Leng, G., Dickson, S.L. Peripheral signals modifying food reward in *Appetite Control*, Springer, (2012), pp. 131-158.

Mercer, J.G., Hoggard, N., Williams, L.M., Lawrence, C.B., Hannah, L.T., Trayhurn, P. Localization of leptin receptor mRNA and the long form splice variant (Ob-Rb) in mouse hypothalamus and adjacent brain regions by in situ hybridization. *FEBS letters*, 387 (1996), 113-116.

Mochida, T., Tohru H., Hiroshi H. The corn protein, zein hydrolysate, administered into the ileum attenuates hyperglycemia via its dual action on glucagon-like peptide-1 secretion and dipeptidyl peptidase-IV activity in rats. *Endocrinology* 151 (2010), 3095-3104.

Monogioudi, E., Faccio, G., Lille, M., Poutanen, K., Buchert, J., Mattinen, M. Effect of enzymatic cross-linking of β-casein on proteolysis by pepsin. *Food Hydrocolloids*, 25 (2011), 71-81.

Morton, G., Cummings, D., Baskin, D., Barsh, G., Schwartz, M. Central nervous system control of food intake and body weight. *Nature,* 443 (2006), 289-295.

Motoki, M., Seguro, K. Transglutaminase and its use for food processing. *Trends in Food Science & Technology,* 9 (1998), 204-210.

Moukarzel, A., Sabri, M. Gastric physiology and function: effects of fruit juices. *Journal of the American College of Nutrition*, 15 (1996), 18S-25S.

Panahi, S., El Khoury, D., Luhovyy, B.L., Goff, H.D., Anderson, G.H. Caloric beverages consumed freely at meal-time add calories to an ad libitum meal. *Appetite*, 65 (2013), 75-82.

Phifer, C.B., Berthoud, H.R. Duodenal nutrient infusions differentially affect sham feeding and Fos expression in rat brain stem. *The American Journal of Physiology*, 274 (1998), R1725-33.

Phillips, R.J., Powley, T.L. Gastric volume rather than nutrient content inhibits food intake. *The American Journal of Physiology*, 271(1996), R766-9.

Raben, A., Agerholm-Larsen, L., Flint, A., Holst, J.J., Astrup, A. Meals with similar energy densities but rich in protein, fat, carbohydrate, or alcohol have different effects on energy expenditure and substrate metabolism but not on appetite and energy intake. *The American Journal of Clinical Nutrition*, 77 (2003), 91-100.

Ramirez, I. Feeding a liquid diet increases energy intake, weight gain and body fat in rats. *The Journal of Nutrition*, 117 (1987), 2127-2134.

Rolls, B., Castellanos, V., Halford, J., Kilara, A., Panyam, D., Pelkman, C., Smith, G., Thorwart, M. Volume of food consumed affects satiety in men. *American Journal of Clinical Nutrition*, 67 (1998), 1170-1177.

Salgado-Delgado, R., Angeles-Castellanos, M., Saderi, N., Buijs, R.M., Escobar, C. Food intake during the normal activity phase prevents obesity and circadian desynchrony in a rat model of night work. *Endocrinology*, 151 (2010), 1019-1029.

Salgado-Delgado, R.C., Saderi, N., Basualdo Mdel, C., Guerrero-Vargas, N.N., Escobar, C., Buijs, R.M. Shift work or food intake during the rest phase promotes metabolic disruption and desynchrony of liver genes in male rats. *PLoS ONE*, 8 (2013), e60052.

Saper, C.B., Chou, T.C., Elmquist J.K. The need to feed: Homeostatic and hedonic control of eating. Neuron, 36 (2002), 199-211.

Schwarz, J., Burguet, J., Rampin, O., Fromentin, G., Andrey, P., Tomé, D., Maurin, Y., Darcel, N. Three-dimensional macronutrient-associated Fos expression patterns in the mouse brainstem. *PLoS ONE*, 5 (2010), e8974.

Simpson, B.K., Rui, X., XiuJie, J. Enzyme-assisted food processing in *Green Technologies in Food Production and Processing* Springer, (2012) pp. 327-361.

Stengel, A., Goebel-Stengel, M., Wang, L., Hu, E., Karasawa, H., Pisegna, J.R. Tache, Y. High-protein diet selectively reduces fat mass and improves glucose tolerance in Western-type diet-induced obese rats. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology,* 305 (2013), R582-R591.

Stull, A.J., Apolzan, J.W., Thalacker-Mercer, A.E., Iglay, H.B., Campbell, W.W. Liquid and solid meal replacement products differentially affect postprandial appetite and food intake in older adults. *Journal of the American Dietetic Association*, 108 (2008), 1226-1230.

Tahara, Y., Shibata, S. Neuroscience forefront review Chronobiology and Nutrition. Neuroscience, 253 (2013), 78-88

Tolhurst, G., Heffron, H., Lam, Y.S., Parker, H.E., Habib, A.M., Diakagiannaki, E., Cameron, J., Grosse, J., Reimann, F., Gribble, F.M. Short-chain fatty acids stimulate glucagon-like peptide-1 secretion via the G-protein–coupled receptor FFAR2. *Diabetes,* 61 (2012), 364-371.

Turek, F.W., Joshu, C., Kohsaka, A., Lin, E., Ivanova, G., McDearmon, E., Laposky, A., Losee-Olsen, S., Easton, A., Jensen, D.R., Eckel, R.H., Takahashi, J.S., Bass, J. Obesity and metabolic syndrome in circadian clock mutant mice. Science, 308 (2005), 1043-1045.

Westerterp-Plantenga, M., Lejeune, M., Nijs, I., Van Ooijen, M., Kovacs, E. High protein intake sustains weight maintenance after body weight loss in humans. *International Journal of Obesity,* 28 (2004), 57-64.

Wisén, O. & Johansson, C. Gastrointestinal function in obesity: Motility, secretion, and absorption following a liquid test meal. *Metabolism*, 41 (1992), 390-395.

Wilkinson, C., Dijksterhuis, G., Minekus, M. From food structure to texture. *Trends in food science and technology* 11 (2001), 442-450.

