We would like the thank the reviewers for their comments and suggestions. The revised manuscript clarifies the issues brought up by the reviewers with several new pieces of data. For clarity, we have noted in this response how the manuscript has been modified.

**Associate Editor's Comments:  
Both reviewers requested dexamethasone measurements and reference to human pharmacology. Ideally these studies might be performed on leftover serum from animals used in these studies. If none is available, measurements on comparably treated animals is acceptable or historical data but only if measured in your laboratory. I note that steroid hormone measurements, including synthetic dexamethasone, must conform to journal policies.**  
**Reviewer Comments:  
Reviewer 1: This is a solid study reporting on the combination of obesity and chronically elevated glucocorticoids leading to exacerbations in metabolic function. I feel this work is potentially worthy of publication with the inclusion of the following data:**  
In order to confidently draw comparisons between the DEX-treated chow-fed versus high-fat fed groups, it is important to demonstrate that the method of DEX administration (via the drinking water) results in comparable elevations in serum DEX. There is a possibility that the exacerbated metabolic function observed in the high-fat fed mice was simply due to increased consumption of the DEX-treated drinking water in these animals. As such, a measurement of serum DEX, in both the DEX-treated chow-fed and DEX-treated high-fat fed groups should be included.

We measured the amounts of dexamethasone these mice were consuming (via measurement of drinking water throughout the study; 1A-C of this response) as well as the serum concentrations (via LC-MS; D). The obese dexamethasone-treated mice did consume modestly more dexamethasone when compared to lean mice when normalized by body weight. To our surprise, as the study went on the HFD mice specifically drank more water (and dexamethasone), even though they started with lower water consumption (Figure 1C of this response). This was reflected in serum concentration which was determined from blood at the end of the study. The increase in dexamethasone consumption may reflect that the obese dexamethasone-treated mice were severely diabetic which may cause increased water intake noted in the third week of treatment, as has been documented previously by others (1). These new data are described in the revised methods:

**Methods for dex intake and quantification:**

**Water intake was measured weekly to determine the concentrations of dexamethasone consumed per cage. Average concentration per mouse was estimated by accounting for number of mice in the cage. Serum from 16 hour fasted mice was acquired prior to euthanizing at the end of the study and sent to the University of Michigan Pharmacokinetics Core for LC-MS analysis of dexamethasone concentration.**

…and results sections:

**Results:**

**Obese dexamethasone-treated mice 2.09-fold higher fluid intake (p=) when compared to lean dexamethasone-treated mice. This was reduced to a 22.01% increase in the obese animals when accounting for the higher weights of these mice. Increased intake resulted in a 7.59-fold increase in serum dexamethasone concentration in the obese dexamethasone-treated mice when compared to lean dexamethasone-treated mice (p=0.031).**

../../../../../../../../Desktop/CushingAcromegalyStudy/manuscript/Obesity-Glucocorticoids/figure%201%20for%20revie

**Figure 1: Dexamethasone intake and serum concentrations.** Amount of dexamethasone consumed per mouse (A), amounts normalized to mouse weight (B) and normalized weekly measures (C) as determined by volume consumed per cage per week for NCD- (n=12) and HFD-fed (n=20) mice. Concentration of dexamethasone in serum of NCD-fed (n=8) and HFD-fed (n=11) at the end of the study as determined by LC-MS (D).

We are grateful to the reviewer for probing us to look into this trend, as this presents a significant limitation to our study. We have addressed in the revised manuscript, although do not believe that this fully accounts for the more phenotype observed in these mice for several reasons now indicated in the revised discussion:

**The obese, dexamethasone treated animals consumed increasing fluids, including dexamethasone as the study progressed (Figure XX) resulting in increased serum dexamethasone at sacrifice (Figure XX). This was unexpected and may be due to the increased urination, and water requirement in severely diabetic animals, as has been documented previously** (1). **This is an important limitation to our study, although we note that several phenotypes including fasting glucose, liver triglycerides, hepatic lipogenic gene expression, and adipose tissue mass changed in different directions in lean and obese animals, and therefore is unlikely due to an increased dose of dexamethasone.**

We also note that we have observed increased blood glucose and glycerol levels with less than one week of dexamethasone exposure in a smaller scale time course experiment (Figure 2 of this response). At this stage, dexamethasone consumption is lower in the HFD group than the NCD group. While we are willing to include these data in the revised manuscript if necessary, the small n (4 animals per group at each time point) is less rigorous than we would prefer, and it will take approximately 3 months to repeat this time course.

Minor points

1. The figures 1A, 1B, 1C and 1D appear to be mislabeled in the legend.

Fixed in both the legends document and main document. There are typographical errors on both lines 278 & 406.  
Fixed 278, removed sentence with typo in 406 as it regarded patients (acknowledgements).   
  
**Reviewer 2: Authors assessed some metabolic effects of increased glucocorticoid in combination with obesity induced by hyper-caloric feeding (in mice). Authors speculate that this combination of events is present in "many individuals". Therefore they propose that pre-clinical studies on this topic are needed. The results are very descriptive, in line with expectation, and no mechanism of action has been identified. Thus, this study is very descriptive and its results expected.**  
  
Main criticisms:  
1) If authors wanted to mimic the clinical glucocorticoid treatment in mice, then was the increase in circulating glucocorticoid content experimentally-induced in mice comparable to the level seen in humans undergoing glucocorticoid therapy?

