# Reviewer 1

The findings are interesting, but not completely novel. Adhikary et al (Steroids 2019) published similar findings before. - Experimental design is good, with multiple readouts for muscle function/strength.  - Statistical analysis is not always clear. The figure legends explains that an arterisk means significant interaction between diet and treatment, but the graphs in Figure 1A and B both show an arterisk. The authors should clarity this.

**We have clarified in the legend for figure one that the asterisk does not indicate an interaction for these panels:**

**Asterisks indicate significant interaction between diet and treatment by two-way ANOVA except for panels A-B where it indicates a difference between treatments (n=5-8 per group).**

It is surprising that dexamethasone treatment in lean mice has so little effect on grip strength, as similar studies with dexamethasone show strong effects of dexamethasone treatment on grip strength in lean mice (Shen et al, Journal of Cachexia Sarcopenia Muslc 2019; doi. 10.1002/jcsm.12393). The authors should elucidate on the discrepancy between their study and the published one

**A critical differences exist between our work and that of Shen *et al.*. One is that they used a much higher dose of dexamethasone (25mg/kg for them, 1 mg/kg for our study). We believe that this explains the reduced loss of grip strength in our model. We have cited the work of Shen et al in our introduction**

In several parts of the manuscript (e.g. line 104) a reference to the figure is missing.

In line 145, Figure 2I should be Figure 2H. –

**We have updated these references, and thank the reviewer for noticing these mistakes.**

The manuscript lacks mechanistic insight. There is no data that shows any insight on why the dexamethasone-induction of atrogenes is more robust in obese mice.

**We agree that we have not defined the mechanism by which obesity modifies glucocorticoid actions in muscle. We believe that the data presented in Figure 3 supports the hypothesis that obesity causes more transactivation of critical GR-dependent genes but as yet do not have a clear biochemical mechanism to why. We are pursuing this question aggressively and look forward to identifying and sharing these answers in forthcoming work. We speculate about a few options in the revised discussion:**

**One hypothesis is that obesity remodels the chromatin landscape, allowing for easier GR access to genes involved in modulating muscle size and function. Indeed, obesity alters the packing and accessibility of DNA in adipocytes [14,21,41] and therefore may have a similar effect in muscle in which Glucocorticoid Response Elements are more easily bound by GR causing increased glucocorticoid action. Another potential mechanism is that the effects of GR-dependent signaling are enhanced by insulin resistance by FOXO dephosphorylation**

# Reviewer 2

The manuscript by Gunder et al. describes and interesting mouse model, which combines obesity and excess of glucocorticoids. They investigate the effect of obesity and dexamethasone on several parameters including water and food consumption, body weight and fat mass, Then, they analyze several muscle features and insulin effect on blood glucose.

The manuscript is well written and well organized. The part dealing with muscle features is sound and rather exaustive.

My only concern is about the poor description of glucose metabolism in the animal model.

In particular, they only performed an insulin tolerance test to evaluate insulin resistance. I do understand that the use of hyperinsulinemic clamp is not easy and obvious to perform, but they should at least further investigate the mechanisms of insulin resistance.

**This is an excellent point and we are providing two new pieces of data to address this important point in the revised manuscript. First we have done glucose clamp experiments in this model and published the results from the obese animals in Harvey et al 2018. To summarize, we found that there was strongly impaired glucose infusion rates, driven by elevated endogenous glucose production and impaired suppression of EGP by insulin. This was concordant with a lack of suppression of NEFA levels by insulin. We observed decreased peripheral 2-deoxyglucose uptake in muscle and adipose tissues. For the lean animals, we did not publish the data because we found a suppression of insulin clearance in lean animals. This was not observed in obese animals. As such the lean animals were chronically exposed to higher insulin levels. These data are presented here, and as you can see there is only mild glucose intolerance in lean dexamethasone treated mice. Future experiments are planned to investigate the phenomena of differential insulin clearance. We have clarified the findings in Harvey et al in the revised discussion section of this manuscript:**

../../../../../../../../Desktop/CushingAcromegalyStudy/manuscript/Obesity-Glucocorticoids/Endo%20clamp%20Respo**Glucose Clamp Data in NCD-fed Mice:** Insulin clearance (A), plasma insulin concentrations (B), area under the glucose infusion rate curve (C), hepatic glucose production (D) and glucose turnover (E) for lean mice during at basal and during euglycemic clamp following 3 weeks of dexamethasone (n=10) or vehicle (n=13) treatment. For clamp experiments, insulin was infused at 4 mU/kg/min following a prime continuous infusion of 40mU/kg bolus. All mice were fasted for 5-6 hours prior to experiments.

