Author's response to reviews

Title: Exploring mechanisms of fatigue during repeated exercise and the dose dependent effects of carbohydrate and protein ingestion: study protocol for a randomised controlled trial

Authors:

Abdullah F Alghannam (A.F.Alghannam@bath.ac.uk)
Kostas Tsintzas (kostas.tsintzas@nottingham.ac.uk)
Dylan Thompson (D.Thompson@bath.ac.uk)
James Bilzon (J.Bilzon@bath.ac.uk)
James A Betts (J.Betts@bath.ac.uk)

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Author's response to reviews: see over
The comments and feedback provided by the reviewer are greatly appreciated. These were certainly the foundation for the revised and improved version of the manuscript. Provided below are point-by-point responses with associated amendments to the manuscript that are highlighted in yellow font and underlined.

Major Compulsory Revisions:

1. Generally the manuscript was interesting and describes two studies that should be of interest to readers with an interest in sport and exercise science/nutrition.
   There is already a substantial amount of literature published on this specific topic (i.e. many studies documenting the effects of different carbohydrate/protein amounts, type and timing on muscle glycogen resynthesis or performance) and I think it would be worth further clarifying what makes the described studies novel.

   Further information to clarify the significance of the study was added in the introduction, as per the comments raised by the reviewer (lines 75-122 in the revised manuscript).

   We agree that a number of investigations were conducted to examine the effects of carbohydrate/protein ingestion on muscle glycogen resynthesis during short-term recovery. Furthermore, several studies examined the effects of carbohydrate/protein on subsequent exercise capacity/performance. However, few studies concurrently examined nutrient intake, muscle glycogen during short-term recovery and subsequent endurance capacity (Casey et al. 2000; Williams et al. 2003; Lunn et al 2012). None of which explored glycogen metabolism during a subsequent bout. The latter being the main outcome of the current trial.

2. There are numerous spelling, punctuation and grammar errors throughout the manuscript and these should be corrected and the manuscript thoroughly proof read prior to subsequent submission. I have not listed all of these as they are too numerous, but one consistent error throughout the manuscript is the authors’ continual switch between future and past tense, at times even within the same sentence.
We appreciate the comment provided by the reviewer. This was addressed in the current manuscript.

3. Do the authors really feel they can assess the dose response effects of carbohydrate ingestion on exercise capacity adequately with their study design? I feel the study design is adequate to examine mechanisms of fatigue, but the order effect and assumptions/expectations that are introduced by the study design means that capacity cannot truly be measured. I.e. by using this design you are telling the subjects they will exercise longer on the high carbohydrate treatment. That said, the data you have presented for phase 1 suggests this is not the case. Did all subjects exercise longer in the second trial than the first? It would appear from the mean (SD) data that this might not be the case.

The assumptions made were based on a wealth of literature (Betts & Williams, 2010), and it was expected that participants would therefore run longer with high-sucrose ingestion. It should be noted that the study included measures in an attempt to minimise/diminish expectancy effects. For example, the familiarisation session was identical to the main protocol (i.e. first run to exhaustion followed by 4-h recovery with only water ingestion and a subsequent exhaustive run) to ensure that participants are familiar with their individual perception of volitional fatigue. The sample of participants recruited were also endurance trained and are accustomed to prolonged running that minimises trial order/learning effects. This was evident from the similar time to exhaustion during the first run between the low sucrose and high sucrose treatments (n=8; 101 ± 18 and 102 ± 19 min, respectively). Furthermore, ratings of perceived exertion in the high sucrose treatment were lower (mean score of 16) at the time point corresponding to fatigue in the low sucrose treatment. Coupled with the substantial enhancement in subsequent endurance capacity obtained from our preliminary results (approximately 30 minutes improvement with high sucrose treatment), it seems reasonable to assert that the extended run times were largely ascribed to the ergogenic benefit of the high sucrose supplement and not merely a psychological artifact of expectancy effects.

With regards the question relating exercise time in Phase I, the reported times to exhaustion in low and high sucrose treatments (101 ± 18 and 102 ± 19 min,
respectively) are based on the first run in each treatment and prior to any nutritional intervention during 4 h recovery period. We can confirm that preliminary data shows that each participant exercised for longer in the second trial. Nonetheless, the times to exhaustion were removed in the revised version of the manuscript as it was deemed more suitable to report times to exhaustion for the entire sample of participants in Phase I once data collection has been completed and disseminated. Therefore, the revised manuscripts (lines 610-614) now reads “further support of this is gained through the initial run times to exhaustion obtained from participants tested thus far in Phase I, indicating comparable times to exhaustion (data not shown) and thus endorsing the notion that intra-individual pre- and post-exercise muscle glycogen contents were at comparable levels between the trials”.

