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:

**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. Dexamethasone standard was used to make a calibration curve from 2.5 to 100 ng/mL. A separate weighing of dexamethasone was used to make quality control standards at 3 and 30 ng/mL. Quality control standards were run in triplicate before and during sample analysis. For each calibration standard and quality control standard, 10 µL of blank plasma, 10 µL of calibration or QC standard, and 40 µL of internal standard were mixed in a 96-well plate. Each analytical sample was prepared by mixing 10 µL mouse plasma, 10 µL acetonitrile and 40 µL internal standard into a well of a 96-well plate. Some samples were below 10 µL in volume. In these cases, the volume collected was diluted to 10 µL and prepared in the same manner as the other samples. The plate was mixed at 1000 rpm for 5 min, then centrifuged at 3500 rpm for 10 min. Four microliters of supernantant were injected for analysis onto a Waters Xevo TQD triple quadrupole UPLC mass spectrometer for analysis.**

…and results section:

**Over the course of the experiment, obese dexamethasone-treated mice consumed more water, starting at a lower amount, which then increased over the duration of the experiment (Figure 3E). Overall this corresponded to a 22% increase when normalized to the animal’s body weight. By the end of the study, this increased intake resulted in a 7.6-fold increase in serum dexamethasone concentration in the obese dexamethasone-treated mice when compared to lean dexamethasone-treated mice (Figure 3F; p=0.031).**

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**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 dexamethasone as the study progressed (Figure 3E) resulting in increased serum dexamethasone at sacrifice (Figure 3F). 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

**../../../../../../../../Desktop/CushingAcromegalyStudy/manuscript/Obesity-Glucocorticoids/ResponseFigure 2: Blood glucose and glycerol levels in NCD- and HFD-fed mice following 2 weeks of dexamethasone treatment:** Plasma glucose (A) and glycerol (B) levels in mice following 3, 7 and 14 days of ~1mg/kg/d dexamethasone in their drinking water or left untreated (controls; time zero). Adult (70 day-old) C57BL/6J mice were provided ad libitum access to NCD or HFD for 8 weeks prior to treatment. Blood was taken prior to euthanasia in a semi-fasted state (food was not removed but time of sacrifice was at the end of the light cycle).

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 higher end of therapeutic doses, 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 (2–4). 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 (5,6), even when accounting for the increased potency of dexamethasone in comparison to cortisol. See revised discussion:

**The dose of dexamethasone received was within the clinical range administered to human patients** (2,3)**, corresponding to approximately 5 mg/day in an averaged sized human. Circulating concentrations of dexamethasone were similar to those observed in Cushing’s Syndrome patients** (5,6) **even after accounting for dexamethasone’s higher potency.**

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 factor to the hyperglycemia and insulin resistance in obese, dexamethasone-treated mice is hepatic glucose production. 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. To expand on our molecular data, we have also added new data in this revision addressing the role of HSL phosphorylation in obese, dexamethasone treated animals. As can be seen in the new Supplementary Figure 2, HSL phosphorylation on PKA sites is attenuated in obese animals. This is described in the revised results section:

**There were no significant increases observed in HSL expression or activation (via phosphorylation) that might explain enhanced lipolysis in the obese, dexamethasone treated mice (Supplementary Figure 1A-B). In fact, phosphorylation of PKA sites on HSL tended to be lower in obese mice when compared to lean, as has been reported previously** (7)**.**

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

**We did not find any significant differences in the effects of diet or treatment on HSL phosphorylation. Interestingly, obesity and dexamethasone treatment appeared to slightly decrease HSL phosphorylation, consistent with previous reports** (7)**. Given these results, we attribute enhanced lipolysis seen in obese dexamethasone-treated mice to upregulated ATGL.**

**Figure 3: ITT normalized to percent change from basal.** Insulin was given via i.p. injection at a concentration of 2.5 U/kg following five weeks of dexamethasone (NCD n=12; HFD n=12) or vehicle (NCD n=12; HFD n=12) treatment and 17 weeks of diet.

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 3 of this response (and the revised Supplementary Figure 1A), 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 1A).**

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 (see Figures 4A and B of this response). This was an unexpected finding, but is concordant with previous reports that dexamethasone may cause impaired insulin degradation (8,9). Importantly this was not observed in the HFD animals (see Supplementary Figure 1F), nor does it strongly impact our interpretation of insulin tolerance tests. This confounding effect made interpretation of NCD glucose clamps problematic because the two groups in the NCD cohort had different effective insulin exposures, and 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 (Figures 4C-E of this response), likely a counterbalance between insulin resistance and insulin turnover. While these data broadly agree 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:

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**Figure 4.Glucose Clamp Data in NCD-fed Mice:** Insulin clearance (A), plasma insulin concentrations (B), area under the glucose 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.

**References**

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. **Medscape.** Decadron, Dexamethasone Intensol (dexamethasone) dosing, indications, interactions, adverse effects, and more. Available at: https://reference.medscape.com/drug/decadron-dexamethasone-intensol-dexamethasone-342741. Accessed March 26, 2018.

5. **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.

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

7. **Gaidhu MP, Anthony NM, Patel P, Hawke TJ, Ceddia RB.** Dysregulation of lipolysis and lipid metabolism in visceral and subcutaneous adipocytes by high-fat diet: role of ATGL, HSL, and AMPK. *Am. J. Physiol. - Cell Physiol.* 2010;298(4):C961–C971.

8. **Protzek AOP, Rezende LF, Costa-Júnior JM, Ferreira SM, Cappelli APG, Paula FMM De, Souza JC De, Kurauti MA, Carneiro EM, Rafacho A, Boschero AC.** Hyperinsulinemia caused by dexamethasone treatment is associated with reduced insulin clearance and lower hepatic activity of insulin-degrading enzyme. *J. Steroid Biochem. Mol. Biol.* 2016;155:1–8.

9. **Ali M, Plas C.** Glucocorticoid regulation of chloroquine nonsensitive insulin degradation in cultured fetal rat hypatocytes. *J. Biol. Chem.* 1989;264(35):20992–20997.