**In our previous study we demonstrated vie euglycemic hyperinsulinemic clamps that obese dexamethasone treated mice were insulin resistant (as determined by suppressed glucose infusion rates), had lowered muscle glucose uptake, and had elevated endogenous glucose production. Based on elevated lipolysis in these mice, we posited that this is due to indirect promotion of glucose production by adipocyte lipolysis [21].**

For instance, Does the lack of In vivo insulin effect relate to defects in muscle insulin action. What about insulin effect on canonical targets (muscle vs liver vs adipose tissue)?

**To the reviewer’s second question, we have added new data about pAkt signaling in these muscles. Consistent with other reports, we did not detect any differences in proximal insulin signaling. We have added these data to the revised manuscript as the new Figure C-D:**

**To test whether proximal insulin signaling was affected in either group, we evaluated muscle lysates from gastrocnemius tissues at the end of a hyperinsulinemic euglycemic clamp. We found that the relative phosphorylation of Akt at Ser 473 was unchanged between water and dexamethasone treatments, in either group (Figure 4C-D). This is consistent with prior work demonstrating that proximal insulin signaling is largely unaffected by glucocorticoids [28,29].**

How the authors address the mechanism of dexa-induced defects in insulin action of obese vs lean animals

**We agree that we have not defined the mechanism by which obesity modifies glucocorticoid actions in muscle. We have made this explicit in the revised description. We believe that the data presented in Figure 3 supports the hypothesis that obesity causes more transactivation of critical GR-dependent genes but as yet do not have a clear biochemical mechanism to why. We are pursuing this question aggressively and look forward to identifying and sharing these answers in forthcoming work. We speculate about a few options in the revised discussion:**

**One hypothesis is that obesity remodels the chromatin landscape, allowing for easier GR access to genes involved in modulating muscle size and function. Indeed, obesity alters the packing and accessibility of DNA in adipocytes [14,21,41] and therefore may have a similar effect in muscle in which Glucocorticoid Response Elements are more easily bound by GR causing increased glucocorticoid action. Another potential mechanism is that the effects of GR-dependent signaling are enhanced by insulin resistance by FOXO dephosphorylation**

# Reviewer 3

Glucocorticoids widely used in clinical medicine but many side effects in skeletal muscle is  serious problem of use this hormone. The present paper is dedicated to the negative side effects of dexamethasone in skeletal muscle, effect of obesity on muscle atrophy and grip strength. The atrophy  and reduction of muscle strength mainly in type II fibers is logical as muscle fibers with low oxidative capacity are more sensitive to the catabolic effect of glucocorticoids.

This paper contain new knowledge of effect of glucocorticoids in skeletal muscle, have theoretical and practical value. Paper is well written and I suggest to publish paper without changes.

**We thank the reviewer for their comments**

# Reviewer 4

Gunder et al. examined the effects of dexamethasone treatment on parameters of skeletal muscle atrophy in mice fed either a High-fat diet or standard chow. This work builds on their previous work by Harvey et al. (2018), where the authors observed impaired glucose tolerance, decrease fat mass, hepatic steatosis, and increased lipolysis. They demonstrate that HFD-dexamethasone animals weight less than their HFD-vechile controls despite consuming significantly more calories.  The discrepancy in the mouse body wright was due to less fat mass and lean mass. They comprehensively demonstrate that dexamethasone treatment decreases muscle strength, fibre type and cross sectional area. However, despite the reductions in muscle mass and strength, the authors did not observe differences in markers of the E3 ligases, MuRF1 and Atrogin-1.  The manuscript is well written, relevant, but could be improved from the addition of some molecular work.

Main comments

1. Please include main effects of the diet and dexamethasone treatment either in text or present on graph, as it is hard to interpret where there are main effects.

