PEER REVIEW HISTORY

BMJ Open publishes all reviews undertaken for accepted manuscripts. Reviewers are asked to complete a checklist review form and are provided with free text boxes to elaborate on their assessment. These free text comments are reproduced below.

ARTICLE DETAILS

Title (Provisional)

Effects of exercise and exercise timing on energy intake and appetite control in Danish individuals with overweight or obesity with and without type 2 diabetes: a protocol for a randomized controlled crossover trial

Authors

Jalking , Lea; Launbo, Natja Poder; Møller Jensen, Marie Møller; Pedersen, Hanne Enghoff; Blond, Martin B; Gerstenberg , Marina Kjærgaard; Finlayson, G; Beaulieu, Kristine; Færch, Kristine; Groth Grunnet, Louise; Quist, Jonas Salling Salling

VERSION 1 - REVIEW

Reviewer	**1**
Name	**Graybeal, Austin J**
Affiliation	**University of Southern Mississippi**
Date	**07-Oct-2024**
COI	**None**

Title:

Minor comment: The title is a little confusing and does not necessarily reflect the aims/hypothesized outcomes of the study. Also, is hedonic “appetite control” the appropriate term since hedonic eating behaviors (probably the more applicable term) are generally a moderator for homeostatic dysregulation (i.e., a lack of control)? An example of a new title could something like “The effect of meal and exercise timing on appetite regulation in individuals with overweight or obesity with and without type II diabetes: a protocol for a randomized controlled crossover trial.”. Finally, you only state obesity in the title but both overweight and obesity in the rest of the study. Please correct.

Introduction:

I think that this introduction takes a lot of time/words to say that appetite regulation is a concern in this group, particularly due to hedonic eating patterns, and because exercise is often a first line recommendation, it is important to know if time of day impacts eating behaviors so that these training programs can be more informed and better support optimal

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eating behaviors. It would be helpful to re-review the introduction and improve its clarity as, at present, it feels like a list of information that does not tie together well. More specific comments below:

Grammatical/punctuation errors throughout that make it somewhat laborious to read. Recommend a thorough re-read before re-submission of the protocol or the final manuscript(s).

Line 95: “continually” or “continuously” increasing.

Line 96: “… while societal structures still fuel the rise…”: this feels like more of an emotional argument. Which societal structures specifically (you could provide parenthesized examples of these institutions/outlets)? By fuel the rise do you mean “contribute to the perpetual/persistent increases in rates of T2D”?

Regarding my first comment, it may be better to lessen the discussion on the influence of insulin resistance to some degree and include this information in the discussion of the final manuscripts. While insulin sensitivity might be improved in the morning hours, there is often less time for daily ambulation to occur (i.e., elevating core body temperature, peak cerebral arousal, etc.) which leads to lower intensity exercise bouts, and there is evidence showing that exercise performance is more optimal in the evening when compared to the morning hours. Though the study is acute, wouldn’t more optimal session performance improve insulin resistance and lead to greater reductions in appetite? This is something to consider. Moreover, if insulin resistance improves in the morning and worsens in the evening (as stated in lines 118-120), and insulin resistance is a primary driver of appetite regulation, then aren’t these individuals more protected from the effects of insulin resistance (regarding appetite) in the morning ; requiring interventions that can aid in the evening hours? You also state that exercise-induced anorexia returns to normal within a few hours. If exercise is performed in the morning hours, this would likely rebound around the times when most eating windows occur (lunch/dinner), which may lead to energy overcompensation in this group (i.e., eat more than they expended during exercise). You state in your hypotheses that reward will be higher for more palatable foods in the evening, but if energy intake is not different (your primary hypothesis), do hedonic mechanisms still “control” eating, or just create an internal environment that is conducive to overconsumption in certain situations? Is it also possible that most adults get hungrier in the evening as a product of lower stress (i.e., work/social/school-related stressors) and a more comfortable environment (i.e., home) with access to the foods they enjoy? I would recommend re-reviewing the introduction, simplifying it, and saving much of the information for future discussion sections.

Line 132: Would this not depend on carbohydrate availability and exercise intensity? Insulin sensitivity is highest in the morning, yes, but I am not sure that reflects evening substrate utilization independent of these factors. This needs more supporting evidence or should be excluded from this section.

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Methods:

Line 179: I think there is too much variance within your groups. While your T2D group is fine, I would recommend stating how this will be verified (i.e., diagnosed T2D or will you perform a metabolic screening test for verification). Moreover, will this group also be required to have obesity?

