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Ketogenic diet in combination with voluntary exercise impacts markers of hepatic metabolism and oxidative stress in male and female Wistar rats

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Abstract

Ketogenic diets (KD) are shown to benefit hepatic metabolism; however, their effect on the liver when combined with exercise are unknown. We investigated the effects of a KD vs a ‘Western’ diet (WD) on markers of hepatic lipid metabolism and oxidative stress in exercising rats. Male and female Wistar rats with access to voluntary running wheels were randomized to three groups (n= 8-14 per group): standard chow (SC; 17% Fat), WD (42% Fat) or KD (90.5% Fat) for 7 weeks. Body fat percentage (BF%) was increased in WD and KD vs SC, although KD females displayed lower BF% vs WD (p≤0.05). Liver triglycerides were higher in KD and WD vs SC, but were attenuated in KD females vs WD (p≤0.05). KD suppressed hepatic markers of de novo lipogenesis (FAS, ACC) and increased markers of mitochondrial biogenesis/content (PGC-1α, TFAM and citrate synthase activity). KD also increased the hepatic GPx1 and lowered oxidized glutathione. Females rats exhibited elevated hepatic markers of mitochondrial biogenesis (TFAM), mitophagy (LC3II/I ratio, ATG 12:5) and cellular energy homeostasis (pAMPK/AMPK) vs males. These data highlight that KD and exercise beneficially impacts hepatic metabolism and oxidative stress and merits further investigation.

- Ketogenic diet feeding combined with exercise improved hepatic oxidative stress, suppressed markers of de novo lipogenesis, increased markers of mitochondrial content vs western diet feeding.
- Males and females responded similarly to combined ketogenic diet feeding and exercise.
- Female rats exhibited elevated hepatic markers of autophagy/mitophagy and energy homeostasis compared with male rats.

Key words: ketogenic diet; mitochondria; oxidative stress; exercise; liver; sex; lipid metabolism
**Introduction**

In recent decades, rates of obesity and its associated conditions, including insulin resistance, type 2 diabetes and nonalcoholic liver disease (NAFLD) have markedly increased and contribute to cardiovascular disease (CVD) (Flegal et al. 2016; Fox et al. 2007). NAFLD is a common chronic disease, comprised of a spectrum of liver injury ranging from simple steatosis to steatohepatitis (NASH), fibrosis and end stage cirrhosis. The prevalence of NAFLD in the general population ranges from 10% to 30% and as high as 80% – 100% in obese populations (Chalasani et al. 2012; Vernon et al. 2011). Currently, there are no pharmacological treatments available for NAFLD. Recently, attention has shifted to alternative dietary regimes to combat metabolic diseases such as NAFLD. Ketogenic diets (KD), low in carbohydrate and very high in fat, have traditionally been used to treat neurodegenerative disorders. More recently, KD have been used therapeutically to treat a multitude of health disorders ranging from obesity to type 2 diabetes and NAFLD (Partsalaki et al. 2012; Samaha et al. 2003; Schugar and Crawford 2012; York et al. 2009). With the increasing therapeutic application of low-carbohydrate diets, further understanding of the range of metabolic responses observed in the liver are timely.

Although KD are accepted as an efficacious approach to weight loss (Hession et al. 2009; Nordmann et al. 2006; Santos et al. 2012; Shai et al. 2008), there still remains significant controversy about their metabolic effects. KD have been shown to improve glucose tolerance, insulin sensitivity and decrease markers of hepatic de novo lipogenesis in male mice compared to mice maintained on a high fat, high sucrose ‘western’ diet (WD) (Badman et al. 2009; Kennedy et al. 2007). Interestingly, improved mitochondrial quantity and quality and increased antioxidant defense systems are potential mechanisms through which KD feeding may also result in physiological benefits (Hutfles et al. 2017; Hyatt et al. 2016; Kennedy et al. 2007; Milder and Patel 2012; Milder et al. 2010). Conversely, others have shown that KD feeding can increase fat mass, reduce lean mass and increase insulin resistance (Garbow et al. 2011). Elevations in hepatic inflammation and steatosis in male rat and mouse models have also been
reported (Asrih et al. 2015; Garbow et al. 2011; Jornayvaz et al. 2010). Clearly, further research is needed in order to address the current disparities in the literature.