We measured intake of dexamethasone weekly throughout the study and found that mice were receiving less than 1mg/kg/d. Though this is at the high end, it is within the clinical range administered to humans, which is generally from 0.75-9mg/d and up to 3mg/kg/d (~210mg for an average American male), depending on the patient’s condition (2,3). As mentioned above, the obese mice had higher intake of dexamethasone and that was matched with elevated serum concentrations; however, these values were within range of serum cortisol concentrations observed in Cushing’s syndrome patients (4,5), even when accounting for the increased potency of dexamethasone in comparison to cortisol.

**Write something for the discussion section**

2) What is the novelty of this study?

To our knowledge this is the first paper to investigate chronically elevated glucocorticoids in the context of pre-existing obesity and compare to the lean phenotype. We show that obesity results in a more dramatic phenotype, including increased insulin resistance and lipolysis, as well as metabolic disturbances not noticed in lean mice given dexamethasone, such as excess hepatic lipid accumulation and pronounced fasting hyperglycemia. Additionally, we provide glucose clamp data that illustrate the main attributing factors to the hyperglycemia and insulin resistance in obese, dexamethasone-treated mice is hepatic glucose production. Lastly, we show that lipolysis is highly correlated with the increased metabolic perturbations both at the physiological (i.e. enhanced glycerol release) and molecular level (elevated ATGL transcripts and protein expression); moreover, obese dexamethasone-treated mice have reduced suppression of lipolysis in the presence of insulin when compared to obese controls. While these data agree with some published studies, we believe that these are valuable data to the research community. We have also added new data in this revision addressing the role of HSL and Perilipin phosphorylation in obese, dexamethasone treated animals. As can be seen in the new Supplementary Figure 2, both HSL and Perilipin phosphorylation on PKA sites is attenuated. This is described in the revised results section:

**Say something in the results**

And mentioned in the discussion in terms of the molecular links between glucocorticoids and lipolysis:

**Say something in the discussion**

3) Fig. 1A: In relative terms, insulin-induced changes in glycemia are similar between the 4 groups. Please, show data as percentage change over basal.

The requested data is presented in Figure 3A of this response (and the revised Supplementary Figure XX), demonstrating impaired insulin response in both lean and obese animals. This is described in the results section as such:

**When normalized to percent change from basal, dexamethasone treatment results in reduced glucose disposal when compared to water controls in lean and obese mice (Supplementary Figure 1X).**



A

4) Fig. 1C-F: What is the effect of glucocorticoid treatment on these parameters in NCD mice? Are these effects exacerbated in HFD?

We did perform glucose clamp experiments on chow-fed (lean) animals, but we observed substantial differences insulin clearance rates between the NCD-control and NCD-dexamethasone groups. This was an unexpected finding, but is concordant with previous reports that dexamethasone may cause impaired insulin degradation. Importantly this was not observed in the HFD animals (see Supplementary Figure 1F), nor does it impact our interpretation of insulin tolerance tests. This made interpretation of NCD glucose clamps problematic because the two groups in the NCD cohort had different effective insulin exposures, so we chose to not include those data. The result of the impaired insulin clearance was that NCD animals appeared have very modest differences when treated with dexamethasone in terms of glucose infusion rate, rate of glucose disposal and endogenous glucose production, likely a counterbalance between insulin resistance and insulin turnover. While this broadly agrees with our overall hypothesis of more impaired glucose homeostasis in obese, dexamethasone treated animals, we thought that this would be confusing and tangential to the reader. We will defer to the editor and reviewers though, if these data deemed to be of value, we are happy to include them in the manuscript, but for now present them below:

1. **Lee SM, Bressler R.** Prevention of diabetic nephropathy by diet control in the db/db mouse. *Diabetes* 1981;30(2):106–111.

2. **Tyrrell JB, Findling JW, Aron DC, Fitzgerald PA, Forsham PH.** An overnight high-dose dexamethasone suppression test for rapid differential diagnosis of Cushing’s syndrome. *Ann.Intern.Med.* 1986;104:180–186.

3. **Fleseriu M, Biller BMK, Findling JW, Molitch ME, Schteingart DE, Gross C, Auchus R, Bailey T, Biller BMK, Carroll T, Colleran K, Fein H, Findling JW, Fleseriu M, Hamrahian A, Katznelson L, Kerr J, Kipnes M, Kirschner L, Koch C, Lerman S, Lyons T, McPhaul M, Molitch ME, Schteingart DE, Vaughan TB, Weiss R.** Mifepristone, a Glucocorticoid Receptor Antagonist, Produces Clinical and Metabolic Benefits in Patients with Cushing’s Syndrome. *J. Clin. Endocrinol. Metab.* 2012;97(6):2039–2049.

4. **Martin NM, Dhillo WS, Banerjee A, Abdulali A, Jayasena CN, Donaldson M, Todd JF, Meeran K.** Comparison of the dexamethasone-suppressed corticotropin-releasing hormone test and low-dose dexamethasone suppression test in the diagnosis of cushing’s syndrome. *J. Clin. Endocrinol. Metab.* 2006;91(7):2582–2586.

5. **Papanicolaou DA, Yanovski JA, Cutler GB, Chrousos GP, Nieman LK.** Distinguishes Cushing ’ s Syndrome from Pseudo-Cushing. *Endocrinol. Metab.* 2009;83(4):1163–1167.

Please also see: https://reference.medscape.com/drug/decadron-dexamethasone-intensol-dexamethasone-342741