Wu, T., Thazhath, S.S., Marathe, C.S. Bound M.J., Jones, K.L., Horowitz, M. Rayner, C.K. Comparative effect of intraduodenal and intrajejunal glucose infusion on the gut–incretin axis response in healthy males. *Nutrition & diabetes* 5 (2015), e156.

Yokoyama, K., Nio, N., Kikuchi, Y. Properties and applications of microbial transglutaminase. *Applied Microbiology & Biotechnology,* 64 (2004), 447-454.

Zhang, M., Gosnell, B.A., Kelley, A.E. Intake of high-fat food is selectively enhanced by mu opioid receptor stimulation within the nucleus accumbens. The Journal of pharmacology and experimental therapeutics, 285 (1998), 908-914.

Zhang, M., Balmadrid, C., Kelley, A.E. Nucleus accumbens opioid, GABaergic, and dopaminergic modulation of palatable food motivation: contrasting effects revealed by a progressive ratio study in the rat. *Behavioral Neuroscience*, 117 (2003), 202-211.

Zheng, Louise D., et al. "Mitochondrial epigenetic changes link to increased diabetes risk and early-stage prediabetes indicator." *Oxidative medicine and cellular longevity* 2016 (2016), art ID 5290638.

Figure legends

Figure 1: Protein crosslinking using transglutaminase solidifies a liquid diet.

The steady state viscosity was measured in duplicate on the liquid and crosslinked diet with an increasing stress range resulting in shear rates from 0.01 to 30 s^{-1}. Transglutaminase treatment gave rise to higher viscosity than a liquid treated with the inactivated TG indicating solidification of the liquid diet. (B) Outline for the three studies performed indicating time for acclimatization, determination of individual food intakes and feeding periods.

Figure 2: Caloric intake at ZT13-15 and ZT20-22 in rats fed a (A) solid, (B) liquid or (C) crosslinked (Xlinked) diet over time after the introduction of scheduled feeding. (D) Caloric intake average over days 3-6 at ZT13-15 and ZT20-22. Two-way ANOVA and *post-hoc* Holm-Sidak test. *P<0.05, ***P<0.001 n=14 per group.

Figure 3: Bodyweight, food intake and body composition in response to scheduled access to 100% of daily caloric intake presented as a solid, liquid or crosslinked form of diet.

(A) Daily caloric intake averaged over the days 3-6 of scheduled feeding of rats fed one of the 3 experimental diets presented as % of average of daily caloric intake on ad-libitum intake. (B) Body weight change throughout the course of the experiment. Arrow indicates start of schedule feeding of experimental diets; (C) Fat mass and (D) Lean mass as determined by Echo MRI. Caloric intake and body weight comparison was carried out by One-way ANOVA and *post-hoc* Holm-Sidak test. Body composition values was compared by paired t-test (ns = not significant, * P<0.05, *** P<0.001). Data is presented as mean and ± SEM. All groups, n=14.

Figure 4: GLP-1, and leptin and Insulin response following food presentation.

(A) Circulating GLP-1 (B) Leptin and (C) Insulin plasma concentrations prior to and 90 minutes after food intake. Statistical comparison carried out by Two-Way ANOVA and *post-hoc* Holm-Sidak test. Data presented as mean and ± SEM. n=7 for each group (* P<0.05, ** P<0.01, *** P<0.001).

Figure 5: Glucose, NEFA and triglyceride profiles of rats schedule fed experimental diets prior to and 90 mins following food presentation.

(A) Circulating Glucose concentrations. (B) Circulating non-esterified fatty acids (NEFAs) concentrations. (C) Circulating triglyceride concentrations prior to and 90 minutes after food intake. ‘a’ indicates lower base line value in rats fed the liquid diet compared to rats fed either the solid or crosslinked diet; ‘b’ indicates overall lower levels in the liquid group P<0.001 at both time points vs. solid and crosslinked forms. Statistical comparison by Two-Way ANOVA and *post-hoc* Holm-Sidak test. Data presented as mean and ± SEM. All groups, n=7; * P<0.05, *** P<0.001.

Figure 6: *Npy, Agrp, Cart* and *Pomc* hypothalamic mRNA expression in rats schedule fed experimental diets.

Relative mRNA expression of appetite regulatory hypothalamic neuropeptides in the arcuate nucleus (ARC) and representative autoradiograph images; (A) *Agrp* mRNA expression; (B) *Npy* mRNA expression; (C) *Cart* mRNA expression and (D) *Pomc* mRNA expression. Effect of form on *Npy* and *Agrp* expression in the liquid group compared to the solid and crosslinked diets. Statistical comparison carried out by Two-Way ANOVA and *post-hoc* Holm-Sidak method. * P<0.05; Data presented as mean and ± SEM. n=7, all groups.

Figure 7: Assessment of early- and late-phase satiety in rats schedule fed a solid, liquid or protein crosslinked diet.

(A) Daily caloric intake averaged over days 3-6 of scheduled feeding prior to testing satiety presented as % of average intake on ad-libitum intake; (B) Testing early-phase satiety – histograms showing caloric intake of test diets in the scheduled food access at ZT13-15 together with caloric intake of stock AIN-39M diet in a 1h period after the scheduled food access at ZT15-16 and subsequent intake of test diets during the second scheduled meal at ZT20-22 presented as % of average intake on ad-libitum intake; (C) Total caloric intake of the three food access events during the satiety test presented as % of average intake on ad-libitum intake; (D) Testing late-phase satiety – histograms showing caloric intake of test diets in the scheduled food access at ZT13-15 together with caloric intake of stock AIN-93M diet during the first hour of the second scheduled feeding event and test diets during the second hour of the second schedule meal presented as % of average intake on ad-libitum intake. (E) Total caloric intake of the three food access events during the satiety test presented as % of average intake on ad-libitum intake. One-Way ANOVA with post-hoc Holm-Sidak test; N=7 per group, * P<0.05, **P<0.01, *** P<0.001.