Line 179: I think the cutoffs for overweight/obesity as they pertain to differences in appetite are the larger issue. For example, a young adult (i.e., ~24 years old) with a BMI of 40 kg/m2 may have worsened appetite than a 50 year old with T2D and a BMI of 29 kg/m2, particularly if they have some indication of impaired insulin at that weight status (i.e., pre-diabetes and/or abdominal obesity). Would you screen for indicators of insulin resistance in those with overweight/obesity and exclude if so? If not, it may also be ideal to use waist circumference and/or fasting blood glucose as an inclusion criteria, particularly for those with overweight. This way, you could argue that each of your groups has some degree of metabolic dysfunction as opposed to a group of muscular people who happen to meet the criteria for “overweight” but do not have impaired insulin or abdominal obesity.

Line 179: Since T2D is a more latent disease, will you control age differences between groups? While the study is primarily a within-subject design, you have between group comparisons, and age will likely be different between these groups. I would recommend reducing the age range to 18-60 years, or controlling for age in your analyses.

Line 179: Aiming for a somewhat equal distribution of males and females as well? Please state if so.

Line 186: Energy intake for how long after exercise (i.e., just before and during the ad libitum test meal)? 24-hour energy intake is listed as a secondary outcome below, so this is somewhat confusing. Please delineate the differences between the energy intake measurements listed for the primary and secondary outcomes.

Line 227: Is 15:00 PM (3:00 PM) considered the evening hours? It would be more ideal to have this occur during normal evening exercise hours (i.e., 5:00 PM) as opposed to what most would consider “mid-late afternoon”.

Line 227: Habitual smokers should be excluded as smoking affects your primary variables of interest (appetite). If you decide not to exclude (I would caution against this), smoking should not be allowed for 24-48 hours prior to testing, though cessation would also likely not be feasible (i.e., increase cravings).

Line 230: Since you are allowing medication, it would be useful to declare whether the participants with T2D are insulin-dependent or not (or either) as this may impact their responses. It is also likely that individuals in your target group will be using, for example, blood pressure medications, which could impact responses to exercise. Will you perform adjustments for medication use in your analyses?

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Line 262: Will you collect markers of subjective appetite at the same timepoints as the blood samples? Please list the timepoints to will collect the VAS measurements. It would be advantageous to collect the VAS measurements to determine whether physiological markers of hunger and satiety align with subjective markers since this is not always the case. Moreover, I recommend collecting both blood samples and subjective appetite markers prior to exercise (i.e., right before the onset; I did not see this listed) and at the midway point during exercise. If it is not feasible to collect the blood samples at the midway point, then at least the VAS markers should be collected.

Line 306: to keep with the ad libitum nature of the study, you could provide them with an excess of water (e.g., 1 L) and let them consume water ad libitum on the first trial, and only give that much water on the subsequent trials. That way they are not being restricted to only 200-250 mL or forced to consume that much if they do not want to. It could also impact appetite if they drink it all in the last 5 minutes of exercise just to meet the instructions for consumption. Same goes for the ab libitum meal. Participants should be able to consume as much water as needed and this should be measured rather than controlled.

Line 313: I do not think that participants should be constrained to a single pie after their first selection. If you hypothesize that desire for more rewarding foods is higher in the evening, and a participant is randomized to an evening visit first, they may select a pie that they find more rewarding that they would not select at other points during the day, and be stuck to that pie they may not want for the remaining visits. It would also be removing the effect of day-to-day changes in food choice, which is a better reflection of how this would occur in the real world. In essence, it would be a more equitable comparison if participants were presented with each pie at each visit and allowed to select. If this was not possible, you should provide a palatability questionnaire at screening and ask each participant to rank their liking of each pie option and use the pie they stated they like the most as the test meal.

Line 335: It would be impressive if you only had a 10% attrition for a study such as this. I would recommend increasing your attrition to 20% but will leave this to the authors discretion.

Statistical analyses: The inferential statistics tests, the correction methods, assumption tests, normality tests, etc. that you plan to run are unclear/not stated (other than GLM or null hypothesis significance tests), and there is no information for how confounding variables will be controlled (or if they will). I would recommend stating the specific analytical plans with the test names and procedures explicitly described. I know that this is a pre-registration so you may want to be broad, but it is better to be specific and then just provide rationale as to why you had to change the plan in the final manuscript(s).

Reviewer **2**

Name **Creasy, Seth**

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Affiliation and diabetes	**University of Colorado School of Medicine, metabolism**
Date	**10-Oct-2024**
COI	**None**

: I commend the authors on this interesting study. There are some items that require further justification/clarification. I look forward to seeing the results of this study.