Regular exercise aids in the management and prevention of obesity, insulin resistance, type 2 diabetes, and NAFLD. Studies investigating the synergistic effects of combining exercise and KD feeding on liver health are limited. To our knowledge only one group has assessed the impact of a KD (67% kcal fat) combined with exercise (Holland et al. 2016), reporting that 6 weeks of resistance training + KD in male rats reduced hepatic steatosis and improved serum insulin, glucose and lipid profiles compared to WD and standard chow (SC) fed groups. However, there is a paucity of research examining the physiological impact of a traditional KD (i.e. > 90% kcal fat) in conjunction with aerobic exercise on markers of hepatic metabolism and any sex differences that may exist. Given the current knowledge gaps, we tested the hypothesis that very high fat KD (> 90% kcal fat) feeding in conjunction with voluntary wheel running exercise would significantly impact hepatic markers of de novo lipogenesis and mitochondrial health compared to high fat, high sucrose WD and SC feeding in male and female Wistar rats.
Materials and Method

**Animal protocol** - Experimental protocols were approved by the University of Missouri Animal Care and Use Committee. Rats were maintained on a 12:12-h light/dark cycle at 21–22 °C. Male and female Wistar rats were pair housed (with same sex) until 7 weeks of age. At 7 weeks of age, male and female rats were randomly assigned to one of three groups (n = 8-14/group), and provided access to standard chow (SC; 26.5%, 56.5%, 17%-PRO, CHO, FAT; #5008; Formulab Diet Purina; St. Louis, MO, USA), a ‘Western-style’ diet (WD; 15.2%, 42.7%, 42% & 0.2% cholesterol by weight; #88137; Envigo Teklad Diets; Madison, WI, USA) or a ketogenic diet (KD; 9.2%, 0.3%, 90.5%; #96355; Envigo Teklad Diets) and water *ad libitum* for 7 weeks. See Table 1 for macronutrient breakdown of diets. Females were matched for body mass at the beginning of the study. However, due to availability of male rats during randomization, the WD male rats had a higher starting body mass compared with SC and KD male rats. Males and females were considered as separate groups for a total of 6 groups. All animals were individually housed for the duration of the study. Rats were fasted 3 to 4 hours prior to euthanasia via CO₂ asphyxiation.

**Wheel Running** - To assess voluntary physical activity, all groups were given free access to voluntary running wheels 24 h/d for the duration of the study (7 weeks). Running distance was monitored daily using BC 800 bicycle computers (Sigma Sport, St. Charles, IL, USA). Running wheels were locked 48 hours prior to euthanasia.

**Body composition** - Body composition was measured within 5 minutes after euthanasia, using a Hologic QDR-1000/W dual-energy x-ray absorptiometry machine.

**Ketone Body measurement** – Whole blood β-hydroxybutyrate levels were measured using a Precision-Xtra ketone body meter (#B075RQS6B2; Abbott Laboratories, Santa Clara, CA) and blood ketone test strips (#B010MPEOOK; Abbott Laboratories) via tail vein stick. Ketone bodies were measured at week 3 and 7 with the average reported.
Hepatic triacylglycerol content and enzyme activity - Livers were quickly excised from euthanized rats, flash frozen in liquid nitrogen and stored at -80°C. Liver triacylglycerol (TAG) content, citrate synthase activity were measured as described previously (Rector et al. 2008).

Reduced glutathione (GSH) and oxidized glutathione (GSSG) - GSH and GSSG concentrations were determined by fluorometric methods as previously described (Rivera et al. 2008), utilizing methods of Hissin and Hilf (Hissin and Hilf 1976).