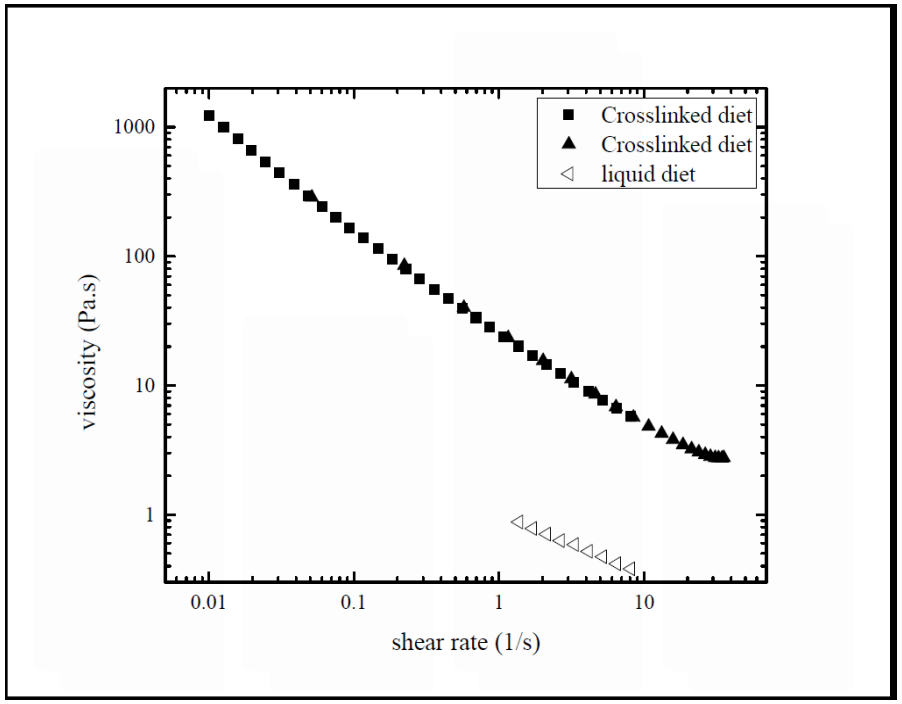
Figure 8: Effect of continuous *ad libitum* access to a solid, liquid or protein crosslinked diet on body weight and body composition

(A) Body weight change prior to and after access to experimental diets. Arrow indicates start of continuous access to experimental diets; (B) Fat mass and (C) determined by Echo MRI prior to receiving experimental diets and at the end of the experiment; Body weight data statistical comparisons carried out by One-way ANOVA and *post-hoc* Hom-Sdiak test. Composition analysis compared by t-test. Data presented as mean and ± SEM. * P<0.05, *** P<0.001, all groups, n=14.

Figure 9: Caloric intake of ad-libutm fed rats during the light phase, dark phase and total of a 24h period.

(A) Total caloric intake over 24h on an unrestricted *ad libitum* intake of the experimental solid, liquid or protein-crosslinked diet; (B) Caloric intake during the dark phase; (C) Caloric intake during the light phase. Statistical comparison carried out by One-Way ANOVA and *post-hoc* Holm-Sidak test. * $P<0.05$, **$P<0.01$, *** $P<0.001$, data presented as mean and ± SEM. All groups, n=7.

A


Crosslinked diet
Crosslinked diet
liquid diet
viscosity (Pa.s)
1000
100
10
1
0.01
0.1
1
10
shear rate (1/s)

