Effect of a 4-week weight maintenance diet on circulating hormone levels: implications for clinical weight loss trials

A. Sainsbury[1,2], I. R. Evans[1], R. E. Wood[3], R. V. Seimon[1], N. A. King[3], A. P. Hills[4] and N. M. Byrne[3,5]

[1]The Boden Institute of Obesity, Nutrition, Exercise & Eating Disorders, Sydney Medical School, The University of Sydney, Camperdown, Australia; [2]Neuroscience Research Program, Garvan Institute of Medical Research, Darlinghurst, Australia; [3]Institute of Health and Biomedical Innovation and School of Exercise and Nutrition Sciences, Queensland University of Technology, Brisbane, Australia; [4]Centre for Nutrition and Exercise, Mater Research Institute, The University of Queensland and Griffith Health Institute, Griffith University, Brisbane, Australia; [5]Bond Institute of Health and Sport, Faculty of Health Sciences and Medicine, Bond University, Gold Coast, Australia

Summary

The majority of weight loss studies fail to standardize conditions such as diet and exercise via a weight maintenance period prior to commencement of the trial. This study aimed to determine whether a weight stabilization period is necessary to establish stable baseline hormone concentrations. Fifty-one obese male participants with a body mass index of 30–40 kg m−2 and aged 25–54 years underwent 4 weeks on an energy balance diet that was designed to achieve weight stability. Blood samples were collected in the fasting state at commencement and completion of the 4-week period, and circulating concentrations of 18 commonly measured hormones were determined. During the 4-week weight maintenance period, participants achieved weight stability within −1.5 ± 0.2 kg (−1.4 ± 0.2%) of their initial body weight. Significant reductions in serum insulin (by 18 ± 6.5%) and leptin (by 21 ± 6.0%) levels occurred, but no significant changes were observed for gut-derived appetite-regulating hormones (ghrelin and peptide YY), nor thyroid, adrenal, gonadal or somatotropic hormones. There were no significant correlations between the change in body weight and the change in circulating concentrations of insulin or leptin over the 4-week period, indicating that the observed changes were not due to weight loss, albeit significant negative correlations were observed between the changes in body weight and plasma ghrelin and peptide YY levels. This study demonstrates the need for baseline weight maintenance periods to stabilize serum levels of insulin and leptin in studies specifically investigating effects on these parameters in the obese. However, this does not apply to circulating levels of gut-derived appetite-regulating hormones (ghrelin and peptide YY), nor thyroid, adrenal, gonadal or somatotropic hormones.

What is already known about this subject?

- The majority of clinical trials in obese participants do not employ a pre-intervention run-in or weight stabilization period.
- The circulating concentrations of many hormones measured during clinical trials in obese participants could be in a state of flux prior to interventions.
- A weight stabilization period with standardized conditions could be particularly important for studies investigating endocrine responses in obese participants, in order to establish stable baseline values at the participant's starting weight, thereby reducing variability in the data and increasing the power to detect any significant effects of energy restriction and weight loss.

What this study adds

- In obese participants, a weight stabilization period results in significant reductions in circulating concentrations of insulin and leptin but not gut-derived appetite-regulating hormones (ghrelin and peptide YY) or thyroid, adrenal, gonadal or somatotropic hormones.
- Weight stabilization periods should be used prior to interventions (e.g. for weight loss) in which circulating insulin and leptin levels are measured in obese participants, with pre-intervention (baseline) values of insulin and leptin being measured at the end of the weight stabilization period. Without this period, changes in insulin and leptin cannot be specifically attributed to the intervention.

- For investigations of circulating concentrations of gut-derived appetite-regulating hormones (ghrelin and peptide YY) or thyroid, adrenal, gonadal or somatotropic hormones in obese participants, a weight stabilization period is not necessary to establish stable baseline values.

Introduction

In recent decades, the prevalence of obesity has increased exponentially giving rise to a global health issue that affects billions of men, women and children [**1, 2**]. Current intervention studies focus not only on achieving weight and fat loss while protecting lean body mass, but also on investigating metabolic health and potential mediators of health benefits associated with weight reduction. This includes assessing circulating concentrations of hormones such as insulin and sex hormones [**3-7**].

