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Appetite-regulatory hormone responses on the day following a prolonged bout of moderate-intensity exercise

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Running Head: Latent Appetite-Regulatory Responses Following Exercise

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Abstract

Exercise increases energy expenditure however acutely this does not cause compensatory changes in appetite or food intake. This unresponsiveness contrasts the rapid counter-regulatory changes seen after food restriction. The present investigation examined whether corrective changes in appetite-regulatory parameters occur after a time delay, namely, on the day after a single bout of exercise. Nine healthy males completed two, two-day trials (exercise & control) in a random order. On the exercise trial participants completed 90 min of moderate-intensity treadmill running on day one (10:30 – 12:00 h). On day two appetite-regulatory hormones and subjective appetite perceptions were assessed frequently in response to two test meals provided at 08:00 and 12:00 h. Identical procedures occurred in the control trial except no exercise was performed on day one. Circulating levels of leptin were reduced on the day after exercise (AUC 5841 ± 3335 vs. 7266 ± 3949 ng\(^{-1}\)·mL\(^{-1}\)·7 h, \(P = 0.012\)). Conversely, no compensatory changes were seen for circulating acylated ghrelin, total PYY, insulin or appetite perceptions. Unexpectedly, levels of acylated ghrelin were reduced on the exercise trial following the second test meal on day two (AUC 279 ± 136 vs. 326 ± 136 pg\(^{-1}\)·mL\(^{-1}\)·3 h, \(P = 0.021\)). These findings indicate that short-term energy deficits induced by exercise initially prompt a compensatory response by chronic but not acute hormonal regulators of appetite and energy balance. Within this 24 h time-frame however there is no conscious recognition of the perturbation to energy balance.

Key words: Exercise, Appetite Regulation, Gut Peptides, Compensation
1. Introduction

The relationship between exercise and appetite regulation has important implications regarding the role of exercise in weight management [1]. In recent years, advancements in scientific understanding regarding the psycho-biological regulation of appetite and food intake have ignited research interest around the interaction between exercise, appetite regulation and energy balance [2]. Within this sphere, one particular issue that has received significant attention is the impact of exercise on hormonal mediators of appetite which are central components of the body’s homeostatic system governing energy balance and weight control [3,4].

The body’s appetite-regulatory system includes several peptides of gastro-intestinal, pancreatic and adipose tissue origin, which communicate acute nutrient status and chronic energy availability to the central nervous system [4]. Leptin and insulin act as chronic mediators of energy balance, with circulating concentrations being present in proportion to stored energy within adipose tissue [5]. Additionally, on a meal-to-meal basis, food intake is regulated by a selection of gastrointestinal peptides, most notably acylated ghrelin, peptide-YY (PYY), glucagon-like peptide-1 (GLP-1), cholecystokinin (CCK) and oxyntomodulin [6]. Ghrelin is secreted from the stomach and remains unique as the only circulating appetite stimulating hormone. Furthermore, the prandial profile of ghrelin is suggestive of an important role in meal initiation [7,8]. Conversely, each of the other short-acting peptides has an inhibitory effect on appetite. Highly prominent is PYY which is secreted chiefly from the distal intestine and colon in direct proportion to the energy content of an ingested meal [9,10]. Within key appetite-regulatory brain centres these afferent signals are
integrated and the summed response initiated which impacts directly up on appetite and eating, as well as thermogenesis and substrate metabolism [11].

Research has demonstrated that single bouts of exercise have a marked impact on the circulating levels of appetite-regulatory hormones with changes occurring rapidly after the initiation of exercise [2]. Notably however, these alterations appear to be transient. For example, circulating levels of acylated ghrelin are distinctly suppressed during exercise of moderate-intensity or higher [12,13,14]. This perturbation however is absent within 30 min after exercise. Similarly, circulating concentrations of PYY increase during moderate- to high-intensity exercise however customary levels are re-established shortly thereafter [2,15,16]. Each of these responses is consistent with an appetite-inhibitory profile which may in part contribute to a well-characterised inhibition of appetite at moderate-high exercise intensities, a phenomenon which has been termed ‘exercise-induced anorexia’ [17].