B

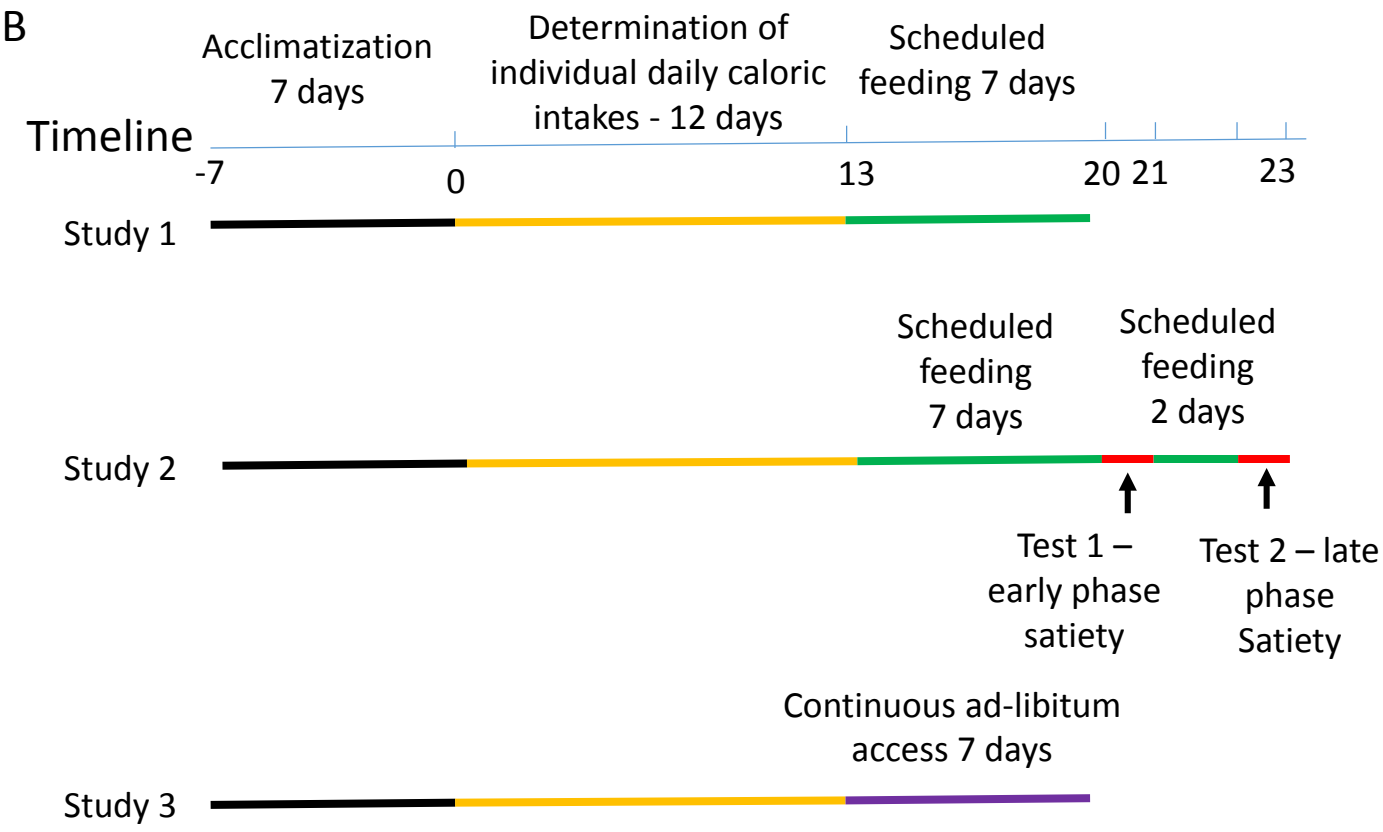

Timeline
Acclimatization 7 days
Determination of individual daily caloric intakes - 12 days
Scheduled feeding 7 days
-7
0
13
20 21
23
Study 1
Scheduled feeding 7 days
Scheduled feeding 2 days
Study 2
Test 1 – early phase satiety
Test 2 – late phase Satiety
Continuous ad-libitum access 7 days
Study 3

Figure 1

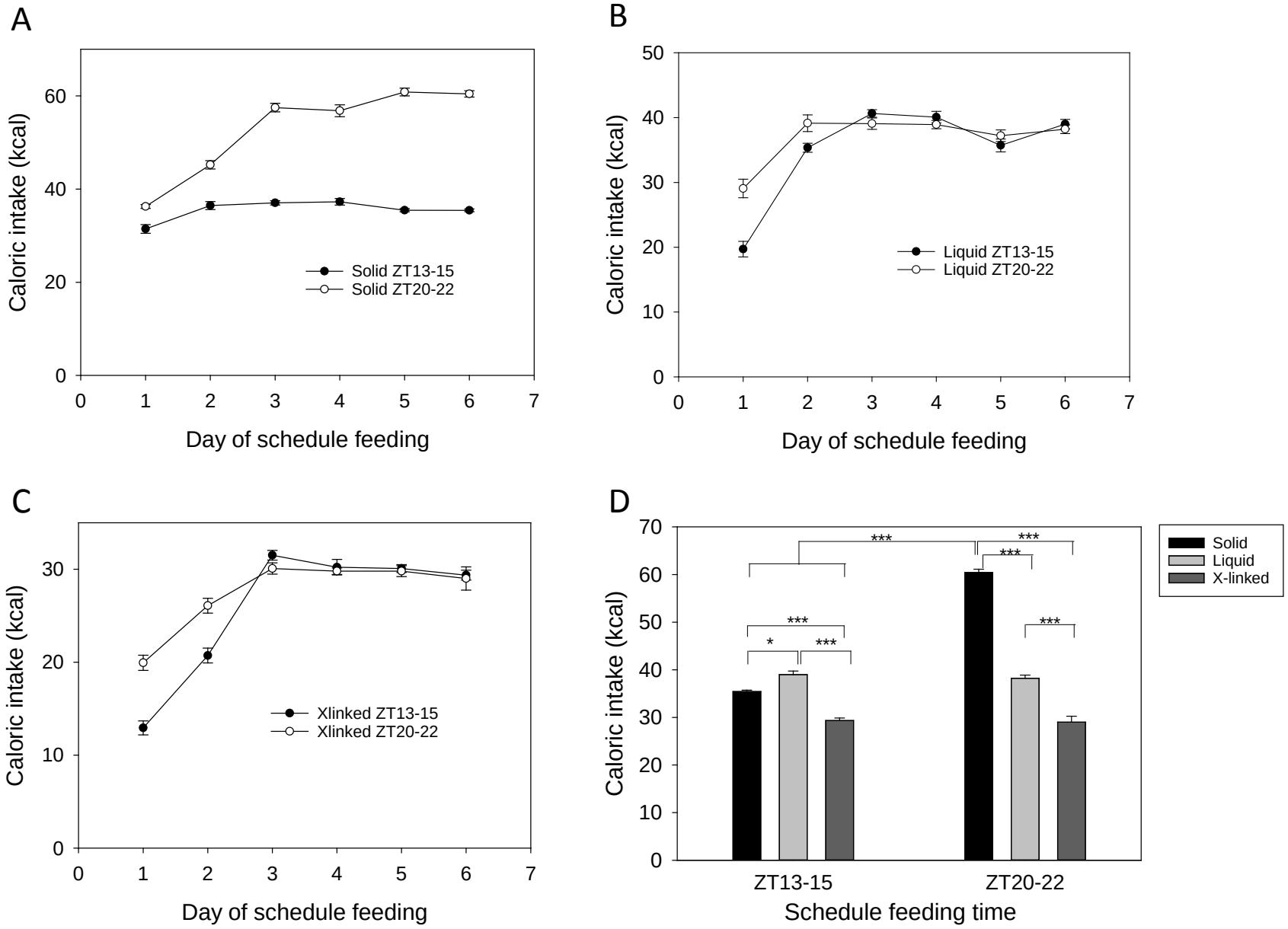

A
Caloric intake (kcal)
Day of schedule feeding
Solid ZT13-15
Solid ZT20-22
B
Caloric intake (kcal)
Day of schedule feeding
Liquid ZT13-15
Liquid ZT20-22
C
Caloric intake (kcal)
Day of schedule feeding
Xlinked ZT13-15
Xlinked ZT20-22
D
Caloric intake (kcal)
Solid
Liquid
X-linked
ZT13-15
ZT20-22
Schedule feeding time