In clinical trials, investigators often employ a run-in or stabilization period to collect preliminary and baseline data. Stabilization periods are used in approximately 19% of randomized controlled trials of new obesity treatments, most notably in pharmaceutical trials where they are incorporated into 29% of studies [**8**]. However, the necessity of including stabilization periods as part of randomized controlled trials of obesity treatments has been questioned in light of a recent systematic review which showed that significantly less weight loss occurred in studies of less than 3 months duration that included a run-in period, as compared with those without this period [**8**]. While the difference in weight loss between studies of a longer duration that did or did not include a run-in period was minimal, this significant finding has created doubt as to the utility of stabilization periods for weight loss interventions [**8**]. Other bodies have also questioned the value of these periods, including the European Medicines Agency and members of the Food and Drug Administration Division of Metabolic and Endocrine Products.

Despite common use of run-in periods in weight loss trials as mentioned above, the majority of weight loss studies fail to standardize conditions such as diet and exercise prior to commencement of the trial. However, we considered that a weight stabilization period with standardized conditions could be particularly important for studies investigating endocrine responses to weight loss, in order to establish true baseline values at the participant's starting weight [**8**]. For example, if a participant has recently gained weight and is in energy excess prior to commencement of a weight loss trial, their circulating concentrations of thyroid hormones [**9, 10**], cortisol [**11**], leptin [**12**] and insulin [**9, 13**] may be increased relative to their usual levels, while those of the appetite-regulating, gut-derived hormones ghrelin [**13**] and peptide YY (PYY) [**14, 15**] may be decreased and increased, respectively. In this case, the apparent effect of the weight loss intervention will be exaggerated because part of the effect is due to the fact that the participant is no longer gaining weight. On the other hand, if a participant has recently lost weight and is in energy deficit prior to commencement of a weight loss trial, circulating levels of thyroid hormones [**9, 10**], leptin [**9, 12, 16, 17**], insulin [**9, 13, 16**] and PYY [**6, 14, 15**] may be reduced, while that of ghrelin [**6, 13**] may be raised. In this case, the true effect of the weight loss intervention will be masked because the parameter will not be in steady state prior to commencement of the weight loss intervention. Thus, establishing stable baseline values for hormone levels could reduce variability in the data and increase the power to detect significant effects of energy restriction and weight loss on these parameters.

Few studies have investigated the impact of weight stabilization periods on circulating hormone levels, undoubtedly because of the cost and additional participant burden. Moreover, many of the weight loss studies that do employ a pre-randomization run-in period sample only at the end of the run-in period, and this value is taken as the participant's baseline for that particular parameter. To our knowledge, no studies have systematically investigated the stability of a wide range of hormones at both points during this period.

Given the added expense of run-in periods and multiple blood sampling for hormone determination, the aim of this study was to determine whether a weight stabilization period (4 weeks on an energy balance diet in obese men [**18**]) is necessary to establish stable baseline values of a wide range of circulating hormones.

Materials and methods

Ethics statement and participants

This study was approved by the Human Research Ethics Committee of the Queensland University of Technology in Brisbane, Australia [**18**]. Fifty-one obese male participants aged between 25 and 54 years (mean 40.5 ± 5.7 years) with a body mass index of 30–40 kg m−2 (mean 34.9 ± 4.9 kg m−2) were enrolled into the study. Participants were required to have been weight stable (±2 kg) in the 6 months prior to the trial, as estimated by self-report during screening interviews that included questions about recent weight history. These questions were asked during the initial telephone screening, and then again at the face-to-face screening interview. No control was exercised by the investigators over dietary intake or energy expenditure during this period. A total of four participants withdrew from the study after the −4-week and before the 0-week time point measures were recorded one participant was unwilling to adhere to the prescribed diet, one participant fell ill and decided not to continue in the trial, one participant had sleep apnoea and was not able to stay awake during the measurement of resting energy expenditure (REE, one of the outcome measures in the overall trial [**18**]), and one participant increased his level of physical activity and had a BMI under 30 kg m−2 prior to the 0-week time point.