Studies have shown that acute energy deficits induced by food restriction lead to rapid and quite striking compensatory alterations to appetite and appetite-regulatory hormones i.e. hormones change in directions expected to stimulate appetite and eating [14,18]. Intuitively, it may be expected that energy deficits induced by exercise would lead to similar responses in appetite-regulatory parameters however several studies have failed to observe any compensatory changes in circulating appetite hormones (acylated ghrelin or PYY) on the day that exercise is performed. This unresponsiveness occurs with bouts of exercise associated with high levels of energy expenditure i.e. large perturbations to energy balance, and over several hours of observation afterwards [13,14]. This is consistent with a lack of change in
energy intake [19]. It remains possible that there is a time-delay before exercise-induced energy deficits manifest as alterations in appetite, appetite regulatory parameters and food intake. This notion was postulated half a century ago [20] and in the absence of altered oro-gastric input, may reflect a greater time-span necessary for the body to detect and respond to exercise-induced energy balance perturbations. This notion is supported by previous evidence which identified latent changes in circulated leptin on the day after exercise [21,22]; and the findings of a recent study which documented an exercise-induced suppression of fasting and meal-stimulated plasma acylated ghrelin response 12 h after undertaking exercise [23].

The present study assessed the latent effects of exercise on appetite and critical mediators of appetite control and energy balance on the day after a single bout of exercise. Specifically, we sought to confirm and extend previous findings by characterising the meal-stimulated (breakfast and lunch) responses of key acute and chronic appetite-regulatory hormones (acylated ghrelin, total PYY, leptin & insulin) on the day after a single bout of exercise. We hypothesised that meal-stimulated acylated ghrelin (suppression) and PYY (elevation) responses would be attenuated on the day after exercise whilst circulating levels of leptin would be reduced. Furthermore, we thought that these changes would be associated with commensurately altered subjective appetite perceptions.
2. Materials & Methods

2.1 Participants

After receiving local ethical advisory committee approval nine young, healthy male volunteers (age 22.0 ± 1.2 y; weight 72.0 ± 6.9 kg; BMI 22.6 ± 1.8 kg·m²; waist circumference 74.4 ± 1.8 cm; estimated basal metabolic rate 7247 ± 405 kJ; \( \dot{\text{VO}}_2 \) max 60.6 ± 7.6 mL·kg·min\(^{-1}\)) gave their written informed consent to participate. Participants were weight stable (< 2 kg change in body mass in the last three months), non-smokers, free of cardio-metabolic disease, had a BMI within the healthy range (18.5 – 24.9 kg·m²) and were not taking any medications or supplements. Participants were active i.e. typically games players, but were not accustomed to undertaking endurance exercise regularly.

2.2 Pre-assessment and Study Familiarisation

Before main trials, participants attended the laboratory where they were familiarised with the study procedures and underwent necessary pre-assessments. Participants completed questionnaires assessing health status and physical activity habits after which measurements of height, weight and waist circumference were taken. Participants then completed two treadmill running tests; 1) a progressive 16 min submaximal test to determine the relationship between treadmill running speed and oxygen consumption; 2) a maximum oxygen uptake test (\( \dot{\text{VO}}_2 \) max). These tests have been described in depth previously [12].

2.3 Main Experimental Trials
In subsequent weeks participants completed two main experimental trials (exercise and control) separated by a washout period of at least seven days. Each main trial spanned across two days and was preceded by a 48 h lead-in phase where diet and physical activity (absence of) were standardised. Within this standardisation phase dietary intake was controlled by the participants i.e. on each participant’s first trial they ate *ad libitum* however participants recorded what they ate and replicated it exactly in the lead up to their second main trial. Adherence to this procedure was confirmed verbally by the study experimenters before main trials. Each main trial was composed of an intervention phase (day one) and a data-collection phase (day two). This design permitted the assessment of appetite-regulatory responses on the day after exercise. The order of main trials was randomised with five participants completing the control trial first and four completing the exercise trial first. Figure 1 provides a schematic illustration of the main trial protocol.

**Figure 1:** Schematic illustration of the main trial protocol. For day two, thin back arrows represent appetite scales; large arrows represent blood samples; black rectangles indicate test meals.

Main trials began on the morning of day one and ended at approximately 15:10 on day two. During this period participants were required to attend the laboratory
between 10:00-13:30 on day one and 07:30-15:10 on day two. In the time away from the laboratory participants were instructed to remain completely inactive and this was checked repeatedly by the study experimenters via telephone. During the study, participants travelled to and from the laboratory via motorised transport unless they lived within 400 meters in which case they were permitted to walk. During main trials participants were provided with all of their food which was consumed at set times that were standardised across trials. Water was permitted *ad libitum* on day one, however to avoid any impact on appetite and/or gastric function during the data-collection phase of trials water consumption was standardised on day two.