Figure 2

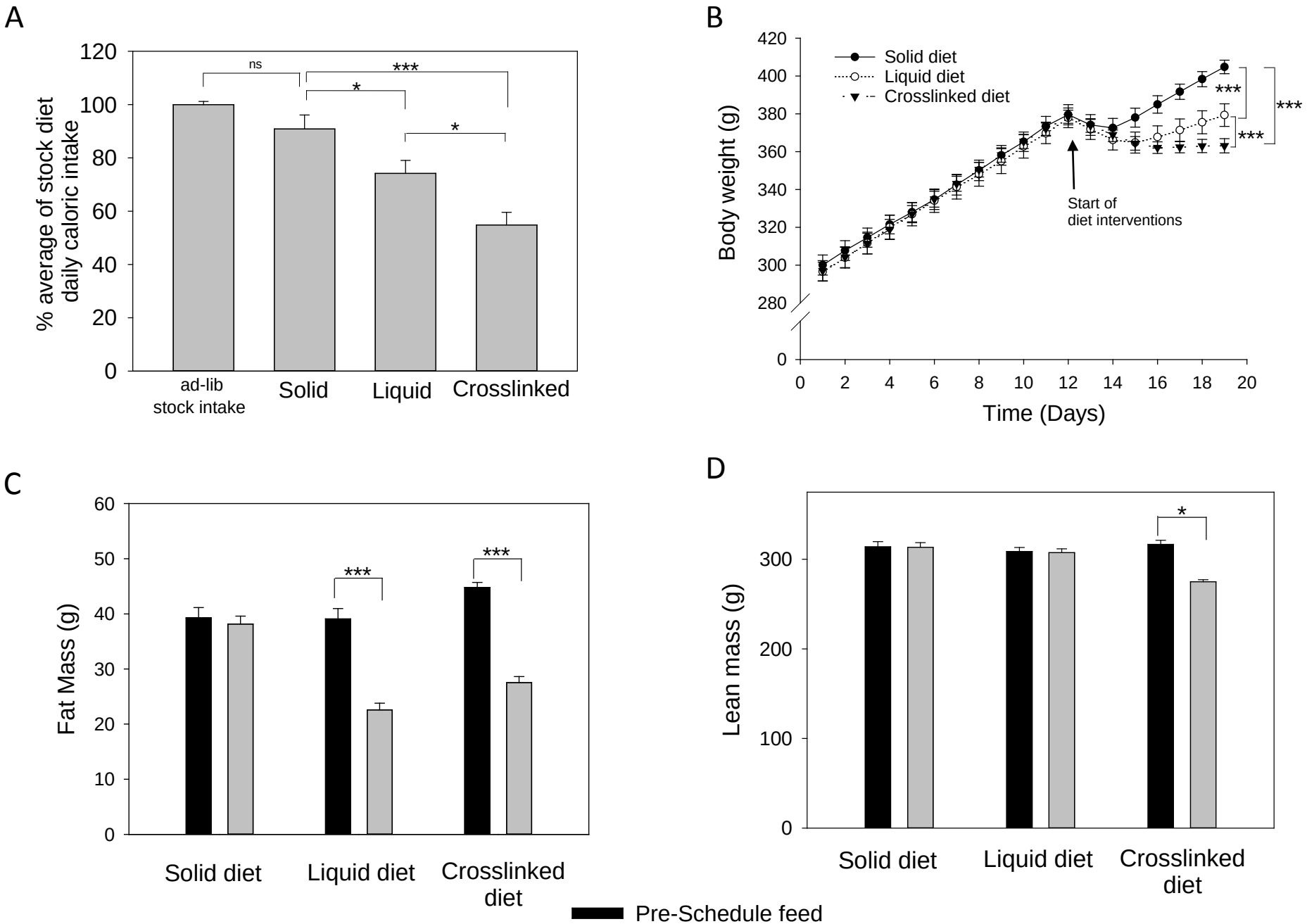

A
% average of stock diet daily caloric intake
120
100
80
60
40
20
0
ns
***
*
*
ad-lib stock intake
Solid
Liquid
Crosslinked
B
Body weight (g)
420
400
380
360
340
320
300
280
0
Solid diet
Liquid diet
Crosslinked diet
***
***
***
Start of diet interventions
0
2
4
6
8
10
12
14
16
18
20
Time (Days)
C
Fat Mass (g)
60
50
40
30
20
10
0
***
***
Solid diet
Liquid diet
Crosslinked diet
D
Lean mass (g)
300
200
100
0
*
Solid diet
Liquid diet
Crosslinked diet
Pre-Schedule feed
Post-Scheduled Feed

Figure 3

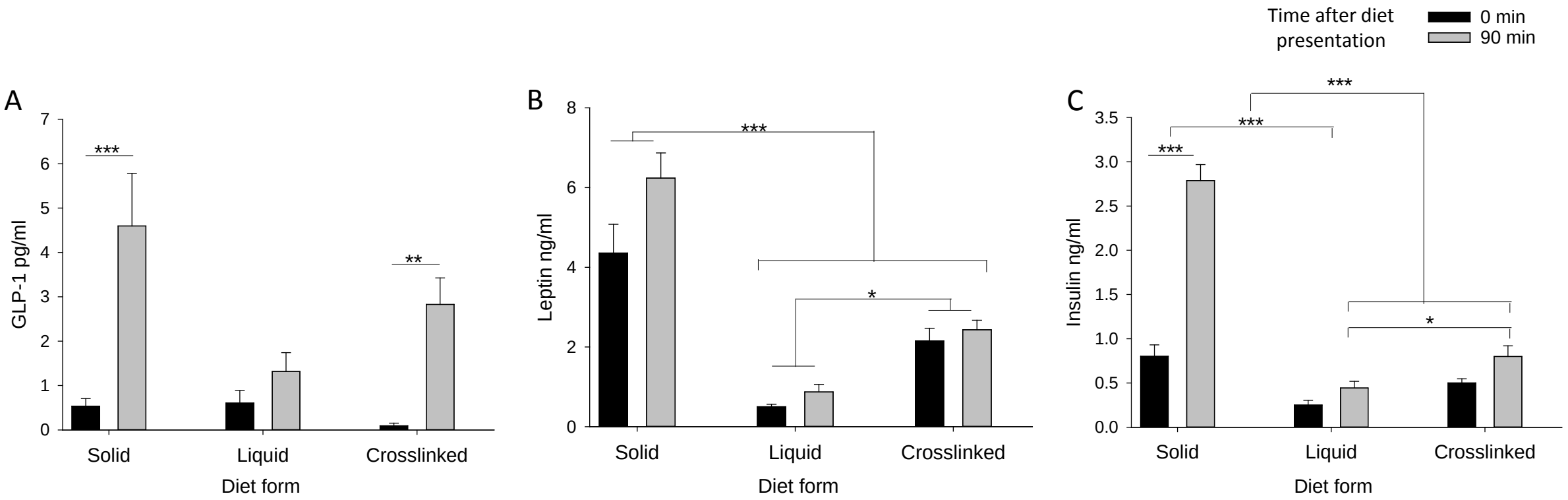

Time after diet presentation
0 min
90 min
A
GLP-1 pg/ml
***
**
Solid
Liquid
Crosslinked
Diet form
B
Leptin ng/ml
***
*
Solid
Liquid
Crosslinked
Diet form
C
Insulin ng/ml
***
***
***
*
Solid
Liquid
Crosslinked
Diet form