Study protocol

Participants were prescribed an energy balance diet for a 4-week period, which consisted of 2 weeks of diet titration and 2 weeks of weight stability. At the beginning of the titration period, the amount of energy required to maintain energy balance was estimated using a physical activity level (PAL) of 1.4 or 1.5 based on self-reported levels and measured REE. REE was measured using a standard 30-min protocol following an overnight fast using a ventilated hood and canopy system (TrueOne 2400 Metabolic System, ParvoMedics, Inc, Sandy, UT, USA), in accordance with the manufacturer's instructions. Participants were provided with ready-made meals (Lite n' Easy, Banyo, QLD, Australia) supplemented with additional food items to match the prescribed energy intake and consisting of 15–20% protein, 25–30% fat and 55–60% carbohydrate. The ready-made menus consisted of five meals each day: breakfast, morning snack, lunch, afternoon snack and dinner. The additional foods were tailored to the preferences of each participant, and were selected to fit in with the structure of the meal plan. Typical foods included Sustagen® (Nestle Health Sciences, Vevey, Switzerland), milk, juice, nuts, biscuits, cheese with crackers and dessert. Participants were asked to keep a food diary to monitor their daily energy intake. The prescribed diet was adjusted throughout the titration period to ensure that weight remained stable during the final 2 weeks. Participants were required to be sedentary and to not commence any exercise programme during the study. In order to verify maintenance of PALs, participants were instructed to keep a physical activity diary throughout the study, by briefly recording the time, duration and mode of any physical activity (e.g. walking to and from work or home when taking public transport, or to or from their vehicle should they park more than a short distance from their workplace), and to record any recreational activities they participated in during the study. One participant lost 6.1 kg during the 4-week period because of an error in the calculation of prescribed energy intake, so plasma and serum samples from that participant were not analyzed and none of his data was included. Apart from this participant, no other body weight data were excluded. Fasting blood samples were collected in the morning at the start (week −4) and end (week 0) of the 4-week weight maintenance period. The fasting period was from 22:00 h the previous evening to 8:00 to 9:00 h on the morning of blood collection (10 to 11 h of fasting). Participants were also weighed in the laboratory at these time points, fasted and wearing only underwear and a pair of gym shorts, using a set of calibrated scales (Cosmed Srl, Rome, Italy).

Blood collection

Samples were taken following an overnight fast and within a 1-h sampling window for all participants as described above at both time points to minimize circadian variability in outcome measures. For thyroid-stimulating hormone (TSH), free triiodothyronine (T3), free thyroxine (T4), reverse triiodothyronine (reverse T3), cortisol, luteinizing hormone (LH), follicle-stimulating hormone (FSH), testosterone, sex hormone-binding globulin (SHBG), insulin-like grown factor-1 (IGF-1), insulin and leptin, blood was collected into serum tubes from Becton Dickinson (BD Australia New Zealand, North Ryde, NSW, Australia) and allowed to clot for 30 min at room temperature. For adrenocorticotropic hormone (ACTH), blood was collected into ice-cold EDTA-coated tubes from BD and placed into wet ice. For PYY and ghrelin, blood was collected into ice-cold tubes from BD containing EDTA plus 20 μL mL−1 whole blood of Aprotinin

(Sigma-Aldrich, St Louis, Missouri, USA) and 10 μL mL−1 whole blood of dipeptidyl peptidase-IV inhibitor (Linco Research, St Charles, MO, USA) and placed into wet ice. Tubes were centrifuged at 4oC and at 1100–1300 g for 10 min in a swing head centrifuge rotor. Aliquots were pipetted into CryoPure® tubes (Sarstedt Australia, Technology Park, SA, Australia) and plunged in liquid nitrogen before being stored at −80°C until subsequent analysis.