On day one of the exercise trial participants consumed their standardised breakfast at home at 07:30. At 10:00 participants arrived at the laboratory ahead of their treadmill run (10:30-12:00). Herein, participants ran on a motorised treadmill (Technogym Excite Med, Cesena, Italy) for 90 min at a speed predicted to elicit 70% of their maximum oxygen uptake. At 15 min intervals oxygen uptake was assessed via expired air collections into a Douglas Bag and the speed of the treadmill was adjusted if necessary to maintain the desired exercise intensity. Ratings of perceived exertion were also assessed using the Borg scale [24]. Following the run participants rested in the laboratory until lunch (13:00). After lunch participants went home where they remained (inactive) until returning to the laboratory the following morning. At 18:00 participants consumed their standardised evening meal which was followed by their evening snack at 20:00.

Participants arrived at the laboratory on the morning of day two at 07:15. A cannula was then inserted into an antecubital vein after which participants rested for 30 min.
At 08:00 the data-collection phase of the trial began whereby baseline blood samples were collected and appetite scales completed. A test meal was then consumed over 10 min. On the final bite a clock was started which ran continuously for seven hours. At 4h a second test meal was consumed. Across this period blood samples were collected for the assessment of appetite-regulatory hormones at 0.5, 1, 1.5, 2, 3, 4, 4.5, 5, 5.5, 6 & 7h. Subjective appetite perceptions (hunger, fullness, satisfaction & prospective consumption) were assessed at 30 min intervals throughout using visual analogue scales [25]. Main trials ended after the final blood sample and appetite scale at 7 h, at which point the cannula was removed and participants left the laboratory.

Identical procedures were undertaken in the control trial except on day one participants did not complete any exercise. Instead, they rested within the laboratory and expired air samples were collected every 15 min in order to permit the calculation of net energy expenditure during exercise i.e. gross exercise energy expenditure minus resting energy expenditure.

2.4 Food Provision & Test Meals

On day one of main trials participants received all of their food pre-packaged from the study team with the food provided being identical in the exercise and control trial. The amount of food (energy) each participant received was calculated as 1.4x their estimated basal metabolic rate [26]. This is an amount of food deemed sufficient to meet the needs of an individual on an inactive day. On day one breakfast consisted of white bread and chocolate spread (carbohydrate 64%, fat 25%, protein 11%, 2029 ± 113 kJ - 20% of daily energy provision). Lunch and dinner was a balanced meal
consisting of a tuna and mayonnaise sandwich, salted crisps, chocolate muffin and green apple (carbohydrate 48%, fat 33%, protein 19%, 3552 ± 197 kJ - each meal 35% of daily energy provision). Finally, participants received a chocolate biscuit for the evening snack (carbohydrate 52%, fat 46%, protein 2%, 1013 ± 59 kJ - 10% of daily energy provision).

On day two of trials participants received two (baseline and 4 h) balanced (48% carbohydrate, 19% protein, 33% fat, 2565 kJ energy) test meals that were identical within and between trials. Each participant received the exact same meal i.e. the meal was not normalised to participants’ daily energy requirements. Each test meal consisted of white bread (109g), cheddar cheese (48g), malt loaf (30g) semi-skimmed milk (100mL) and strawberry milkshake powder (7.5g). Each meal was consumed within 10 min. To ensure euhydration on day two, participants consumed 500 mL of water up on rising. Participants also drank 250 mL of water one hour after each test meal (1 h and 5 h).

2.5 Blood Biochemistry

During day two of main trials venous blood samples were collected via a 21G cannula (Venflon, Becton Dickinson, Helsingborg, Sweden) that was kept patent throughout by flushing with isotonic saline (0.9% w/v sodium chloride). Samples were collected into ice-cooled EDTA monovettes for the determination of plasma leptin, acylated ghrelin, insulin and glucose. To preserve the integrity of the acylated ghrelin sample, monovettes for this peptide were pre-treated with a serine protease inhibitor as described previously [12]. Samples for total PYY were collected into ice-cooled syringes containing 10µL/mL di-peptidyl peptidase-4 inhibitor (Millipore,
Watford, UK) and after mixing were immediately dispensed into EDTA tubes containing aprotinin (Nordic Pharma Ltd, Reading, UK) (500 KIU/mL). Plasma was obtained after spinning whole blood samples at 1600 g for 10 min in a refrigerated centrifuge (4°C) and was stored at -80°C until analysis. At baseline and 4 h measurements of haematocrit and haemoglobin were taken to estimate changes in plasma volume using the method described by Dill & Costill [27].