Figure 4

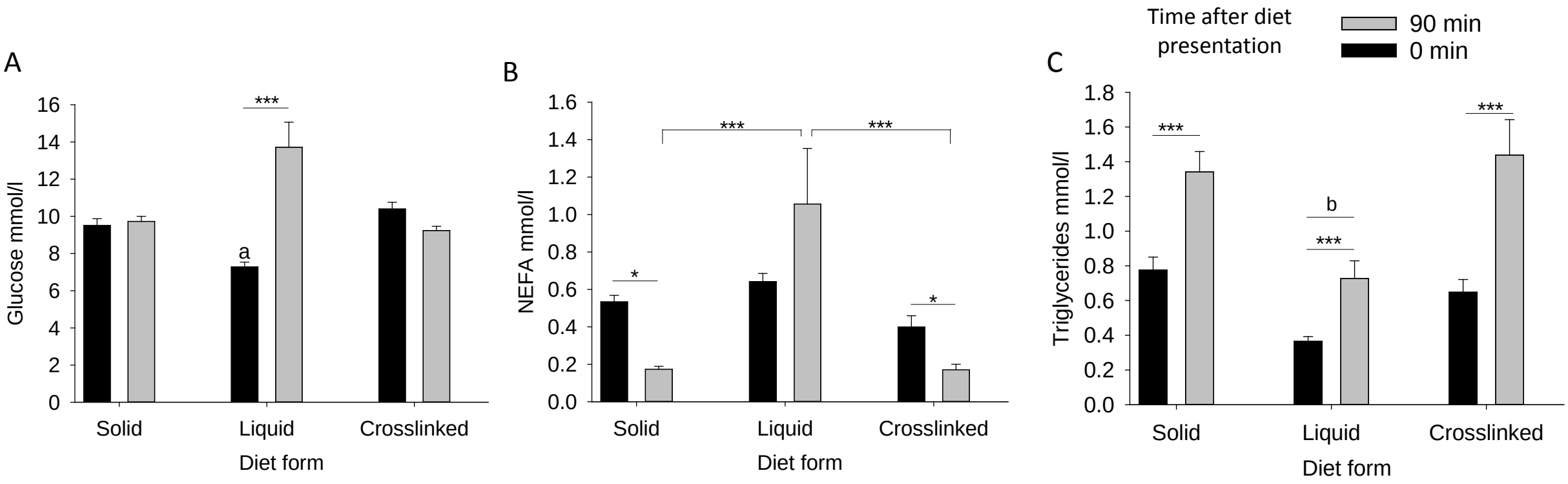

A
Glucose mmol/l
16
14
12
10
8
6
4
2
0
***
a
Solid
Liquid
Crosslinked
Diet form
B
NEFA mmol/l
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
***
*
*
Solid
Liquid
Crosslinked
Diet form
C
Time after diet presentation
90 min
0 min
Triglycerides mmol/l
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
b
***
***
Solid
Liquid
Crosslinked
Diet form