**After consultation with our statistical team, we think it can be misleading to report main effects when there is a significant interaction term. Since our primary outcome is the interaction between glucocorticoids and diet, that is what we reported. All statistical tests are reported in our online data supplement**

1. Change the title as it currently a bit misleading. I think switching “promotes” to “exacerbates” or “augments” is more suitable, as there appears to be some main effects of treatment with the dexamethasone for loss of muscle strength, CSA and mass.

**We have changed the title to augments, and thank the reviewer for this clarification.**

1. Add westerns for MuRF1, Atrogin-1, FOXO3, and LC3BII/I. In its current state, the manuscript is only descriptive and would benefit from the addition of molecular explanations to the changes observed.

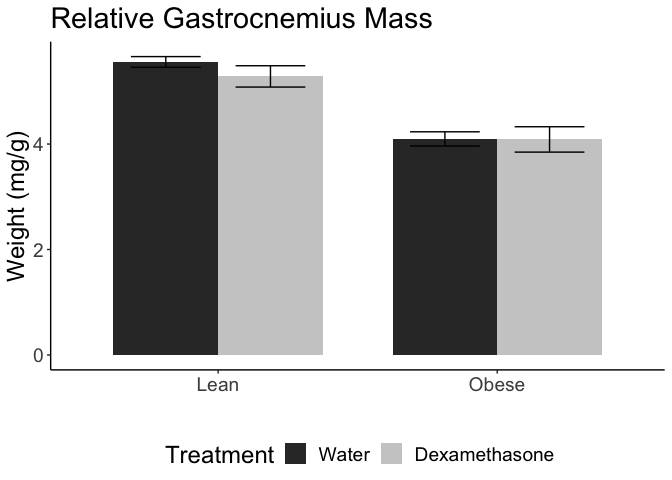
**We have added western blots for Akt in Figure 4C-D but were unable to complete blots for the other factors in time for this revision due to limited laboratory access by our researchers. We have noted this as a caveat in the discussion.**

Other:

1. Please include % fat free mass and lean mass at sacrifice on table 1. While this data is available to some degree on figure 2A, it would be more comprehensive and clear to also list the data at time of sacrifice on table 1 for a complete overview of body composition.

**We thank the reviewer for this suggestion. Both percent fat mass and lean mass at sacrivice have been added and referred to in the text.**

Also please include the gastrocnemius weights normalized to body weight, as the reduction in mass could be attributable to the decrease in body weight in the dexamethasone treated mice.



Gastrocnemius weights normalized to body weight at sacrifice. No significant differences in relative gastrocnemius weights once normalized to total body weight.

**This is an excellent clarification and this information has now been included in the text of the results section. Indeed, the reductions in gastric weight are proportional to reductions in both body weight and lean mass, and as such after normalizing for lean mass there are no significant effects of dexamethasone (p=0.386 or interactions of dexamethasone treatment with diet (p=0.486). There is of course a main effect of diet, due to dramatically different body weights (p<0.001). These data are presented here graphically and described in the revised results section:**

**There were no significant changes in relative gastrocnemius weights due to dexamethasone treatment after normalization to total body weight (pinteraction=0.486, pdexamethasone=0.386). We interpret this to indicating that the individual muscle mass changes were proportional to changes in total body weight, and that this was largely driven by reductions in lean mass.**

1. There is a formatting error on table 1 for fluid intake per day.

**This formatting error has been fixed**

1. Were mice activity levels recorded? Could changes in physical activity account for some of the differences observed?

**No physical activity was not assessed. It is plausible that reduced activity could affect glucose uptake or body weight changes. We have noted this as a potential caveat.**

1. Is the fluid intake for HFD-water vs. Chow-water animals significant? Could this potential increase in fluid intake be do to impaired glucose tolerance?

**No the effect on water intake is not statistically significant. We reported in Harvey et al 2018 that longer dexamethasone did result in increased water intake but we posit that this is due to extreme hyperglycemia and excessive urination. As such, in this study we used a shorter time course to limit this potential confounding possibility.**

1. “In NCD animals, the force generated by nerve stimulation was reduced 10% when treated with dexamethasone.” Is this significant? As Figure 1C does not reflect this. Same for the 11% reduction for muscle force figure 1D. If not statistically significant, I think it would help to list the p values of the main effects of diet and dexamethasone for clarification.