Concentrations of plasma acylated ghrelin (SPI BIO, Montigney le Bretonneux, France), total PYY (Millipore, Watford, UK), leptin (R and D Systems Europe Ltd., Abingdon, UK) & insulin (Mercodia, Uppsala, Sweden) were determined using enzyme-linked immunosorbant assay kits. Plasma glucose concentrations were determined using an enzyme-linked assay kit (Life Technologies Ltd, Paisley, UK). The associated within-batch coefficient of variation for the assays was as follows: acylated ghrelin (7.8%), leptin (6.3%), insulin (3.5%), total PYY (7.1%) and glucose (2.5%).

2.6 Statistical Analysis
Data were analysed using the Statistical Package for the Social Sciences (SPSS) software version 21.0 for Windows. Two-way repeated measures ANOVA were used to examine responses over time for appetite-regulatory hormones and appetite perceptions. Where significant differences were found these were explored using post hoc analysis using the Bonferroni correction for multiple comparisons. When significant main effects were found area under the curve was calculated using the trapezoid method. Statistical significance was accepted at the 5% level. Repeated measures ANOVA (trial x time) showed no differences in plasma volume within (P =
0.504) or between ($P = 0.834$) trials therefore unadjusted plasma hormone concentrations are presented. Results are presented as Mean ± SD unless stated otherwise.

The sample size for this investigation was determined using data derived from the authors’ previous research which detected compensatory acylated ghrelin responses to food restriction [14]. Based on total trial AUC data (control vs. food restriction), with alpha set at 5%, beta at 80%, and a previously observed mean difference and standard deviation of 315 and 260 pg·mL$^{-1}·$h$^{-1}$ - it was determined that at least eight participants were required to provide sufficient statistical power for the present investigation.

3 Results

3.1 Exercise Responses

The 90 min run undertaken on day one was completed at 11.1 ± 1.7 km·h$^{-1}$ which elicited 67.8 ± 4.3% of participants’ maximum oxygen uptake. This induced a net energy expenditure of 4908 ± 523 kJ which was derived predominantly from carbohydrate oxidation rather than fat (74 ± 14 vs. 26 ± 14%). A reported RPE value of 15 ± 1 indicated that participants perceived the run to be ‘hard’.

3.2 Appetite Hormone & Glucose Responses

On the morning of day two, plasma acylated ghrelin concentrations were no different between the exercise and control trial ($P = 0.56$) (Figure 2 upper panel).
Figure 2: Plasma acylated ghrelin (upper panel) & PYY (lower panel) concentrations in the control (▲) and exercise (■) trials. For clarity values are mean ± SEM, n = 9. Black squares indicate test meals (-10 min and 4 h).

Two-way repeated measures ANOVA (trial x time) revealed significant time (P < 0.001) and interaction (P = 0.009) main effects for acylated ghrelin indicating divergent changes over time between trials. Following correction for multiple comparisons using the Bonferroni method no differences at individual time points
were found. Further analysis of the acylated ghrelin AUC identified significantly reduced levels (14%) on the exercise trial following consumption of the second test meal at 4 h (Table 1). However, when normalised to pre-lunch values at 4 h this difference did not remain significant ($P = 0.290$). Moreover, the percentage suppression of circulating acylated ghrelin was no different between trials within the 90 min after eating at the meals consumed at baseline (0 h) or 4 h (both $P > 0.05$).