Figure 5

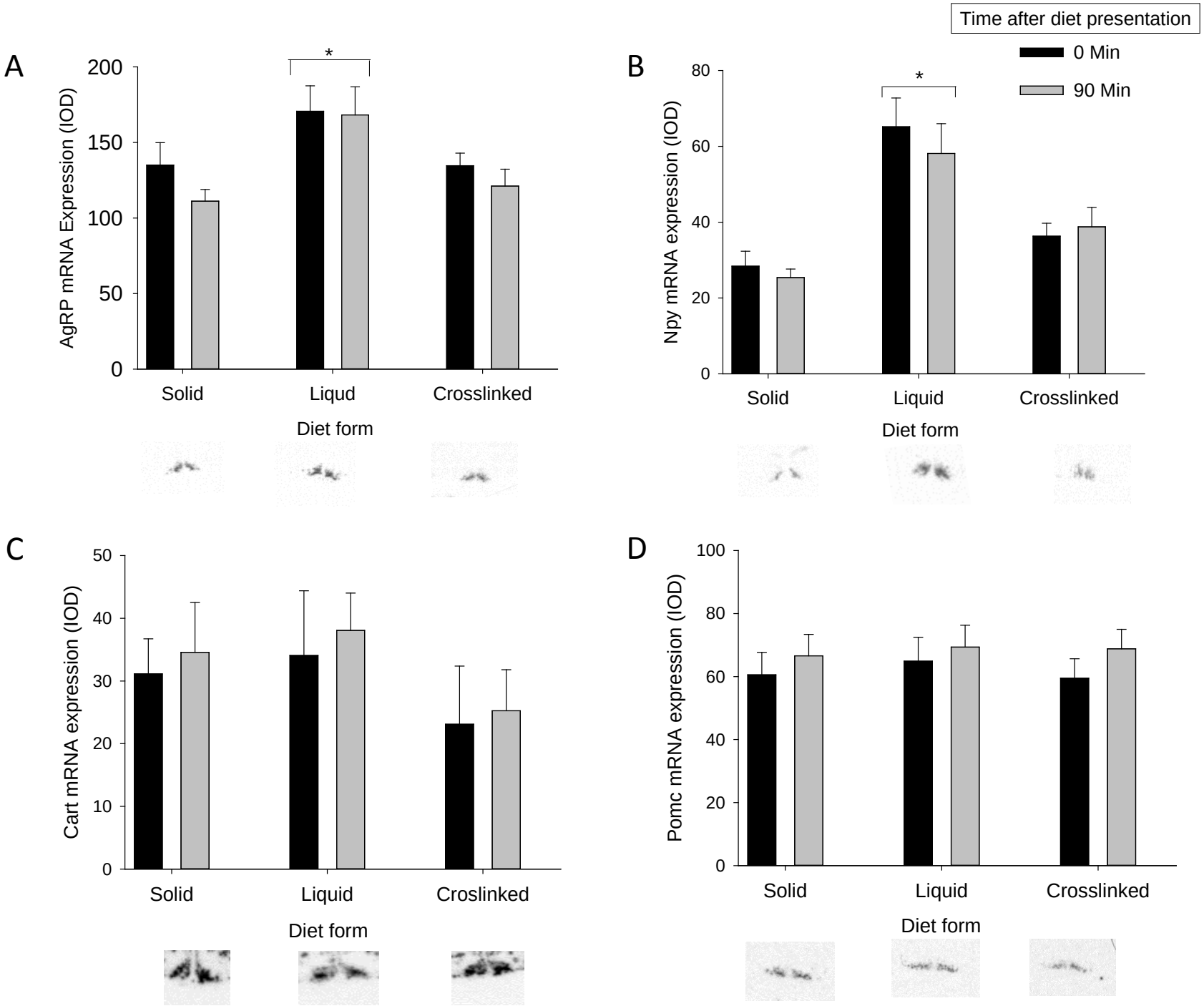

A
AgRP mRNA Expression (IOD)
200
150
100
50
0
*
Solid
Liqud
Crosslinked
Diet form
B
Time after diet presentation
0 Min
90 Min
Npy mRNA expression (IOD)
80
60
40
20
0
*
Solid
Liquid
Crosslinked
Diet form
C
Cart mRNA expression (IOD)
50
40
30
20
10
0
Solid
Liquid
Croslinked
Diet form
D
Pomc mRNA expression (IOD)
100
80
60
40
20
0
Solid
Liquid
Crosslinked
Diet form

Figure 6

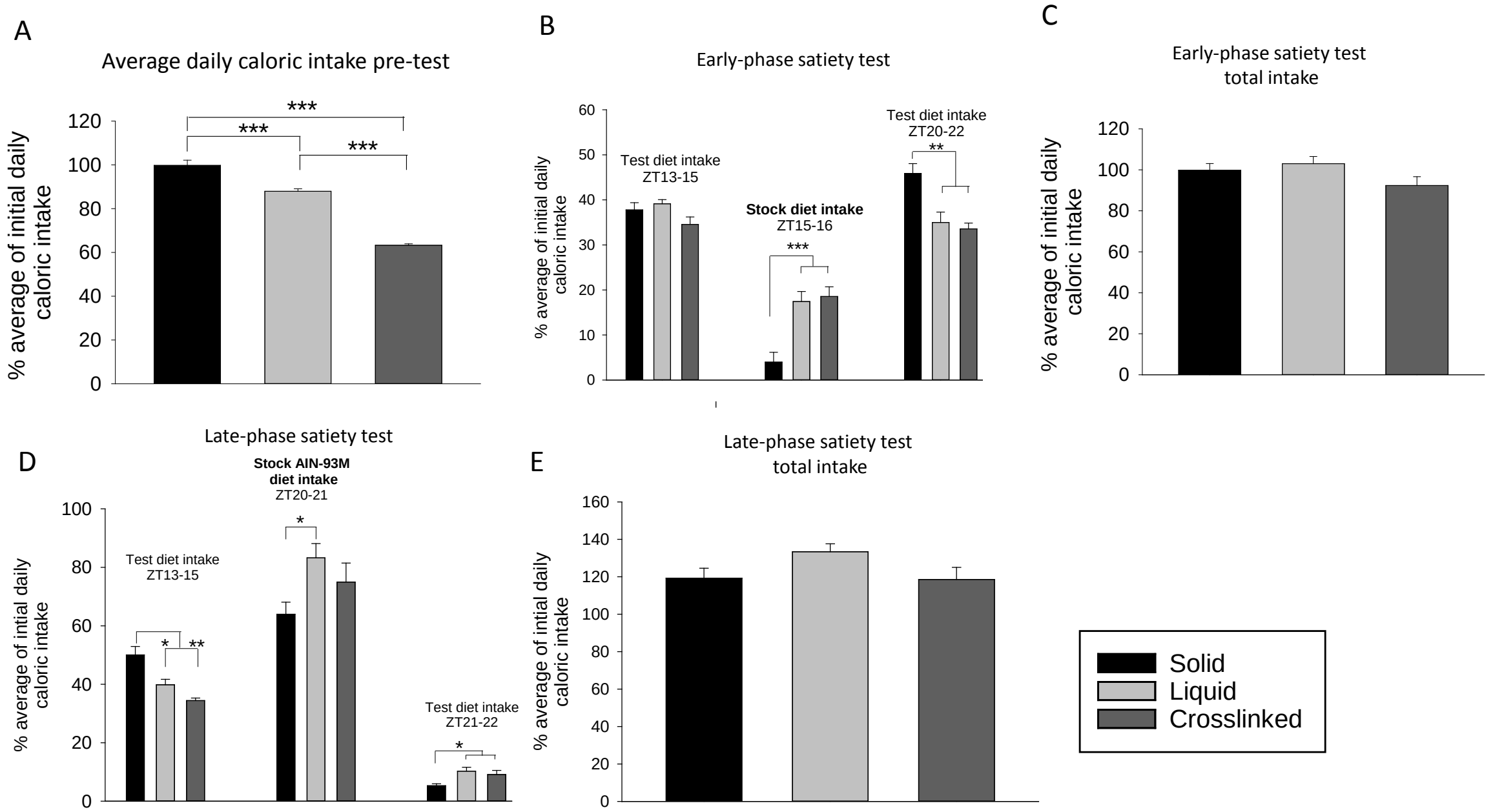

A
Average daily caloric intake pre-test
% average of initial daily caloric intake
120
100
80
60
40
20
0
***
***
***
B
Early-phase satiety test
% average of initial daily caloric intake
60
50
40
30
20
10
0
Test diet intake ZT13-15
Stock diet intake ZT15-16
***
Test diet intake ZT20-22
**
C
Early-phase satiety test total intake
% average of initial daily caloric intake
120
100
80
60
40
20
0
D
Late-phase satiety test
% average of intial daily caloric intake
100
80
60
40
20
0
Test diet intake ZT13-15
*
**
Stock AIN-93M diet intake ZT20-21
*
Test diet intake ZT21-22
*
E
Late-phase satiety test total intake
% average of intial daily caloric intake
160
140
120
100
80
60
40
20
0
Solid
Liquid
Crosslinked