Biochemical analyses

Plasma ACTH was measured using an Immulite immunoanalyzer (Diagnostic Products Corporation, Inc, Los Angeles, CA, USA) by a solid-phase enzyme-labelled chemiluminescent immunometric assay [**19**]. Circulating cortisol, TSH, free T4 and free T3 were measured by UniCel® DxI 800 automatic particle enzyme immunoassay (Beckman Coulter, Inc, Brea, CA USA) [**20**]. Reverse T3 was measured in-house using a radioimmunoassay kit from Adaltis Italia (Rome, Italy). LH, FHS, testosterone and SHBG were measured by chemiluminescent enzyme immunoassay (Immulite 2000 Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA) [**21**]. Free testosterone was calculated using the Sodergard method [**22**]. The free androgen index (FAI) was calculated as 100 × (total testosterone) nmol L−1/(SHBG) nmol L−1. Plasma ghrelin and PYY, as well as serum insulin and leptin concentrations, were measured in-house using commercial radioimmunoassay kits (Merck Millipore, Billerica, MA, USA) according to the manufacturer's instructions. IGF-1 was determined in-house with a radioimmunoassay kit from MeDiagnost (Tuebingen, Germany). There was a technical problem with the IGF-1 radioimmunoassay: following centrifugation, pellets slipped out of a large proportion (~30%) of tubes during the decanting step, leading to very low radioactivity counts and hence extremely high and non-physiological [**23, 24**] readings for IGF-1 concentrations (equal to or greater than 1000 ng mL−1) specifically in those tubes. Cross-checking between each radioimmunoassay tube and its corresponding data read out showed that pellets were intact and IGF-1 concentrations were within the physiological range for humans (less than 1000 ng mL−1) in the remaining ~70% of the tubes, so we are thus confident of the reliability of results for IGF-1 concentrations for the remaining samples.

Statistical analysis

Statistical analysis was performed on data for body weight and circulating hormone levels at week −4 and week 0. The means and standard error of the means (SEMs) of age, body mass index, body weight and hormone levels were calculated using Statistical Package for the Social Sciences (SPSS) version 12.0 (SPSS Inc, Chicago, IL, USA). Values 4 standard deviations above and below the mean were treated as outliers and were excluded from analysis. This resulted in the exclusion of 1, 2, 3 and 2 data points from the data sets for TSH, SHBG, PYY and leptin, respectively. In addition, 26 × IGF-1 values above 1000 ng mL−1 were excluded, as levels exceeding this value are outside the accepted range for humans [**23, 24**]. This high number of exclusions was due to the technical problem with the IGF-1 radioimmunoassay as described above. Differences in body weight and circulating hormone concentrations at weeks −4 and 0 were analyzed with the paired Student's *t*-test using SPSS, with statistical significance set at $P < 0.05$. Correlation coefficients (r) between changes in body weight and changes in circulating hormone levels over the 4-week period, and associated P-values, were calculated using Prism version 6.0 d (GraphPad Software Inc, La Jolla, CA, USA).

Results

During the 4-week energy balance diet, there was a small but significant ($P < 0.0001$) weight change of −1.5 ± 0.2 kg (−1.4 ± 0.2%), with an average coefficient of variation of 0.86. However, this change is well within an accepted range of weight stability in clinical trials as shown in other papers [**25-27**]. Differences in serum insulin and leptin levels between the two time points (weeks −4 and 0) are shown in Fig. 1. The 4-week maintenance period resulted in significant decreases in mean serum insulin (by 18 ± 6.5%) and leptin (by 21 ± 6%) levels (Fig. 1). Table 1 displays the full results of all biochemical assays and shows that no significant changes were seen for circulating hormone concentrations, other than insulin and leptin as mentioned above.