Abstract

1. Please include the age range in the abstract

2. Was an upper range of BMI used? If so, please clarify in abstract.

Introduction

1. Line 129 – I believe the Sato study suggests that fatty acid metabolism was increased at ZT15 (early in the dark phase = equivalent to morning exercise) and glucose metabolism was increased in ZT3 (early in the light phase = equivalent to evening exercise). Please double check.

2. Line 134 – Please change the word “preferable” to rather indicate direction of results

Recruitment

1. Line 217 – Do they need to meet 1 of 3 criteria? Please clarify

2. Line 226 – Can justification be provided regarding differences in fasting duration and how this could affect results?

3. Line 303 – Why was interval training selected?

4. Line 308 – Will any physio measures been conducted during exercise (heart rate, energy expenditure, etc.)?

5. If someone cannot complete exercise, the workload will be reduced but how will this be considered in statistical analyses?

6. Will individuals with T2D be age, sex and weight matched to individuals without? If so I missed this. If not, why not?

Statistical Methods

1. Line 335 – What if attrition is higher than anticipated? Will sample size be increased?

Table 2. Please list out the appetite hormones that will be measured

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VERSION 1 - AUTHOR RESPONSE

Reviewer #1:

Dr. Austin J Graybeal, University of Southern Mississippi
Comments to the Author:
Title:

Minor comment: The title is a little confusing and does not necessarily reflect the aims/hypothesized outcomes of the study. Also, is hedonic "appetite control" the appropriate term since hedonic eating behaviors (probably the more applicable term) are generally a moderator for homeostatic dysregulation (i.e., a lack of control)? An example of a new title could something like "The effect of meal and exercise timing on appetite regulation in individuals with overweight or obesity with and without type II diabetes: a protocol for a randomized controlled crossover trial.". Finally, you only state obesity in the title but both overweight and obesity in the rest of the study. Please correct.

Answer: Thank you for the suggested changes to the title. We have changed the title to: "*Effects of exercise and exercise timing on energy intake and appetite control in Danish individuals with overweight or obesity with and without type 2 diabetes: a protocol for a randomized controlled crossover trial"*, as we find that this reflects the aims of the study better (page 1, lines 1- 3). The title now also includes overweight, and we have checked the document to make sure both overweight and obesity is mentioned throughout.

Introduction:

I think that this introduction takes a lot of time/words to say that appetite regulation is a concern in this group, particularly due to hedonic eating patterns, and because exercise is often a first line recommendation, it is important to know if time of day impacts eating behaviors so that these training programs can be more informed and better support optimal eating behaviors. It would be helpful to re-review the introduction and improve its clarity as, at present, it feels like a list of information that does not tie together well. More specific comments below:

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is more optimal in the evening when compared to the morning hours. Though the study is acute, wouldn't more optimal session performance improve insulin resistance and lead to greater reductions in appetite? This is something to consider. Moreover, if insulin resistance improves in the morning and worsens in the evening (as stated in lines 118-120), and insulin resistance is a primary driver of appetite regulation, then aren't these individuals more protected from the effects of insulin resistance (regarding appetite) in the morning ; requiring interventions that can aid in the evening hours? You also state that exercise-induced anorexia returns to normal within a few hours. If exercise is performed in the morning hours, this would likely rebound around the times when most eating windows occur (lunch/dinner), which may lead to energy overcompensation in this group (i.e., eat more than they expended during exercise). You state in your hypotheses that reward will be higher for more palatable foods in the evening, but if energy intake is not different (your primary hypothesis), do hedonic mechanisms still "control" eating, or just create an internal environment that is conducive to overconsumption in certain situations? Is it also possible that most adults get hungrier in the evening as a product of lower stress (i.e., work/social/school-related stressors) and a more comfortable environment (i.e., home) with access to the foods they enjoy? I would recommend re-reviewing the introduction, simplifying it, and saving much of the information for future discussion sections.

Line 132: Would this not depend on carbohydrate availability and exercise intensity? Insulin sensitivity is highest in the morning, yes, but I am not sure that reflects evening substrate utilization independent of these factors. This needs more supporting evidence or should be excluded from this section.