Table 1: Day two circulating acylated ghrelin and leptin area under the concentration-time curve profiles

<table>
<thead>
<tr>
<th></th>
<th>Total Trial (0-7 h)</th>
<th>Test Meal 1 Response (0-4 h)</th>
<th>Test Meal 2 Response (4-7 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>units 7 h</td>
<td>units 4 h</td>
<td>units 3 h</td>
</tr>
<tr>
<td><strong>Acylated Ghrelin</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>698 ± 298 units</td>
<td>371 ± 166 units</td>
<td>326 ± 136 units</td>
</tr>
<tr>
<td>Exercise</td>
<td>623 ± 312 units</td>
<td>344 ± 179 units</td>
<td>279 ± 136 units</td>
</tr>
<tr>
<td><strong>Leptin</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>7266 ± 3949 units</td>
<td>3697 ± 3068 units</td>
<td>3570 ± 2006 units</td>
</tr>
<tr>
<td>Exercise</td>
<td>5841 ± 3335* units</td>
<td>3068 ± 1626* units</td>
<td>2773 ± 1725* units</td>
</tr>
</tbody>
</table>

Values are pg·mL·unit time and ng·mL·unit time for acylated ghrelin and leptin (mean ± SD, $n = 9$). *different from control ($P < 0.05$)

At baseline on day two the fasting plasma concentration of total PYY was no different between the exercise and control trial (Figure 2 lower panel). Two-way repeated measures ANOVA (trial x time) revealed no differences between trials (all $P > 0.05$).

On day two, baseline circulating levels of plasma leptin were significantly lower on the exercise trial compared with control ($P = 0.03$) (Figure 3 upper panel).
Figure 3: Plasma leptin (upper panel) & insulin (lower panel) concentrations in the control (▲) and exercise (■) trials. For clarity values are mean ± SEM, n = 9. Black squares indicate test meals (-10 min and 4 h).

For circulating leptin, two-way repeated measures ANOVA (trial x time) revealed significant trial (P = 0.016), time (P < 0.001) and interaction (P = 0.009) main effects. After correction for multiple comparisons using the Bonferroni method no differences were found at individual time points between trials. The plasma leptin AUC showed
significantly reduced circulating levels across the entirety of day two (Table 1). At baseline on day two there was no difference in the fasting plasma concentration of insulin between the exercise and control trial (Figure 3 lower panel). Two-way repeated measures ANOVA (trial x time) revealed no between trial differences for plasma insulin (all \( P > 0.05 \)). For circulating glucose there was no difference in the fasting plasma concentration between trials on the morning of day two (\( P = 0.233 \)). Furthermore, two-way repeated measures ANOVA (trial x time) revealed no differences between trials for plasma glucose (all \( P > 0.05 \), data not shown).

To examine alterations in insulin resistance on the morning after exercise HOMA-IR was calculated using fasting concentrations of glucose and insulin [28]. At baseline on day two of the exercise trial HOMA-IR was significantly lower (31%) compared with control (\( P = 0.031 \)). On day two, further analysis revealed significant positive associations between HOMA-IR and circulating leptin AUC within the exercise trial for the total trial \( (r = 0.678) \) and 4-7 h AUC \( (r = 0.699) \) (both \( P < 0.05 \)). No significant relationships between leptin and insulin resistance were apparent in the control trial. Furthermore, there were no significant relationships between HOMA-IR and circulating acylated ghrelin or total PYY.

3.3 Appetite Responses

There were no significant differences in fasting appetite perceptions on day two (hunger, fullness, satisfaction and PFC) between the exercise and control trial (all \( P > 0.05 \)) (Figure 4). For each appetite perception two-way repeated measures ANOVA (trial x time) revealed a main effect of time (all \( P < 0.001 \)) representing changes in
response to test meals. However, no significant trial (all $P > 0.05$) or interaction (all $P > 0.05$) main effects were found.

**Figure 4:** Subjective ratings of hunger (top left), prospective food consumption (top right), fullness (bottom left) and satisfaction (bottom right) in the control (▲) and exercise (■) trials. For clarity values are mean ± SEM, $n = 9$. Black squares indicate test meals (-10 min and 4 h).

### 4 Discussion

Several studies have shown that there are no acute compensatory changes in appetite or appetite-regulatory hormones on the day during which an acute bout of exercise is performed [19,29]. This investigation extended the period of observation in order to determine whether compensatory changes in appetite-regulatory parameters may occur after a time delay, namely, on the day after exercise. Based on previous research suggesting that alterations in appetite regulatory parameters may occur after a time-delay [20,21,23], we hypothesised that meal-stimulated acylated ghrelin (suppression) and PYY (elevation) responses would be attenuated
on the day after exercise whilst circulating levels of leptin would be reduced. Furthermore, we thought that these changes would be associated with commensurately altered subjective ratings of appetite. In contrast to our hypotheses, the novel findings from this study are that acute exercise did not lead to compensatory fasting or prandial acylated ghrelin, total PYY or subjective appetite responses on the day after exercise. Paradoxically, circulating levels of acylated ghrelin were actually lower following a lunchtime meal consumed 24 h after the end of exercise. In addition to these novel outcomes, this study has also re-affirmed previous findings documenting a delayed reduction in circulating leptin after a single bout of exercise (with a large associated energy deficit) [21,22,30].