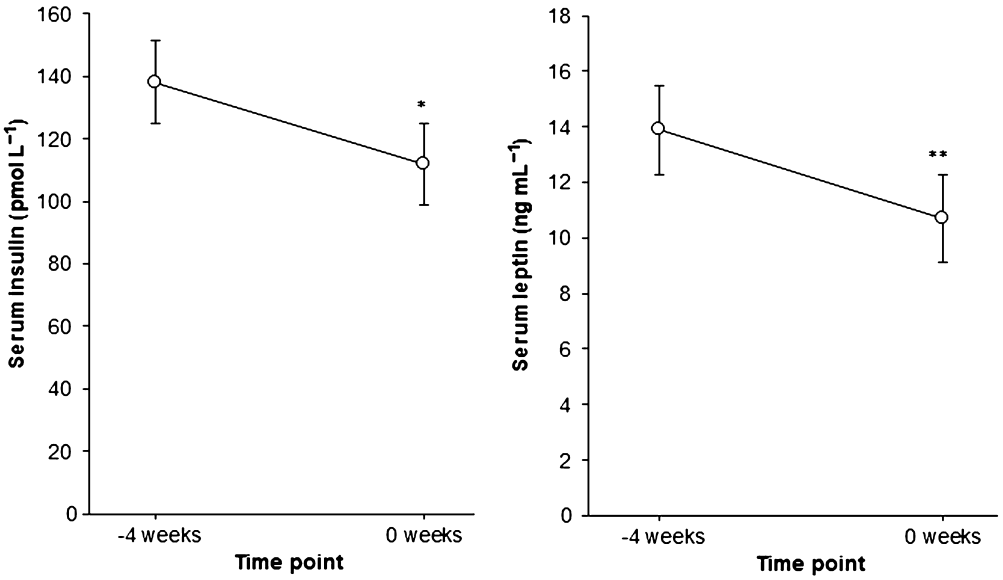

Figure 1 Hormonal changes throughout the weight maintenance period. Serum insulin and leptin concentrations in obese men before and after a 4-week energy balance diet designed to achieve weight maintenance. Data are means ± SEM of 44–46 obese men. *$P < 0.05$ and **$P < 0.01$ for comparisons between 0 and –4 weeks.

Table 1 Circulating hormone concentrations in obese men before (–4 weeks) and after (0 weeks) a 4-week energy balance diet designed to achieve weight maintenance, and the correlation coefficient of changes in hormone concentrations with changes in body weight over this period

	n	–4 weeks	0 weeks	–4 to 0 weeks	*P*-value	Correlation coefficient (*r*)	*P*-value
Hypothalamo-pituitary-thyroid axis							
TSH (mIU L^{-1})	45	1.89 ± 0.1	1.84 ± 0.1	–0.05 ± 0.1	0.51	–0.25	0.10
Free T4 (pmol L^{-1})	46	11.1 ± 0.2	11.3 ± 0.2	0.2 ± 0.2	0.26	–0.11	0.48
Free T3 (pmol L^{-1})	46	4.85 ± 0.1	4.89 ± 0.1	0.04 ± 0.1	0.55	–0.04	0.78
Reverse T3 (ng mL^{-1})	44	0.25 ± 0	0.25 ± 0	0 ± 0	0.90	–0.30	0.06
Hypothalamo-pituitary-adrenal axis							
ACTH (pmol L^{-1})	46	6.93 ± 0.5	6.91 ± 0.4	–0.02 ± 0.5	0.97	0.10	0.52
Cortisol (nmol L^{-1})	46	252 ± 11	267 ± 11	14 ± 12	0.25	0.15	0.33
Hypothalamo-pituitary-gonadal axis							
LH (IU L^{-1})	46	3.9 ± 0.3	3.7 ± 0.3	–0.2 ± 0.2	0.47	–0.08	0.61
FSH (IU L^{-1})	46	5.4 ± 0.5	5.1 ± 0.5	–0.3 ± 0.1	0.05	–0.29	0.05
Testosterone (nmol L^{-1})	46	11.7 ± 0.5	12.1 ± 0.5	0.4 ± 0.3	0.13	–0.26	0.09
SHBG (nmol L^{-1})	44	20.4 ± 1.2	20.9 ± 1.2	0.5 ± 0.4	0.26	–0.23	0.15
Free testosterone (pmol L^{-1})	46	333 ± 15	339 ± 14	6 ± 10	0.53	–0.15	0.33
% free testosterone (%)	46	2.89 ± 0.1	2.85 ± 0.1	–0.04 ± 0	0.21	0.13	0.38
FAI (%)	46	59.7 ± 3.4	59.9 ± 3	0.2 ± 1.9	0.92	–0.02	0.87
Hypothalamo-pituitary-somatotropic axis							
IGF-1 (ng mL^{-1})	25	207 ± 39	231 ± 43	24 ± 25	0.35	0.10	0.61
Gut-derived appetite- regulating hormones							
Ghrelin (pg mL^{-1})	45	1030 ± 43	1020 ± 44	–5 ± 41	0.90	–0.38	0.01⁺
PYY (pg mL^{-1})	43	329 ± 20	314 ± 17	–15 ± 19	0.43	–0.37	0.02⁺
Insulin and leptin							
Insulin (pmol L^{-1})	46	137 ± 11	112 ± 9	–25 ± 9	0.01*	–0.11	0.46
Leptin (ng mL^{-1})	44	13.5 ± 1.1	10.7 ± 0.8	–2.8 ± 0.8	0.002**	0.17	0.29