Figure 7

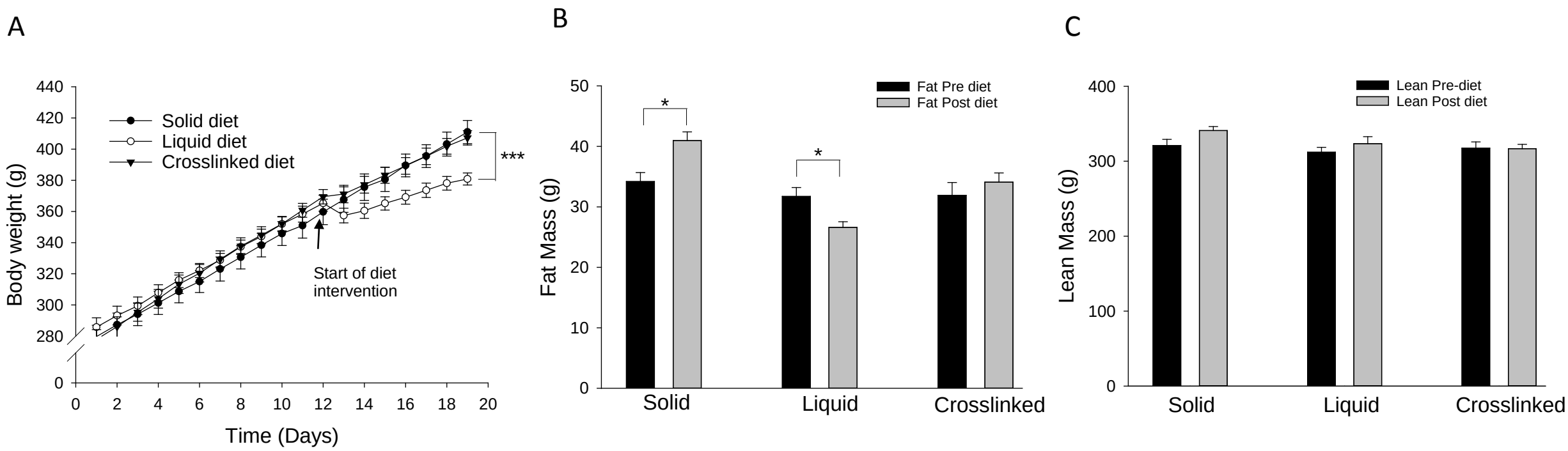

A
Body weight (g)
Time (Days)
Solid diet
Liquid diet
Crosslinked diet
***
Start of diet intervention
B
Fat Mass (g)
Fat Pre diet
Fat Post diet
*
*
Solid
Liquid
Crosslinked
C
Lean Mass (g)
Lean Pre-diet
Lean Post diet
Solid
Liquid
Crosslinked

Figure 8

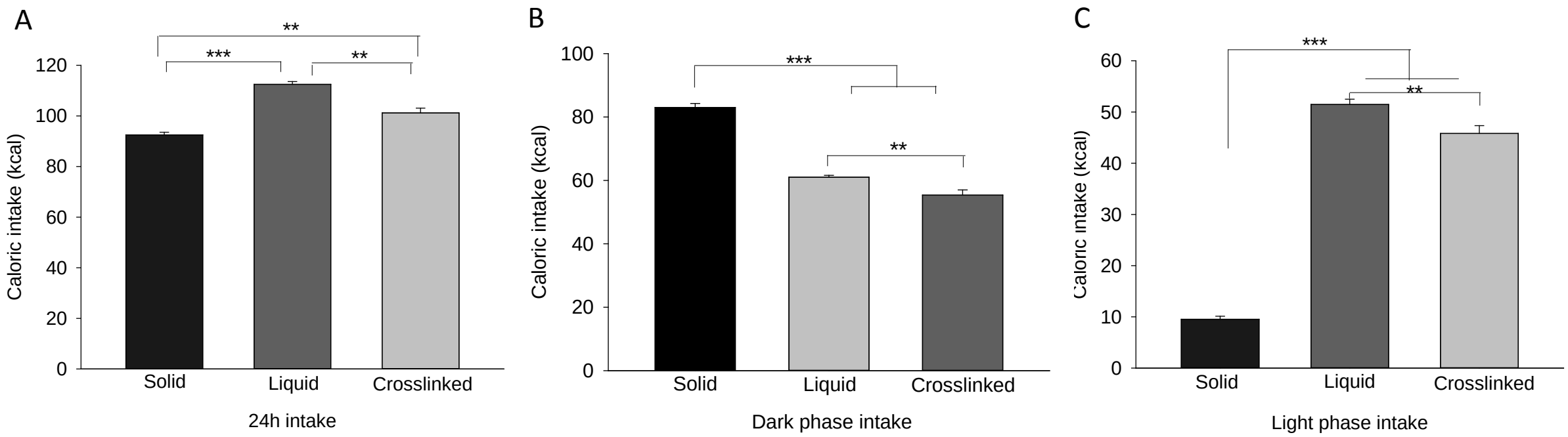

A
B
C
Caloric intake (kcal)
120
100
80
60
40
20
0
**
***
**
Solid
Liquid
Crosslinked
24h intake
Caloric intake (kcal)
100
80
60
40
20
0
***
**
Solid
Liquid
Crosslinked
Dark phase intake
Caloric intake (kcal)
60
50
40
30
20
10
0
***
**
Solid
Liquid
Crosslinked
Light phase intake

Figure 9