Serum analyses – Blood was collected via cardiac puncture immediately after euthanasia and allowed to clot before centrifugation at 1,300rpm for 10 minutes. Serum was extracted and stored at -80°C. Serum glucose, insulin, triglycerides (TAGs), free fatty acids (FFA) and cholesterol concentrations were measured as previously described (Laye et al. 2009a; Rector et al. 2008). Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) assays were performed by Comparative Clinical Pathology Services (Columbia, MO) using commercially available assays. Serum TBARS (Item No. 10009055, Cayman Chemical, Ann Arbor, Michigan) and Total Antioxidant Capacity (TAC; Item No.709001, Cayman Chemical) were measured according to the manufacturer’s instructions.

Western Blotting - Western blot analysis was completed in liver homogenate. Triton X-100 cell lysates were prepared and Laemmli gel loading buffer (#161-0737, Bio-Rad Laboratories, Hercules, CA) was added to the lysate to produce Western blot-ready Laemmli samples. The microtube containing this mixture was then boiled at 100 °C for 10 minutes. For each sample, 10-20 µg of protein was added to each well when running a Western blot gel. Proteins were separated by tris-glycine sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a PVDF transfer membrane (#88518, Thermo Scientific, Rockford, IL). The membrane was then incubated overnight in blocking solution (5% dry milk in Tris-buffered saline (TBS-) Tween 20 buffer) followed by overnight incubation in the primary antibody. After incubation, the membranes were washed with TBS-Tween 20 buffer and placed in a horseradish peroxidase-conjugated secondary antibody (#7074, #7076, Cell Signaling Technology, Danvers, MA). Blots (n = 8/group) and individual protein bands were quantified using a densitometer.
(Image Lab 3.0). For Western blot analysis, each sample was adjusted to the average intensity of all samples on the gel. The total protein staining for each lane, quantified by laser densitometry was used to correct for any differences in protein loading or transfer of all band densities. The primary antibodies used are as follows: acetyl coenzyme A carboxylase (ACC; #3662, Cell Signaling, Danvers, MA), S79 phosphorylation-specific ACC (p-ACC; #3661, Cell Signaling); fatty acid synthase (FASN; #3189, Cell Signaling), autophagy-related gene 12 (ATG12, #4180, Cell Signaling), 5’ adenosine monophosphate activated protein kinase (AMPK; #2603, Cell Signaling), T172 phosphorylated AMPK (p-AMPK; #2531, Cell Signaling), nucleoporin 62 (p62; #5114, Cell Signaling), nuclear factor E2–related factor 2 (NFE2L2/NRF2; #137550, Abcam), Kelch-like ECH-associated protein 1 (Keap1; #12721, Cell Signaling), Light chain 3 (LC3) A/B (#12741, Cell Signaling), cluster of differentiation 36 (CD36; #133625, Abcam, Cambridge, MA), mitochondrial transcription factor A (TFAM; #8076, Cell Signaling), oxidative phosphorylation (OXPHOS; #110413, Abcam, Cambridge, MA), peroxisome proliferator activated receptor-α (PGC-1α, #3242, Millipore, St. Louis, MO), Sirtuin 1 (SIRT1; #1540, Santa Cruz, ), Sirtuin 3 (SIRT3; #2627, Cell Signaling), superoxide dismutase 1 (SOD1, #13498, Abcam), superoxide dismutase 2 (SOD2, #13194, Cell Signaling), Cytochrome c (COX1; #4280, Cell Signaling), Glutathione peroxidase 1 (GPx1; #22604, Abcam), Sterol regulatory element-binding protein 1 (SREBP1; #367, Santa Cruz), Tumor necrosis factor-α (TNF-α; #11948; Cell Signaling), CD11b ( #NB110-89474; Novus Biologicals; Centennial, CO, USA), CD68 (#17832; Santa Cruz).