Answer: Thank you very much for these comments about the introduction. We recognize that the scope of the introduction was not in line with the research questions. We have now rewritten the entire introduction, and simplified it to include: A discussion on exercise-induced anorexia (debates and influencing factors), and whether this is impacted in response to exercise timing, as well as the impact of T2D status (as people with and without T2D have different metabolic and circadian profiles).
The revised introduction is as follows (page 6, line 97 – page 7, line 143):

"*The role of exercise in appetite control and weight management, as well as in the treatment of metabolic disorders such as type 2 diabetes (T2D), has been a prominent focus in numerous research studies. The phenomenon of exercise-induced anorexia was investigated and described by King, Burley & Blundell in 1994 (1), who demonstrated that subjective appetite ratings were lower immediately following an acute bout of exercise compared with pre-exercise levels. Although energy intake after the exercise was not suppressed, relative to the energy expended, a long duration, high intensity exercise bout led to a lower relative energy intake (1). Consistent findings have since shown that post-exercise energy intake, relative to the energy expenditure during exercise, is often reduced compared to resting condition. This indicates that exercise can create an energy deficit that is not fully compensated for by short-term increases in energy intake. However, the effects of exercise on absolute energy intake remain inconclusive, with reports of increased, decreased and no difference in energy intake compared to rest (2–4). These discrepancies may be attributed to methodological variations, including differences in exercise intensity, the timing of the post-exercise meal, and the characteristics of the study population.*

Circadian or diurnal rhythms may influence appetite control and consequently, energy balance. Exercise has been identified as a circadian time cue, with exercise-induced signals shown to affect molecular clock genes (5). The diurnal timing of exercise also seems to impact body weight, with greater losses in body weight observed with morning compared to evening exercise (6,7). However, research on the effects of exercise timing on appetite and energy intake has yielded mixed results. Some studies

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found no significant differences in energy intake at any meal or overall during 26 hours post-exercise or macronutrient preferences between morning and evening exercise (8). However, others reported that morning exercise may lead to greater perceived satiety 15 min post-exercise in women with overweight, although 24-hour post-exercise energy intake was similar (9). Exercise timing can also affect food preferences, with greater wanting for low-fat sweet foods after morning compared with evening exercise and greater wanting for high-fat sweet foods after evening compared with morning exercise (10). The impact of exercise timing may be influenced by individual chronotype, with early chronotypes experiencing greater hunger suppression after morning exercise and late chronotypes after evening exercise (10); however, there is a need for a better understanding of the interaction between chronotype and exercise timing for appetite control and weight management (11).

Furthermore, whether exercise affects appetite control differently in people living only with overweight/obesity or with overweight/obesity and type 2 diabetes is currently unknown. In healthy individuals, glucose tolerance to identical meals is greater earlier compared to later in the day (12). However, in people with T2D, insulin sensitivity has been shown to improve throughout the day and worsen throughout the night into the morning (13). Whether this diurnal variation affects appetite control remains unclear. A few small studies in people at risk of or with T2D, have investigated effects of exercise timing on glycaemic control, with some suggesting that afternoon/evening exercise may lead to better glucose regulation compared to morning exercise (14–16). However, other studies have not observed significant differences (17). Additionally, people living with T2D are often prone to late-night eating (18), which could serve as a potential target for exercise interventions. If evening exercise could suppress appetite and reduce energy intake during the hours following the exercise bout, it may provide a viable strategy to mitigate late-night eating behavior in this population.

Understanding "when" to eat and exercise is of great importance as most clinical guidelines and interventions in prevention and treatment of obesity and related diseases focus on "what" and "how much" to eat or "how" and "how much" to exercise. This study will investigate how exercise and exercise timing affects appetite control and subsequent energy intake after an acute, high intensity exercise bout in people with overweight/obesity and with and without T2D."

Methods:

Line 179: I think there is too much variance within your groups. While your T2D group is fine, I would recommend stating how this will be verified (i.e., diagnosed T2D or will you perform a metabolic screening test for verification). Moreover, will this group also be required to have obesity?

Answer: Type 2 diabetes diagnosis was verified through the participants official medical records. We did not perform metabolic screening. The T2D sub-group was also be required to have overweight/obesity.

Line 179: I think the cutoffs for overweight/obesity as they pertain to differences in appetite are the larger issue. For example, a young adult (i.e., ~24 years old) with a BMI of 40 kg/m2 may have worsened appetite than a 50 year old with T2D and a BMI of 29 kg/m2, particularly if they have some indication of impaired insulin at that weight status (i.e., pre-diabetes and/or abdominal obesity). Would you screen for indicators of insulin resistance in those with overweight/obesity and exclude if so? If not, it may also be ideal to use waist circumference and/or fasting blood glucose as an inclusion criteria, particularly for those with overweight. This way, you could argue

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that each of your groups has some degree of metabolic dysfunction as opposed to a group of muscular people who happen to meet the criteria for "overweight" but do not have impaired insulin or abdominal obesity.