**As above, our primary outcome throughout the paper was the interaction between diet and treatment. The asterisks in Figure 1C-D indicate a significant interaction between diet and treatment. As to the question of whether the pairwise effects of dexamethasone are true in each of the subgroups, these do reach statistical significance for both all groups. For clarity we have added this to the revised results section:**

**Dexamethasone had significant effects in both groups for both muscle (p=0.016 for NCD and p=0.005 for HFD via Student’s *t*-tests) and nerve stimulation (p=0.015 for NCD and p=0.003 for HFD).**

1. It would be better to present the muscle CSA data before presenting the muscle force-CSA regression.

**We appreciate this comment, but in order to keep all the muscle structure data together we have elected to keep CSA in Figure 2. To assist with interpretation we have now mentioned differences in CSA earlier in the results section:**

**In order to examine whether changes in muscle strength were proportional to declines in muscle size, we plotted a regression of force versus whole-muscle cross-sectional area (CSA; Figure 1E-F). The quadriceps CSA was significantly lower for the dexamethasone treated groups and this was enhanced by obesity (Figure 2C).**

1. Please mention in text that the stain for fibre type assesses SDH activity.

**We added this clarification to the revised results section:**

**In order to evaluate any changes in the ratio of oxidative versus non-oxidative fiber-types, we stained muscle sections and quantified the muscle fibers based upon their oxidative capacity. We used NADH/NBT staining which is responsive to succinate dehydrogenase activity.**

Is there a main effect of diet/obesity for decreased type IIa/IIb?

**Based on mixed linear models to account for repeated measures within a sample, and removing the interaction term we found that there was a significant main effect of treatment (p=0.001) but not diet (p=0.159) in medium stained fibers. In light stained fibers, we similarly observed main effects of treatment (p=0.004) and diet (p=0.01). We have added this clarification to the revised results section**

**There was a main effect of dexamethasone treatment in all fiber types except oxidative (p=0.001 for light, p=0.004 for medium and p=0.449 for dark stained fibers). There was a significant main effect of diet reducing fiber size in light (p=0.01) but not medium (p=0.125) or dark stained fiber (p=0.425).**

1. Include the 15 day time point of gene data in figure 3 as bar graphs that show the 4 groups. Also the asterisks are missing on the current figure 3 to what is significant. From the text it looks like 7 days of treatment increases FOXO3, MuRF1 and Atrogin, but this is not reflected in the figure.

**The data for the 15 day time point is presented in Figure 3. None of these comparasons reached statistical significance, though several were close. This is why there is an abence of asterisks. This is clarified in the revised results text**

**After one week of dexamethasone treatment, we observed induction of *Foxo3* and the atrogenes, *Trim63* (Atrogin-1) and *Fbxo32* (MuRF1), to be greater in obese mice compared to their lean counterparts, though the interaction between obesity status and dexamethasone treatment did not reach statistical significance for these transcripts (Figure 3).**

1. It would be interesting to include western blots for MuRF1, Atrogin, phosphor and total FOXO3. Along the same lines, it would be good to include a marker of autophagic flux such as LC3II/I, as changes in autophagy could contribute to the reductions in muscle mass.

**We agree these are interesting questions, but unfortunately are unable to complete these experiments in time. We are perusing the role of mTORC1, obesity and GC-dependent changes in autophagy and hope to publish that work once research operations are fully re-established.**

1. In the discussion, it is mentioned that the mechanisms contributing to selective fibre type loss following dexamethasone treatment is unclear. It would be good if the authors expanded on their current data set to include markers involved in pathways known to induce fibre type switching such as ERK1/2, MAPK etc.

**In Figure 2G we present data that total oxidative fiber type proportions are not significantly, though selective fiber type loss does remain a possibility, especially if there is fiber-type specific turnover with both myogenesis and myoatrophy occurring. A separate study is being done to evaluate the single-cell dependent changes underlying this phenotype and we look forward to evaluating those markers in that work. We thank the reviewer for the excellent idea.**

1. The primer sequence for NR3c1 is missing.

**This has been added, we apologize for the oversight.**