4. It might be useful to the reader for you to state what the practical relevance of this study is. I.e. in what situation might someone exercise to fatigue (maximally) twice within 4 hours? Also, is it not likely that in practice athletes or occupational exercisers would have a substantial meal between exercise sessions? I understand the point of the experiments is to examine mechanisms of fatigue, but there are not many situations under which this exercise scenario might occur, which questions whether it is worth looking at mechanisms of fatigue in the current study design.

A brief section detailing the relevance of the study has been included the in updated manuscript (lines 75-83 in the revised manuscript).

Taking part in physical exercise at moderate to high-intensity places an increasingly high demand on the body’s finite carbohydrate stores. The onset of fatigue during such activities corresponds to depletion of muscle glycogen to critically low levels, and therefore the capacity for exercise is largely dependent on the availability of glycogen prior to exercise. Indeed, the energetic cost of a training session usually exceeds that of endogenous CHO stores, which in turn emphasise the importance for post-exercise nutrition restore glycogen effectively and consequently restore the capacity for subsequent physical exercise whether during training and/or competition.
Many athletes are required to train and compete with minimal recovery (<8 hours) and therefore strive to maximise their recovery. Furthermore, occupational exercisers and individuals undertaking less intensive exercise regimens from any effective and rapid recovery as this will likely increase their participation. Having said that, the reviewer is entirely correct that the reader would benefit from the stating the practical relevance of the study, albeit this should be viewed in the context of maximising muscle glycogen and its relation to subsequent exercise capacity and not in an applied sports/event setting, as these are the main outcomes of the study.

To that end, we agree with the reviewer that in practice athletes or occupational exercisers would have a substantial meal between exercise sessions and that there may not be many situations where athletes would run to exhaustion twice within 4 hours. Nevertheless, the rationale for using carbohydrate and/or protein fractions only was to isolate any confounding variables (i.e. other macro and micronutrients) that could affect glycogen storage/recovery and exercise capacity. In line with the same rationale and in keeping with the main outcomes of the study, it was deemed necessary to experimentally induce glycogen depletion in 2 exercise bouts to assess the capacity for physical exercise interspersed with a period of short-term recovery to introduce the nutritional intervention, which theoretically could provide a snapshot of exercise-induced and insulin-induced glucose uptake to the muscle while also providing a time frame that is comparable with the majority of the available literature in this domain.

5. Does the study design truly explore whether a dose-response relationship exists? You only have two levels of carbohydrate intake (0.3 g/kg/h and 1.2 g/kg/h). It would have made more sense, to me at least, if phase 1 had involved at least three levels of carbohydrate intake to establish the dose-response relationship (e.g. 0.4 g/kg/h, 0.8 g/kg/h and 1.2 g/kg/h) and this would then also be consistent with phase 2.

We agree with the reviewer that obtaining more than two carbohydrate intake levels will be important to examine the extent to which a dose-response relationship is linear. However, we chose the aforementioned carbohydrate intakes for a different rationale than establishing a dose-response. Namely, 0.3 g/kg/BM was chosen to provide substrate for liver glycogen resynthesis due to the first pass extraction of glucose by the liver, while minimally resynthesising muscle glycogen, while the
second treatment (1.2 g/kg/BM) would allow maximal muscle glycogen resynthesis rates during short-term recovery. These intakes were mainly manipulated to examine the importance of muscle glycogen (or indeed liver glycogen) in restoring the capacity for repeated exercise, while also examining muscle glycogen metabolism during subsequent exhaustive bout. We therefore aimed to examine the highest (i.e. optimal) and lowest (i.e. sufficient only to replenish liver) dose. If the current study determines different glycogen degradation/fatigue mechanisms during a subsequent exercise bout, future work would certainly be warranted to further explore whether the dose-response effect is linear.

Nonetheless, the preliminary results from our current study, together with our previous work (Betts et al. 2007), which employed a similar protocol and cohort of participants with comparisons of 0.8 and 1.1 g of sucrose/kg/BM, showed a dose-response relationship between carbohydrate intake and subsequent endurance capacity. Based on this, the authors feel it is reasonable to infer that a dose-response relationship appears to be present between the amount of carbohydrate provided and subsequent endurance capacity.