Answer: Thank you for your comment. We did not specifically screen for indicators of insulin resistance. However, our inclusion criteria have always required a waist-to-height ratio and waist circumference thresholds to ensure we did not include "athletic/muscular" individuals with a BMI ≥25 kg/m². We sincerely apologize for the oversight in not including these criteria in the initial manuscript. We have now corrected this and specified that participants needed a waist-to-height ratio ≥0.5 or a waist circumference of ≥88 cm for women and ≥102 cm for men to be included. These criteria are now clearly listed in *Box 1 (page 24)*.

Line 179: Since T2D is a more latent disease, will you control age differences between groups? While the study is primarily a within-subject design, you have between group comparisons, and age will likely be different between these groups. I would recommend reducing the age range to 18-60 years, or controlling for age in your analyses.

Answer: Thank you for this very good point about age differences. We would ideally have matched the groups to be of similar age, and that will be a learning point for future studies. Unfortunately, we can not adjust the age range or match the groups on age as we have completed data collection for all participants. Consequently, in the analyses where we compare the subgroups with and without type 2 diabetes, we will adjust for age (page 15, lines 361 – 363).

Line 179: Aiming for a somewhat equal distribution of males and females as well? Please state if so.

Answer: Ideally, we would like to have a equal distribution of males and females, but we chose to go with a more pragmatic solution to include participants as they signed up for the trial. We will adjust for sex in the analyses, where we compare the subgroups with and without type 2 diabetes (page 15, lines 361 – 363).

Line 186: Energy intake for how long after exercise (i.e., just before and during the ad libitum test meal)? 24-hour energy intake is listed as a secondary outcome below, so this is somewhat confusing. Please delineate the differences between the energy intake measurements listed for the primary and secondary outcomes.

Answer: We have specified the outcomes on page 9, lines 189 - 190: "*The primary outcome is the difference in energy intake during an ad libitum meal after 45 minutes of acute exercise bout compared to rest. The ad libitum meal will be served 15 min post-exercise*", and line 193: "*(comprised of the energy intake from the ad libitum meal and self-reported energy intake 24-hours post-visit)*".

Line 227: Is 15:00 PM (3:00 PM) considered the evening hours? It would be more ideal to have this occur during normal evening exercise hours (i.e., 5:00 PM) as opposed to what most would consider "mid-late afternoon".

Answer: Thank you for this perspective. Our participants arrived at 3 or 3.30 PM and the exercise bout commenced at 3:30 - 4 PM. The ad libitum meal was served at approximately 4:30 - 5 PM.
We have changed the name of the "evening" visits to "late afternoon", as you are right, these hours would not be considered "evening" per se. We also corrected 15:00 PM to 3:00 PM (page 10, line 230).

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Line 227: Habitual smokers should be excluded as smoking affects your primary variables of interest (appetite). If you decide not to exclude (I would caution against this), smoking should not be allowed for 24-48 hours prior to testing, though cessation would also likely not be feasible (i.e., increase cravings).

Answer: Thank you for your input. This is an important learning point for us, and we will incorporate smoking as an exclusion criterion in future studies. Unfortunately, we have already completed data collection for all participants in our study; therefore, it is no longer feasible to implement the proposed changes. Fortunately, only 3 of 55 participants (who completed the study) were habitual smokers. Therefore, we think that the effect of habitual smoking will be very minimal to our overall results. Furthermore, participants will serve as their own controls, which will further reduce the potential confounding effects of smoking.

Line 230: Since you are allowing medication, it would be useful to declare whether the participants with T2D are insulin-dependent or not (or either) as this may impact their responses. It is also likely that individuals in your target group will be using, for example, blood pressure medications, which could impact responses to exercise. Will you perform adjustments for medication use in your analyses?

Answer: We have gathered detailed information on participants' medication use and will provide a descriptive analysis of the number of participants using each type of medication. Potential participants receiving mediciation that is known to affect appetite have been excluded. Participants receiving medicine that may influence exercise performance (e.g. beta-blockers) have not been excluded. As participants are their own control and receive the same medication during the trial we will not adjust for medicine use in the analyses.