Data are means ± SEM of 25–46 obese men.

*$P < 0.05$ and **$P < 0.01$ for comparisons between 0 and –4 weeks.

⁺$P < 0.05$ for the correlation coefficient between changes in circulating hormone levels and changes in weight.

ACTH, adrenocorticotropic hormone; FAI, free androgen index; FSH, follicle-stimulating hormone; IGF-1, insulin-like growth factor-1; LH, luteinizing hormone; PYY, peptide YY; SHBG, sex hormone-binding globulin; T3, triiodothyronine; T4, thyroxine; TSH, thyroid-stimulating hormone.

To address whether the significant changes in average circulating insulin and leptin levels may have emanated from individuals who lost the greatest amount of weight during the weight stabilization period, we calculated the correlation coefficient between individual weight changes and hormonal changes over the 4-week period. However, apart from a significant negative correlation between weight change and plasma ghrelin or peptide YY levels, there were no significant correlations between weight change and hormone levels over the 4-week period, as shown in Table 1.

Discussion

The aim of the present study was to determine the effect of a 4-week weight stabilization period on circulating hormone concentrations in obese men and to assess the necessity of this period in establishing stable baseline values of a variety of hormones typically investigated in weight loss studies. This study shows that significant reductions in circulating insulin and leptin concentrations occurred during the 4-week weight maintenance period. This effect was not due to weight loss, since the average weight loss during this period was small (less than 1.5% of body weight), and moreover, there was no significant correlation between weight change and changes in circulating insulin or leptin levels. No significant changes occurred in circulating concentrations of other hormones studied, namely TSH, free T4, free T3, reverse T3, ACTH, cortisol, LH, FSH, testosterone, SHBG, free testosterone, % free testosterone, FAI, IGF-1, ghrelin and PYY. Taken together, these data suggest that the use of a weight maintenance period prior to commencement of a weight loss intervention may be necessary for establishing stable baseline values in studies investigating the effects of dietary interventions on circulating insulin and leptin levels, but not in studies investigating circulating thyroid, adrenal, gonadal and somatotropic hormone levels, nor gut-derived appetite-regulating hormones.

There are several reasons why insulin and leptin levels may have significantly decreased during the weight stabilization period. As participants were free-living in the weeks prior to consenting to and enrolling in the trial, the fluctuations in circulating insulin and leptin levels during the weight maintenance period may have been due to changes in energy intake and/or dietary composition compared to during the preceding, unregulated period. Thus, if a participant were to overfeed prior to the trial, their insulin and leptin levels may have been on an incline at the beginning of the trial [**13, 17, 28**], giving the illusion of a higher than usual level. The subsequent decline observed in these hormones after commencing the energy balance diet may reflect a relative decrease in energy intake and return to the participants' true energy balance values. Additionally, diet composition may have differed during the energy balance diet compared with the participants' habitual diet. For example, research has shown that a decrease in dietary carbohydrate content will decrease circulating levels of insulin and leptin to a greater extent than a decrease in dietary fat content [**29-32**]. While any such changes in energy intake or dietary composition may have contributed to the significant decreases in circulating insulin and leptin levels observed, these effects or others were not strong enough to affect other hormones under investigation in this study.

This study demonstrates the need for inclusion of a weight stabilization period in trials involving obese participants where circulating concentrations of insulin and leptin are under investigation. Such a period is likely to be of particular importance in trials involving dietary treatments that differ in composition to participants' normal diets. Without normalization, any changes detected at the conclusion of the intervention cannot be attributed to the intervention *per se*, as they may be partly attributable to fluctuations that occurred before the trial commenced.