6. I liked the section related to the use of exercise capacity and completely agree with the authors. I would add, in many settings lots of athletes actually undertake exercise that is very similar to exercise capacity testing. For example pace is often set by the faster runners/ cyclists etc. with the majority of athletes attempting to hold onto the pace set for as long as possible before slowing their pace and dropping off the back of a group/ athlete.

We would like to thank the reviewer for their appreciation for the rationale behind the relevance of time to exhaustion as measure to investigate mechanistic perturbations to fatigue. With permission from the reviewer, this valuable addition has been included in the revised manuscript (lines 258-262).

7. Line 91. Please clarify why you chose this protein fraction.

The 2 paragraphs below have been added to the revised manuscript (lines 157-176):
An important factor determining the rate of muscle glycogen resynthesis is insulin-mediated glucose uptake by the muscle cells (Jentjens & Jeukendrup, 2003). A proposed mechanism for the potential benefit of protein co-ingestion in enhancing the rate of glycogen storage is the synergistic effect of this substrate on insulin secretion (Zawadzki et al. 1992; van Loon 2000). It has been recently demonstrated that plasma insulin response increases to greater extent in whey than in casein protein in its intact form (Reitelseder et al., 2011). The ingestion of a protein hydrolysate facilitates greater digestion and absorption compared with its intact protein, resulting in more rapid increase in circulating insulin concentrations (Koopman et al., 2009). This was further confirmed by the finding of greater insulinotropic properties when whey protein hydrolysate was ingested as opposed to whey protein in humans (Morifuji et al., 2010a).

In terms of glycogen storage, the ingestion of whey protein has been shown to stimulate this process more rapidly both in liver and skeletal muscle tissues than when casein was ingested (Morifuji et al., 2005). Furthermore, it appears that ingesting whey protein hydrolysate with carbohydrate augments glycogen resynthesis to a greater extent than when carbohydrate is co-ingested with intact whey protein, casein or intact branched-chain amino acids (Morifuji et al., 2010b). Taken together, these results indicate that a hydrolysed whey protein fraction may have a profound role in stimulating insulin secretion and concomitant muscle glycogen storage, and thus forming the basis for the inclusion of this protein fraction in the current study.

8. Line 98. Why sucrose and not glucose?

This following paragraph has been added to the revised manuscript (lines 145-155):

The use of the disaccharide sucrose was chosen on the basis of its potential positive contribution to liver and/or muscle glycogen resynthesis by virtue of equimolar amounts of glucose and fructose. Following exhaustive exercise, sucrose and glucose ingestion seem to elicit similar muscle glycogen resynthesis rates (Blom et al. 1987). However, resting intravenous (Nillson and Hultman, 1974, Bergstrom and Hultman, 1974).
1967) and oral ingestion (Delarue et al. 1993) studies indicate that fructose preferentially stores liver glycogen relative to glucose, while glucose infusion favours muscle glycogen resynthesis. Given the importance of both liver and muscle glycogen replenishment during short-term recovery and subsequent endurance capacity (Casey et al. 2000), sucrose was deemed a preferable source of carbohydrate to undergo predominant hepatic metabolism (i.e. fructose) to optimise liver glycogen resynthesis alongside a glucose source to maximise muscle glycogen storage (Wallis and Wittekind, 2013).

9. Line 159-162. Please include more information here. What was analysed and by whom?

Further information has been added to the manuscript to address the questions raised by the reviewer (234-236 in the revised manuscript).

10. Line 229. It is not clear here whether all subjects do all trials or whether 8 do phase 1 and 8 do phase 2. Please clarify.

The entire research trial aims to recruit 16 participants (8 participants in each phase of testing). This has been updated in the revised manuscript (lines 307-308).

11. Line 254. Why is there an SD for how long subjects will abstain from caffeine?

The reported was not a SD but an allowable tolerance. We felt that the statement was in line with many other papers relating to controls imposed in an experimental protocol. Each participant arrived to the laboratory at 08:00 ± 1 h following an overnight fast (≥ 10 hours). Participants were requested to abstain from caffeine consumption at precisely 17:00 on the day before any trial. Consequently, the allowable tolerance was intended to reflect the inter-individual time differences of when the exercise protocol commenced (i.e. those who abstained from caffeine for 15 hours would have commenced their protocol at 08:00). The number of hours and allowable tolerance have now been removed and changed in the revised manuscript to indicate the precise time (i.e. 17:00 were caffeine will be abstained; lines 334-335).
12. Line 294. Venous blood sample?