Line 262: Will you collect markers of subjective appetite at the same timepoints as the blood samples? Please list the timepoints to will collect the VAS measurements. It would be advantageous to collect the VAS measurements to determine whether physiological markers of hunger and satiety align with subjective markers since this is not always the case. Moreover, I recommend collecting both blood samples and subjective appetite markers prior to exercise (i.e., right before the onset; I did not see this listed) and at the midway point during exercise. If it is not feasible to collect the blood samples at the midway point, then at least the VAS markers should be collected.

Answer: Yes, we will collect VAS scores at the same time points, with an additional measurement immediately following the ad libitum meal (time point 30). We have included the following in the 'Subjective Appetite' section (page 11, lines 268 – 269): "*We will collect the VAS measurements in the fasted state on the test visits and at 5 subsequent timepoints (0, 15, 30, 45, and 60 minutes after the completion of the exercise bout/rest)*".

Blood samples were collected 5–20 minutes before exercise. We recognize the potential benefit of drawing samples immediately before and during exercise. However, based on practical experience from acute exercise studies, we decided against collecting blood during the exercise period, as the limited time between high-intensity intervals would have made this challenging. Our experience suggests that drawing blood during exercise may interfere with participants' performance and that reduced blood flow to the arms during exercise often complicates sample collection.

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Collecting VAS scores during the exercise session would have been feasible and could yield interesting insights. We will certainly consider this approach in future trials.

Line 306: to keep with the ad libitum nature of the study, you could provide them with an excess of water (e.g., 1 L) and let them consume water ad libitum on the first trial, and only give that much water on the subsequent trials. That way they are not being restricted to only 200-250 mL or forced to consume that much if they do not want to. It could also impact appetite if they drink it all in the last 5 minutes of exercise just to meet the instructions for consumption. Same goes for the ab libitum meal. Participants should be able to consume as much water as needed and this should be measured rather than controlled.

Answer: We acknowledge your point regarding ad libitum water; however, we approached this aspect somewhat differently in the study design. Our intent was to ensure participants could replace the water lost during the exercise session without overhydrating. We considered that participants might have a stronger 'reward' response to drinking water immediately post-exercise, as hydration is often prioritized over food after physical exertion. Therefore, we opted not to provide ad libitum water, aiming to meet a physiological need rather than a psychological one. This way, participants would prioritize water if they were overly thirsty, preventing potential interference with the ad libitum meal.

Participants were provided with 250 mL (men) and 200 mL (women) of water during the exercise session and the same amount during the meal (15 minutes post-exercise), totaling 500 mL for men and 400 mL for women.

Line 313: I do not think that participants should be constrained to a single pie after their first selection. If you hypothesize that desire for more rewarding foods is higher in the evening, and a participant is randomized to an evening visit first, they may select a pie that they find more rewarding that they would not select at other points during the day, and be stuck to that pie they may not want for the remaining visits. It would also be removing the effect of day-to-day changes in food choice, which is a better reflection of how this would occur in the real world. In essence, it would be a more equitable comparison if participants were presented with each pie at each visit and allowed to select. If this was not possible, you should provide a palatability questionnaire at screening and ask each participant to rank their liking of each pie option and use the pie they stated they like the most as the test meal.

Answer: Thank you for this comment. We implemented a similar approach. During the screening visit, participants were presented with various pie options and selected the one they would consume at all test visits (without knowing if they would begin with a morning or evening test visit). We maintained consistency in pie type to minimize variations in energy intake, as the different pies had different macronutrient composition. By having participants consume the same pie across all four test visits, we controlled for variations in both energy content and pie 'liking.' This approach helped to avoid situations where a preference for one pie, such as a pesto pie over a leek pie, might influence intake more than the exercise/rest conditions.

From a food reward and preference perspective, it would indeed be interesting to evaluate participants' pie choices across different conditions, which could be relevant in future studies.

Line 335: It would be impressive if you only had a 10% attrition for a study such as this. I would recommend increasing your attrition to 20% but will leave this to the authors discretion.

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Answer: Our attrition rate was 7% (4 out of 58 participants withdrew before completing all test visits). Please note that we replaced participants who were randomized but did not attend any test visits - a detail we apologize for not disclosing in the original manuscript. We have now included this information in the 'Sample Size' section (page 14, lines 349 – 350): "*Participants who withdraw after randomization, but before completing any test visits will be replaced.*".

Statistical analyses: The inferential statistics tests, the correction methods, assumption tests, normality tests, etc. that you plan to run are unclear/not stated (other than GLM or null hypothesis significance tests), and there is no information for how confounding variables will be controlled (or if they will). I would recommend stating the specific analytical plans with the test names and procedures explicitly described. I know that this is a pre-registration so you may want to be broad, but it is better to be specific and then just provide rationale as to why you had to change the plan in the final manuscript(s).