**Statistical Analysis** - Main effects of diet (standard chow vs western diet vs ketogenic diet) and sex (male vs female) were examined via a two-way ANOVA in SPSS (IBM SPSS Statistics for Windows, Version 24.0, Armonk, NY), and significant main effects ($p \leq 0.05$) were followed up with Fisher's LSD *post hoc* comparisons. When a significant interaction explained a main effect(s), only the interaction was interpreted. All values are reported as means ± SE, and a $p$ value of ≤0.05 denotes a statistically significant difference.
Results

*Animal and Serum Characteristics* – Male rats weighed significantly more than females (main effect of sex, \( p = 0.009 \); Fig. 1A). WD fed males were heavier than SC and KD fed males at the beginning of the study (Fig. 1A). WD and KD males gained less weight across the 7 week intervention compared with SC males (Fig. 1B). Final body mass of SC and WD males were greater than KD males (main effect of diet, \( p = 0.0009 \); Fig. 1A). Body fat percentage at the end of the study was significantly higher in WD and KD fed rats vs SC in both sexes (\( p<0.001 \)). Interestingly, KD fed females had a significantly lower body fat percentage vs WD females (\( p\leq0.05 \); Fig. 1C) and this was not observed in the males. Food and energy intake are presented as grams/day (Fig. 1D) and kcal/day (Fig. 1E), respectively. Food and energy intake were greater in male vs female rats (main effect of sex, Fig. 1D and E; \( p \leq 0.041 \)). Energy intake (kcal/day) was significantly higher in the WD males vs SC and KD fed males, while WD and KD fed females consumed more energy vs SC females (sex*diet interaction, \( p = 0.0106 \); Fig. 1E). Food and energy intake were not significantly different between KD fed males and females (Fig. 1D and E; \( p > 0.05 \)). In addition, female rats ran significantly more than their male counterparts (main effect of sex, \( p < 0.0001 \); Fig. 1G) and WD male ran significantly more than SC males.

As expected, blood ketone bodies were significantly elevated in KD fed rats (main effect of diet, \( p < 0.0001 \)), with greater elevations observed in males vs females (sex*diet interaction, \( p < 0.0001 \); Table 2). Serum TAGs, cholesterol, glucose, and insulin concentrations were higher in WD fed animals vs SC and KD fed groups (main effect of diet, \( p < 0.0001 \); Table 2). No differences in serum NEFA concentrations were observed among groups (Table 2). We also assessed serum markers of liver injury, inflammation, and oxidative stress. There was no effect of diet on serum AST or ALT concentrations (\( p > 0.05 \); Table 2), although male rats had higher serum AST concentrations vs females (main effects of sex, \( p = 0.0064 \); Table 2). Surprisingly, serum TBARS concentrations were elevated in females vs males (main effect of sex, \( p=0.0005 \)), which was driven by dramatic elevations in KD fed females (sex*diet interaction, \( p=0.0008 \); Table 2). No differences in TAC levels were observed among groups (\( p > 0.05 \), Table 2).
**Liver Phenotype** - To further explore the impact of a KD on the liver in exercising male and female rats, we assessed hepatic markers of lipid content, oxidative stress and inflammation. Both WD and KD feeding displayed significantly elevated liver triglycerides (TAGs) (~4.6 and 4.5-fold vs SC, respectively; main effect of diet, p < 0.0001; Fig. 2A) even though rats were voluntary wheel running; whereas, hepatic TAGs were 30% lower in KD females vs WD females (p<0.05) and significantly lower vs KD fed males (sex*diet interaction, p = 0.0235; Fig. 2A). Although hepatic TAGs were elevated, KD feeding decreased oxidized glutathione (GSSG) levels vs SC (main effect of diet, p = 0.0378; Fig. 2C) and increased glutathione peroxidase 1 (GPx1) protein content vs SC and WD in both sexes (main effect of diet, p < 0.0001; Fig. 2D). Hepatic nuclear factor E2–related factor 2 (NRF2) content, another protein involved in the oxidative stress response was significantly lower in KD fed males vs SC fed males (sex*diet interaction, p ≤ 0.05), while no differences in NRF2 protein content was noted within female groups. Furthermore, male rats exhibited elevated hepatic NRF2 protein content compared with females (main effect for sex, p = 0.0008; Fig. 2E). The repressor of NRF2, Kelch-like ECH-associated protein 1 (KEAP1), did not differ based on diet, but was significantly elevated in the liver of female vs male rats (p < 0.0001, Fig. 2E). There were no differences in hepatic superoxide dismutase (SOD)1 or SOD2 protein content (p > 0.05; Supplementary Figure S11).