Within the acute appetite-regulatory system acylated ghrelin remains unique as the only circulating peptide that stimulates appetite and eating. Specifically, on a meal-to-meal basis, levels of acylated ghrelin rise and fall in timing with prandial changes in hunger, a pattern suggesting an important role in regulating meal initiation and/or termination [7,8]. Alongside this acute action, significant attention has also been given to understanding the extended role that acylated ghrelin plays within the regulation of energy balance and body weight. In this scenario, acylated ghrelin responds dynamically to changes in energy balance with increases in circulating levels during periods of energy deficit being a key homeostatic response to defend body weight [31,32].

In the present investigation we hypothesised that exercise completed on day one would lead to higher circulating levels of acylated ghrelin on day two as a counter-regulatory response to the energy deficit. Conversely, on day two, we saw no
changes in circulating levels of acylated ghrelin at rest or in response to the morning test meal. Interestingly, however, after consumption of the second test meal consumed at lunch (13:00), circulating levels of acylated ghrelin (AUC) were actually lower on the exercise trial. In an exercise context, previous studies have described an attenuated postprandial acylated ghrelin response, i.e. a less marked suppression, after individuals have completed multiple bouts of exercise across several days [33,34]. Furthermore, one recent study with a similar design to the present study also reported an attenuated postprandial ghrelin response on the day after participants had performed 1 h of moderate-intensity exercise [23]. This physiological change reflects an impaired satiety response and in theory would be associated with a more rapid onset of subsequent eating and potentially a greater energy intake at meals. It is not entirely clear why the findings differed in the present investigation. In the studies of Hagobian et al [34] and Mackelvie et al [33] it is possible that the attenuated meal-related change in acylated ghrelin reflects the accumulated energy deficit created over multiple days; however it should be noted that in the former study this response was only seen in women and not men. The present investigation studied the more short-term impact of a single bout of exercise on acylated ghrelin and this difference may contribute to the divergent outcome. The reason for the difference in findings between the present investigation and that of Heden et al [23] is less clear given the similarity in study design, participants examined and test meals implemented. Specifically, Heden et al [23] reported lower fasting levels of acylated ghrelin on the morning of day two after exercise and observed an attenuated meal-related (breakfast) suppression. Conversely, we did not see any difference in fasting levels of acylated ghrelin on day two, nor did we detect any difference in the postprandial acylated ghrelin AUC (0-4 h). This lack of change in
fasting acylated ghrelin concentration is consistent with what we have previously found when fasting samples were collected on the day after a 90 min treadmill run (22.5 h post-exercise) in young, healthy, males [13]. Although the precise mechanisms are unknown, key differences between studies may explain the divergent outcomes, including the elapsed time before sampling (12 h vs. 20 h), intensity of exercise (moderate vs. moderate-vigorous) and energy expenditure elicited (1800 vs. 4908 kJ). Furthermore, in the study conducted by Heden et al [23] participants remained fasted overnight after performing exercise that evening, whilst in the present investigation participants consumed two meals and a snack in-between exercise and the beginning of blood assessments the next day. This protocol difference may have impacted on acylated ghrelin via alterations in substrate metabolism. Nonetheless, despite these alterations in acylated ghrelin, neither investigation observed any change in subjective appetite perceptions indicating that this physiological change did not translate into altered behaviour.