Answer: We have made substantial changes to the section: "Statistical Analysis Plan" (page 14, line 352 – page 15, line 374). The new section is presented here:

"*All outcomes will be reported as summary of the raw data using appropriate summary statistics. Descriptive data are presented as mean ± SD if normally distributed, as median [IQR] if non-normally distributed and as n (%) if categorical.*

Outcomes measured on the test days will as a general rule be analysed using a linear mixed model (LMM). LMMs will be used to assess energy intake differences across conditions (rest vs. exercise), time of day (morning vs. late afternoon), and their interactions, as well as the randomized visit sequence, while accounting for within-subject variability with a random intercept on participant level. We will calculate levels for each condition and the contrasts between them (estimated levels and differences with 95%CI will be calculated and presented). The interaction with T2D status on the outcomes will also be explored using T2D as an interaction term in the model. In the LMM with T2D interaction we will adjust for age, sex, and BMI to account for potential differences between groups.

Model assumptions will be assessed using graphical methods, including Q-Q plots, residual vs. predicted plots, and histograms of residuals. If needed outcomes will be log-transformed for analysis and results back-transformed for presentation. Outcomes that do not fit the model will be analysed using a generalized mixed model or by comparing the observed data using nonparametric null-hypothesis tests.

Modelled outcomes will be presented as estimated levels (95% confidence intervals (95%CI)) on test days and comparisons between test days will be presented as estimated differences (95%CI, p-values).

P-values < 0.05 will be regarded as statistically significant. The false positive rate related to the hypothesis for the primary outcome will be controlled by using a hierarchal testing procedure. Secondary/descriptive outcomes will not be adjusted for multiplicity, apart from omics outcomes (proteomics, metabolomics and lipidomics) where a false discovery rate cut-off (<0.1) will be applied."

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Reviewer #2:

Seth Creasy, University of Colorado School of Medicine

Comments to the Author:

: I commend the authors on this interesting study. There are some items that require further justification/clarification. I look forward to seeing the results of this study.

Answer: Thank you very much.

Abstract

1. **Please include the age range in the abstract**

Answer: Now included (page 3, line 54)

2. Was an upper range of BMI used? If so, please clarify in abstract.

Answer: We did not set an upper BMI limit; however, we included an exclusion criterion requiring participants to be capable of completing a 45-minute cycle ergometer exercise bout. In determining eligibility, we considered BMI, current physical activity level, overall health status, and any physical injuries that might hinder their ability to complete the exercise.

Introduction

1. Line 129 – I believe the Sato study suggests that fatty acid metabolism was increased at ZT15 (early in the dark phase = equivalent to morning exercise) and glucose metabolism was increased in ZT3 (early in the light phase = equivalent to evening exercise). Please double check.

2. Line 134 – Please change the word "preferable" to rather indicate direction of results

Answer: Thank you for these comments. We have chosen to revise the whole introduction based on comments from Reviewer #1. Consequently, the Sato study is no longer mentioned, and line 134 has been deleted altogether.

Recruitment

1. Line 217 – Do they need to meet 1 of 3 criteria? Please clarify

Answer: We have revised the criteria wording by changing "*or*" to "*and*" to clarify that **all** three criteria must be met (page 10, line 222).

2. Line 226 – Can justification be provided regarding differences in fasting duration and how this could affect results?

Answer: We chose to have different fasting durations due to severeal different factors: 1) We wanted participants to do an overnight fast to be able to have "true" fasting blood samples from the morning conditions as well as do bioimpedance body composition measurements., and 2) We wanted to mimick daily life, and we did not find it both feasible and realistic to have participants fast throughout the whole day before their late afternoon visits. We will not be able to have the exact same conditions in the morning and late afternoon, as we will not be able to replicate sleep, waking time, physical activity etc. to match these precicely in both timing conditions. Results can be affected by these fatcors and also fasting duration, but this is why we chose to have a rest condition, where the participants are their own control to account for some of these potential differences.

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3. Line 303 – Why was interval training selected?