The proinflammatory mediators, tumor necrosis factor-α (TNF-α) and cluster of differentiation (CD)11b protein content were significantly decreased in KD vs WD, with decreases in CD11b for KD vs SC fed animals also observed (main effect of diet, p ≤ 0.05; Fig. 2F). Interestingly, females displayed greater hepatic CD11b (driven by SC) and CD68 (driven by SC and KD) protein content, while males displayed greater TNF-α content, largely due to the dramatic elevations in WD fed males (main effect of sex, p ≤ 0.05; Fig. 2F).

5′ AMP-activated protein kinase (AMPK) is a marker of cellular energy homeostasis and regulator of both hepatic fatty acid oxidation and de novo lipogenesis. Total hepatic AMPK content was

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1 Supplementary data are available with the article through the journal Web site.
significantly elevated with WD feeding and suppressed with KD feeding (main effect of diet, p<0.0001; Fig. 3A). pAMPK/AMPK ratio, an indicator of AMPK activation, was significantly lower for WD males vs SC and KD fed males (sex*diet interaction, p = 0.0458; Fig. 3A). Interestingly, Thr 172 phosphorylated pAMPK and pAMPK/AMPK ratio was significantly elevated in females vs males (main effect of sex, p = 0.0118 and p < 0.0001, respectively; Fig. 3A). Total SREBP-1c (120kDa), an upstream transcriptional regulator of de novo lipogenesis, was elevated in WD females vs SC females and KD females; these differences were not observed in males (sex*diet interaction, p = 0.004; Fig. 3B). Furthermore, females displayed elevated levels of the mature transcriptionally active fragment (68kDa isoform) of SREBP-1c vs males (main effect for sex, p ≤ 0.05; Fig. 3B). KD feeding dramatically suppressed downstream markers of de novo lipogenesis, including ACC and FASN, as well as increasing pACC/ACC ratio (marker of inactivation) (main effect of diet, p = 0.001; Fig. 3B). Additionally, the lipid transporter CD36, was significantly lowered in females vs males (main effect of sex, p = 0.0007). Finally, KD females displayed elevated hepatic CD36 content vs. SC females (sex*diet interaction, p≤0.05; Fig. 3C), which was not observed in males.

**Hepatic Mitochondrial Markers**- Hepatic citrate synthase activity, a surrogate measure of mitochondrial mass was significantly higher with KD and WD feeding compared with SC in male and female rats (main effect of diet, p = 0.015; Fig. 4A). Cytochrome c protein content, a marker of mitochondrial content, was also higher with a KD, driven by KD fed males (main effect of diet, p = 0.015; Fig. 4B). To further examine mitochondrial content, we assessed the protein content of individual complexes in the electron transport chain (OXPHOS). In contrast to the above, complexes I, II and III were lowered with a KD vs SC, while complex IV was elevated (main effect of diet p ≤ 0.05; Fig. 4C). Additionally, KD fed animals displayed decreased complex I and II content vs WD (main effect of diet p≤0.05; Fig. 4C). No differences in complex V were observed among groups.

PGC-1α and mitochondrial transcription factor A (TFAM), markers of mitochondrial biogenesis, were significantly increased with a KD and WD vs SC, with greater increases in KD vs WD for PGC-1α
(main effect of diet, \( p \leq 0.05 \); Fig. 5A). Males displayed greater PGC-1\( \alpha \) vs females (main effect of sex, \( p = 0.026 \); Fig. 5A), while females displayed greater TFAM protein content vs males (main effect of sex, \( p \leq 0.05 \); Fig 5A). Sirtuin (SIRT)1 content, a protein responsible for deacetylating and increasing PGC-1\( \alpha \) activation was significantly lower in KD vs SC and WD fed rats (main effect of diet, \( p = 0.0018 \); Fig. 5A). SIRT3, another sirtuin involved in mitochondrial biogenesis regulation, displayed a significant main effect for diet (\( p = 0.0001 \); Fig 5A), likely driven by WD males (not observed in females) (sex*diet interaction, \( p = 0.0497 \); Fig 5A).