A 2 ml venous blood sample was obtained during the walking intervals of the exercise protocol. This has been updated in the revised manuscript (line 376).

13. Line 292-294. This section needs additional information. How many times will they be permitted to do this? Does the 2 minutes count in their capacity score?

Walking will be permitted on 2 separate occasions, and fatigue will be accepted on the 3rd occasion where participants indicate they can no longer sustain the prescribed running speed/intensity. The 2 x 2-minute walk times will not count in their capacity score. This has been added to the revised manuscript (lines 375-380).

14. Line 327. How will expired gas be collected at the point of fatigue. More information needed here. If they’ve had 2 min walking, this gas sample may not capture steady state conditions.

Line 413 in the revised manuscript provides additional information regarding the collection of expired gas at the point of fatigue.

Expired gas will be collected during the final minute of exercise. Although the running capacity test was interposed by 2 x 2 minutes walking, participants were able to maintain running following the second walk for at least 4 minutes. Capturing a steady state would require 2-3 minutes of exercise on an ergometer (Taylor et al. 1955) and therefore the current study design is likely to capture a steady state at the point of fatigue.

15. Line 414. You stated earlier that the first morning urine sample will be collected, but here you say 30 min pre-exercise. Will both be collected or are these the same sample?

These are the same sample. The earlier statement is now modified clarify that only a baseline urine sample is collected 30 min before testing (lines 365-367 in the revised manuscript).
16. Line 491. It would be good to know what metabolites were going to be measured.

The names of metabolites have been included in the text (line 576 in the revised manuscript).

17. Line 588-591. Why do the scales have inconsistent anchors? The top of the scale is termed “extremely” for GI comfort and “very very” for fullness and thirst. For me, “extreme” is more than “very very” and thus the scales have different end points.

We recognise that different individuals will understand the semantics of these terms differently and truthfully have no firm rationale for the precise terminology not matching between the scales other than the fact that these scales were developed separately for studies focused on the different outcomes. We applied the scales only to gain some insight about these responses, so were therefore reluctant to adapt from the originals to at least contract results directly with previous literature for each variable (even if this means the actual variables cannot be contracts i.e. GI comfort and thirst).

18. Table 1. Are the energy density values correct here? I make the energy density of the sucrose-protein solution 514 kcal/L given the additional lactose and fat contained in the protein supplement. Why not adjust the amount of sucrose/protein added to the drinks to match energy density.

The reviewer raises a valid comment. As indicated in the note below the table, the assay is unable to detect values for lactose and fat below 3.5 and 2.2 g/l. Accordingly, the presence of lactose and/or fat would be in the range of 0-3.5 and 0-2.2 g/l, and the precise amounts within these ranges cannot be quantified by the assay. Given the potential minor disparities in energy density between supplements (0-34 kcal), the caloric content for lactose and fat was assumed negligible.
Discretionary revisions

1. Line 233. Possibly a little pedantic, but are you sure you will measure a true max and not just peak? It is unlikely all subjects will plateau.

The reviewer comment is greatly appreciated. Indeed, provided that the determination of oxygen uptake was based on an incremental exercise protocol indicates that a plateau may not be observed consistently. However, a plateau in \( \text{VO}_2 \) response was suggested not to be an obligatory consequence of incremental exercise. Day et al. (2003) demonstrated that the \( \text{VO}_{2\text{peak}} \) value attained during an incremental exercise was not different from the plateau during a constant-load test, and therefore may be a valid index of \( \text{VO}_{2\text{max}} \) despite no evidence of a plateau.

The above information in addition to our requisite for the observation of at least 3 of the criteria of the British Association of Sport and Exercise Sciences for establishing maximal oxygen uptake (Bird & Davison, 1997) supports the authors’ reporting of a max value, although one cannot be unequivocally certain.

2. Line 272. Possibly a little pedantic again, but this section is titled “environmental control”, but you didn’t situate subjects in a controlled environment. You just measured the conditions of the environment they were in. Please amend.

The authors would like to thank the reviewer for this suggestion. The section titled “environmental control” now reads “environmental measurements” in the revised manuscript (line 352).