Answer: The exercise protocol was inspired by a previous study in a similar population (Pilmark et al., 2021), in which they used high intensity interval training in blocks of 4 min at 70% $Watt_{max}$. We wanted to ensure that the release of appetite hormones were sufficiently stimulated during the exercise, and consequently we chose to do a 4 x 4 min at 85% $Watt_{max}$ with 3 min active rest (50% $Watt_{max}$) in between intervals. Both exercise mode, duration, and intensity influence post exercise energy intake (Schubert et al., 2013). Intensities above 70% of VO_2max can reduce hunger feelings in the first hours post exercise, but not nesseary energy intake. As high intensites cannot be maintained for long due to metabolite accumulation, high intensity intervals are combined with active rest periods. After testing on study personnel and completing the first 4 participants, we learned though, that the intensity was too high. We changed the high intensity intervals from 85% $Watt_{max}$ to 75% $Watt_{max}$ and the remaining participants did that protocol. We added this information in the section: "Execise Bout" (page 13, lines 310 - 312): "*Initially the high intensity intervals were set at 85% Wattmax, however, after 4 participants we changed the workload to 75% Wattmax since participants were not able to complete the exercise at the 85% Wattmax*".

4. Line 308 – Will any physio measures been conducted during exercise (heart rate, energy expenditure, etc.)?

Answer: We will collect heart rate, workload, rpm, and rates of perceived extertion during the exercise bout. It has been added to the section "Exercise Bout" (page 13, lines 313 - 316): "*During the exercise bout, heart rate, workload, and revolutions per minute (RPM) be measured continuously. Participants will be asked to rate their perceived exertion (using the Borg Scale) at the end of the warm-up, the end of the last low-intensity and high-intensity interval, and at the end of the cool-down period."*

5. If someone cannot complete exercise, the workload will be reduced but how will this be considered in statistical analyses?

Answer: Thank you for this very relevant comment. If someone could not complete the exercise at the planned workload, the workload was reduced. Participants who completed a "reduced" exercise bout on their first exercise visit would do that same protocol for their second exercise visit. Therefore, the exercise protocols were the same between the morning and late afternoon exercise conditions. We will do a supplemental analysis to investigate what characterized participants, who could not complete the original exercise protocol.

6. Will individuals with T2D be age, sex and weight matched to individuals without? If so I missed this. If not, why not?

Answer: Thank you for this relevant comment. Looking back, it would have been ideal to match individuals with and without type 2 diabetes on age, sex and body weight (or BMI). Unfortunaly, this was not incorporated into the initial design, but it will for sure be something to consider and incorporate in future studies. Consequently, in the analyses where we compare the subgroups with and without type 2 diabetes, we will adjust for age, sex and BMI.

Statistical Methods

1. **Line 335 – What if attrition is higher than anticipated? Will sample size be increased?**

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Answer: We planned to replace participants who were randomized to the trial but did not attend any test visits - a detail we apologize for not disclosing in the original manuscript. We have now included this information in the 'Sample Size' section (page 14, lines 349 – 350): "*Participants who withdraw after randomization, but before completing any test visits will be replaced.*". The attrition rate for the study was 7%. While we had provisions to increase the sample size if attrition was unexpectedly high, we did not anticipate this to be a significant issue.

Table 2. Please list out the appetite hormones that will be measured

Answer: The hormones are now listed in Table 2: "*Ghrelin, Glucagon, Gastric inhibitory polypeptide (GIP), Glucagon-like peptide-1 (GLP-1), Peptide Tyrosine Tyrosine (PYY), Pancreatic polypeptide (PP), Cholecystokinin (CCK), Fibroblast Growth Factor 21 (FGF21), Growth Differentiation Factor 15 (GDF15)"*, and in the "*Laboratory Analysis*" section (page 15, line 388 – page 16, line 390).

References

Pilmark, N. S., Lyngbæk, M., Oberholzer, L., Elkjær, I., Petersen-Bønding, C., Kofoed, K., Siebenmann, C., Kellenberger, K., van Hall, G., Abildgaard, J., Ellingsgaard, H., Lauridsen, C., Ried-Larsen, M., Pedersen, B. K., Hansen, K. B., & Karstoft, K. (2021). The interaction between metformin and physical activity on postprandial glucose and glucose kinetics: a randomised, clinical trial. *Diabetologia*, *64*(2), 397–409. https://doi.org/10.1007/s00125-020-05282-6

Schubert, M. M., Desbrow, B., Sabapathy, S., & Leveritt, M. (2013). Acute exercise and subsequent energy intake. A meta-analysis. *Appetite*, *63*, 92–104. https://doi.org/10.1016/j.appet.2012.12.010

VERSION 2 - REVIEW

Reviewer	**1**
Name	**Graybeal, Austin J**
Affiliation	**University of Southern Mississippi**
Date	**20-Dec-2024**
COI	

The authors have addressed all of my concerns and I commend them for their efforts in making these revisions.

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