Finally, we investigated whether markers of hepatic autophagy and mitophagy were altered by KD feeding. Light chain 3 (LC3) II/LC3 I ratio, an indicator of autophagosome formation, was significantly decreased in WD vs SC groups (main effect of diet, \( p = 0.0308 \); Fig. 5B), but was not different for KD vs SC groups. Both WD and KD increased Sequestosome-1 (p62), a scaffold binding protein for LC3, vs SC (main effect of diet, \( p < 0.0001 \); Fig. 5B). In addition, LC3II/LC3I ratio and ATG12:5 conjugate were elevated in female compared with male rats (main effect for sex, \( p < 0.0001 \) and \( p = 0.0446 \), respectively; Fig 5B).
Discussion

High fat, very low carbohydrate, ketogenic diets (KD) have received recent attention in the management of obesity, type 2 diabetes, and NAFLD (Pantsalaki et al. 2012; Samaha et al. 2003; Schugar and Crawford 2012; York et al. 2009). Hepatic ketogenesis plays an important role in maintaining whole body TCA cycle homeostasis and supplies extrahepatic organs with energy substrates in glucose-limiting states. However, limited studies have examined the impact of KD on hepatic health, particularly when combined with exercise training, and none to date have examined potential sex differences in the responses. Here, we report a number of unique and novel observations pertaining to KD feeding when combined with wheel running exercise in male and female Wistar rats. Namely, KD feeding did not abnormally elevate serum lipids, insulin or glucose concentrations compared with a lower fat (though still high fat), western diet. KD feeding also reduced markers of hepatic oxidative stress and inflammation despite elevating hepatic TAGs. KD feeding also dramatically suppressed markers of hepatic de novo lipogenesis, as well as increased select markers of hepatic mitochondrial content and biogenesis. On the other hand, KD feeding increased serum lipid peroxidation in females, increased hepatic TAG levels, and downregulated several mitochondrial electron transport chain proteins in the liver.

Here we report that a KD resulted in a more favorable body composition vs WD feeding in exercising female rats, without observing similar improvements in male rats. Others have also reported improvements in body composition vs WD feeding in exercising rats (Holland et al. 2016). Though not assessed in the current study, it has been speculated that mechanisms behind these observations are caused by factors such as a reduced appetite, hormonal changes caused by a KD (Gibson et al. 2015; Paoli et al. 2015) and increased energy expenditure (Kennedy et al. 2007). In addition, we report that KD feeding attenuated elevations in serum TAGs, cholesterol, glucose, and insulin concentrations compared with WD feeding in exercising male and female rats. These improvements in glucose and insulin are in agreement with some (Holland et al. 2016; Kennedy et al. 2007) but not all (Garbow et al. 2011; Grandl et al. 2018) previous studies. The exact explanation for the discrepancies in the literature are unknown, but could be related to the % fat in the KD and/or the duration of the feeding studies. Further research is...
needed to determine what a healthy adaptation to KD feeding entails (de Oliveira Caminhotto and Lima 2013).

The previously reported effects of KD on measures of oxidative stress and antioxidant benefits seem to be largely equivocal (Greco et al. 2016; Hyatt et al. 2016; Jarrett et al. 2008; Kephart et al. 2017; Sullivan et al. 2004). Here, KD feeding reduced hepatic GSSG (the oxidized form of glutathione) and increased the antioxidant enzyme GPx1 with a corresponding decrease in NRF2 in KD males, suggesting a reduced need for a NRF2 antioxidant response. Similarly, long-term KD feeding (75% kcal fat) has been shown to up-regulate GSH biosynthesis and enhance mitochondrial antioxidant status in the hippocampus of male rats vs SC (Jarrett et al. 2008). Conversely, a recent study demonstrated no improvements in liver, skeletal muscle, or brain oxidative stress markers in KD fed rats (67% kcal fat) vs SC (Parry et al. 2018). In addition to improvements in oxidative stress measures, we found that KD feeding reduced hepatic inflammatory markers TNFα and CD11b compared with WD feeding, findings which were in agreement with other reports of reduced hepatic inflammation (phosphorylated/pan-p65) and serum ALTs in exercised male rats fed a KD (67% kcal fat) vs WD and SC (Holland et al. 2016). Collectively, these data suggest that a KD improves antioxidant defense and reduces oxidative stress and inflammation. Further research is warranted to elucidate the mechanisms involved.