The mechanisms responsible for alterations in circulating acylated ghrelin in response to exercise are not well understood. In the present study, closer scrutiny of the acylated ghrelin data shows that the lower AUC (4-7 h) in the exercise trial was primarily due to a reduced level of acylated ghrelin before the afternoon test meal (at 4 h). This was evidenced by the negation of difference between trials (4-7 h AUC) when values were normalised to those at 4 h; and by the lack of difference in postprandial suppression. Consequently, the lower acylated ghrelin values identified appear to be due to an attenuated pre-prandial rise leading up to the second test meal. Indeed, each of the nine participants all had lower circulating acylated ghrelin concentrations at 4 h on the exercise trial as compared with control. This information
suggests that exercise altered the regulatory mechanisms governing the pre-meal ghrelin surge rather than post-ingestive factors. Unfortunately the present study cannot delineate the specific mechanisms responsible for this finding therefore further research is needed to advance this work and to better understand the broader impact of exercise on the regulation of ghrelin.

PYY is an anorectic peptide secreted primarily by the distal intestine in response to nutrient intake [9,35]. Circulating levels of PYY typically peak 1-2 h postprandially in relation to the energy and macronutrient content of the meal with levels remaining elevated for several hours [10,36]. PYY has a critical role in the short-term regulation of energy intake due to its important role in promoting satiation, satiety and delaying gastrointestinal transit [36-38]. A more long-term influence of PYY on energy homeostasis has also been suggested by associations that have been found between PYY, substrate oxidation and resting metabolic rate [39-40].

Short-term food restriction [14,41] and reductions in body weight [42] have each been shown to lower fasting and/or postprandial circulating levels of PYY. This response is likely to be part of an adaptive mechanism defending energy homeostasis. The impact of exercise on circulating PYY has been examined in several studies with the consensus suggesting that exercise transiently elevates levels of PYY [2,15,16]. A potential limitation of the present study was that circulating levels of total PYY were measured rather than those of PYY\textsubscript{3-36}. The latter variant is the modified peptide that confers the specific inhibitory effect of PYY on appetite, and although the two correlate well [43], it is possible that PYY\textsubscript{3-36} may have responded differently to the intervention. Despite this, the present study is the first to
characterise prandial total PYY responses on the day following an acute bout of exercise. Specifically, we examined whether an acute energy deficit induced by exercise would reduce fasting and/or postprandial levels in the circulation on the following day. The results clearly show that exercise on the prior day had no impact on plasma total PYY responses to meals and these findings therefore demonstrate that total PYY is not sensitive to exercise-induced energy deficits of this magnitude within this time-frame.

In the present investigation one of the most marked changes induced by exercise was a decrease in circulating levels of leptin on the day afterwards. Specifically, in the exercise trial fasting plasma concentrations on day two were a third lower compared with control. Furthermore, across the whole of the day, circulating levels of leptin were reduced by 20% (total trial AUC) after having completed exercise. These data confirm previous reports which have documented reductions in leptin in response to single bouts of exercise. Notably, the consensus arising from previous work, and supported here, are that substantial reductions in circulating leptin occur after exercise when associated with sufficiently high energy expenditure (> 3348 kJ) and following a latency period of ~12-24 h [21,22]. Existing work has shown that circulating levels of leptin are highly responsive to alterations in energy balance/availability [44,45]. Consequently, the change observed in the current study is likely to be related to the energy deficit imposed by exercise (~ 5020 kJ) which was maintained going forward into day two due to strict dietary and physical activity control. It is perhaps interesting to note that comparatively the magnitude of this decrease in leptin is approximately half of that which occurs in response to fasting
over a similar period [46]. The change seen with exercise in this study therefore reflects the less severe perturbation to energy balance.

In concert with leptin, insulin also functions as a chronic regulator of energy homeostasis, providing information to the central nervous system regarding stored energy within adipose tissue [47]. Unlike leptin however, in the short-term, insulin is also a critical regulator of circulating glucose and responds dynamically to systemic perturbations in glycaemia. Additionally, both fasting and postprandial insulin concentrations are mediated at a higher level by insulin sensitivity within peripheral tissues, such as skeletal muscle, liver and adipose tissue. In the present investigation we did not detect any significant changes in fasting or postprandial concentrations of insulin or glucose in the circulation. The nature of the imposed exercise was therefore insufficient to induce alterations in these parameters in this population of young, healthy, and relatively fit men. This information suggests that insulin and glucose had no direct effect on circulating acylated ghrelin or leptin. Despite this, although not statistically different, lower fasting levels of glucose and insulin on day two produced a statistically significant improvement in insulin sensitivity when HOMA-IR was calculated ($P = 0.031$) [28]. Given previous links discovered between appetite hormones with insulin resistance [48,49], we chose to look closer at the relationships between these factors. No relationships were observed between HOMA-IR and acylated ghrelin or total PYY, however significant positive correlations were found between leptin AUC and HOMA-IR across the exercise trial, but not within control. These data indicate that the relationship between insulin resistance/sensitivity and leptin become stronger after exercise however the mechanisms mediating this association are unknown. The improvement
in insulin sensitivity after acute exercise is well defined [50] however the mechanisms governing changes in leptin after exercise are not well understood. The alteration in energy flux may be significant with a potential link to insulin action [51,52] however additional work is needed to better understand these relationships.