While circulating ghrelin and PYY and levels were not significantly affected by the weight-stabilizing period, it is noteworthy that there were significant negative correlations between the change in body weight and the change in these hormone concentrations during this 4-week period. In other words, the more weight that was lost during the 4-week weight stabilization period (i.e. the smaller or more negative the number for weight change), the greater or more positive the number for the change in circulating ghrelin or PYY levels between −4 and 0 weeks. This finding could reflect causal relationships in either direction (e.g. greater weight losses might be expected to trigger greater compensatory increases in circulating levels of the hunger-promoting hormone ghrelin [**6, 13**]; or people who exhibit the smallest reductions in circulating levels of the satiety-promoting hormone PYY might be expected to have a higher level

of satiety and hence higher weight losses [**6, 14, 15**]). However, since overall average weight loss was small during the weight stabilization period (less than 1.5% of body weight), this correlation was not sufficient to cause any overall differences in average circulating ghrelin or PYY levels over the 4-week weight stabilization period. We thus conclude that a weight stabilization period is not required to establish stable baseline values of circulating ghrelin and PYY.

This study has several strengths and weaknesses. It benefits from the incorporation of a large panel of hormones and the rigor with which the weight stabilization was conducted. As this study only included males, future studies involving female participants may be required to determine whether similar changes in hormones across this time period occur in this group. A further limitation of the study is the length and sampling times of the 4-week energy balance diet. Increasing the sampling duration and frequency may inform as to the shortest necessary weight maintenance period for insulin and leptin. However, we hypothesize that no longer than 2 weeks of weight stabilization would be required to stabilize hormone levels because previous research has shown that changes in circulating concentrations of the thyroid hormones T3 and reverse T3 that occur in response to significant weight fluctuations were completely normalized after 10 days on a weight maintenance diet [**33**], and because – unlike the study cited above – participants in the current study did not undergo any major weight changes in the 6 months preceding the trial, as determined by self-report. Despite the above-mentioned limitations, this study has conclusively demonstrated that weight stabilization does not provide any benefit to the stabilization of most of the hormones under investigation here (the gut-derived appetite-regulating hormones ghrelin or PYY, or thyroid, adrenal, gonadal or somatotropic hormones). This finding provides evidence-based options to obesity researchers, because studies where these hormones are primary outcomes do not need to include a weight stabilization period in order to establish stable baseline hormone levels. In contrast, for studies where circulating insulin and leptin levels are primary outcomes, a weight stabilization period may be necessary, albeit more work would be required to determine the minimum time required.

In sum, this study demonstrated that significant decreases in circulating levels of insulin and leptin (but not that of gut-derived appetite-regulating hormones, as well as those under control of the hypothalamo-pituitary-thyroid, -adrenal, -gonadal and -somatotropic axes) occurred when obese men underwent an energy balance diet designed to achieve weight maintenance for 4 weeks. Thus, future trials to determine the effects of interventions on circulating insulin and leptin levels in obese adult males should use a weight maintenance period prior to intervention, in order that any changes in insulin or leptin levels are attributed to that intervention *per se*. Conversely, in studies that aim to investigate the effects of interventions in obese participants on the other hormones listed above, it would seem unnecessary to incorporate a weight stabilization period in order to establish the participant's normal value.

Conflict of Interest Statement

No conflict of interest was declared.

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Author contributions

AS planned and oversaw the hormone and data analyses for this study as well as the writing of the manuscript, and participated in designing and obtaining funding for the overall study. IRE analyzed the data, contributed to some hormone analyses and wrote the manuscript. REW

undertook the intervention and blood sampling in the participants in this trial and provided critical feedback on the overall study design, data analysis and manuscript preparation. RVS analyzed some hormones for this study, and provided critical feedback on data analysis and manuscript preparation. NAK and APH provided critical inputs into the overall study design and participated in obtaining funding for the study, as well as providing critical feedback on the manuscript. NMB took overall responsibility for the parent trial, from leading the overall study design and funding bid, overseeing and participating in the intervention, and providing critical feedback on the manuscript.

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