The effect of exercise on subjective appetite perceptions has received widespread attention within psycho-biological research over the last 20 years. The most consistent finding within this body of literature is that single bouts of exercise transiently suppress appetite, a phenomenon that has been termed exercise-induced anorexia [17]. This effect is brief, typically lasting no more than 30 min, and does not typically affect food intake when measured for several hours afterwards [13,19,53]. This response to an exercise-induced energy deficit is in direct contrast to that observed when food restriction is used as a method to induce negative energy balance. In this scenario, rapid and marked compensatory increases in appetite and food intake are noted [14,18]. Although in the immediacy a rather loose coupling exists between exercise-induced energy expenditure, appetite and food intake, one study has suggested an association may begin to emerge after a delay of approximately two days [20]. In the present investigation we sought to explore this relationship further within a controlled laboratory setting by assessing changes in subjective appetite parameters on the day after exercise. In the present study, at no point within day two did exercise affect subject ratings of hunger, fullness, satisfaction or prospective food consumption. These results are consistent with those from two previous investigations with similar study designs with either similar [54] or lower exercise-induced energy deficits [23]. Interestingly, in the recent study conducted by Heden et al [23], although no changes in appetite perceptions (hunger
and fullness) were detected in lean participants, prior exercise led to lower postprandial ratings of fullness in a group of obese individuals. This discrepancy may suggest that obese individuals are more sensitive to exercise-related perturbations in energy balance than lean individuals. Nonetheless, in the present population a period of negative energy balance cannot continue indefinitely and although reductions in energy-expending processes are expected to occur, at some point it is likely that a compensatory increase in appetite will manifest. The results from the current study suggest that this lag phase endures for more than 24 h, however further research is needed to determine the exact time-scale of this response.

This study has some limitations which should be recognised. Firstly, although we are confident that participants remained inactive during the time away from the laboratory in-between days one and two, the lack of objective physical activity data prevents us from being 100% certain. Secondly, we studied a small, homogenous group of healthy males and this limits the ability to generalise findings to a wider population. Finally, we did not assess the impact of this intervention on other important appetite regulatory peptides such as glucagon-like-peptide-one, pancreatic polypeptide or oxyntomodulin. We therefore do not know how the current intervention impacted on this wider network of appetite regulatory signals. Future studies that attend to these factors will provide important scientific and translational insight. In particular, extending this work into obese individuals would be valuable.

In conclusion, this study has shown that a large (4908 ± 523 kJ) exercise-induced energy deficit leads to a compensatory decrease in circulating levels of leptin on the day afterwards. Conversely, circulating levels of acylated ghrelin, total PYY and
subjective appetite perceptions do not display counter-regulatory responses within this time-frame. Interestingly, exercise actually led to a reduction in circulating levels of acylated ghrelin in the afternoon on the day following exercise. These data suggest that short-acting appetite-regulatory hormones do not couple strongly to exercise-induced energy deficits within the 24 h after exercise. Instead, exercise-induced perturbations in energy balance of this magnitude manifest within this time-frame as a notable reduction in circulating leptin. This physiological change shows that exercise-induced energy deficits are initially sensed within 24 h however the lack of change in subjective appetite perceptions suggests that this signal does not reach consciousness at this time.

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Author Contributions
JAK and MAN conceived the study. JAK, JOG, BMK and SX performed the experimental procedures. APJ, JAK and SX conducted the biochemical analysis. JAK and MAN wrote the manuscript. All authors reviewed the final version of the manuscript before submission.

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Highlights

- Circulating acylated ghrelin concentrations were reduced on the day after exercise
- Circulating leptin concentrations were reduced on the day after exercise
- Exercise did not affect circulating insulin or total PYY on the day after exercise
- Appetite perceptions were unaltered on the day after exercise