KD (93.3% kcal fat) feeding is known to reduce hepatic de novo lipogenesis and increase hepatic fatty acid oxidation, while high-fat (~40%kcal), high sucrose, WD feeding increases hepatic de novo lipogenesis and impairs fatty acid oxidation (Garbow et al. 2011; Kennedy et al. 2007). Similarly, we demonstrate that KD feeding downregulates markers of hepatic de novo lipogenesis (ACC, pACC, FASN) vs WD feeding. On the other hand, six weeks of KD (67% kcal fat) feeding in resistance trained male rats failed to alter hepatic markers of lipogenesis when compared with SC and WD (Holland et al. 2016). These differences are likely due to differing macronutrient composition (23% PRO, 10% CHO, 67% FAT) compared with the current study.

In the current study, KD feeding increased measures of mitochondrial mass (citrate synthase activity and cytochrome c protein content), despite downregulation in hepatic protein content of the
electron transport chain complexes I, II and III. The observed increases in mitochondrial mass are consistent with past literature in which KD feeding (67% kcal fat) (Hyatt et al. 2016) and exercise (Rector et al. 2011) alone are shown to increase hepatic mitochondrial volume and function. The impact of KD diet on hepatic electron transport chain complex content is mixed (Hutfles et al. 2017; Hyatt et al. 2016; Kephart et al. 2017), with further research needed in this area.

The mitochondrial deacetylase enzyme, SIRT 3, has been shown to be induced by increases in PGC-1α and normally functions to catalyze NAD+ dependent deacetylation of numerous substrates in the mitochondrial matrix in order to maintain mitochondrial function (Ansari et al. 2017; Kim et al. 2010). Chronic high fat, high sucrose, WD feeding has been associated with increases in mitochondrial protein hyperacetylation, reductions in deacetylase activity, in particular SIRT3, and subsequent decreased mitochondrial function in sedentary mice with a fatty liver (Kendrick et al. 2011; Morris et al. 2013). Here, we observe that a high fat, high sucrose, WD resulted in a significant increases in hepatic SIRT3 protein content vs SC in exercising male rats. These differences in findings may possibly be due to the confounding effect of exercise in our study. Conversely, a KD did not result in significant changes in hepatic SIRT3 protein content vs SC fed exercising male rats, despite increases in PGC-1α protein content in response to a KD. Although acetylation status and sirtuin activity were not assessed in the current study, the reductions in ETC complex protein content, in particular complex I and II, in KD fed animals vs SC and WD fed animals may be associated with decreased SIRT3 mediated deacetylation at the mitochondrial matrix in response to a KD. Future studies should explore the impact of a KD on acetylation status and sirtuin activity on the liver and their subsequent downstream effects on mitochondrial function.

PGC-1α and TFAM coregulate multiple transcription factors involved in mitochondrial biogenesis and content (Wu et al. 1999), and we have previously reported that PGC-1α and TFAM expression are increased in the liver with exercise training in SC fed rats (Laye et al. 2009b). Here, hepatic mitochondrial biogenesis markers PGC-1α and TFAM were upregulated with WD and KD.
compared with SC feeding in exercising rats. While some studies have reported increased PGC-1α expression in response to a KD (Jornayvaz et al. 2010), others have reported no effects in sedentary male rodents (Kennedy et al. 2007; Parry et al. 2018). These differing observations compared to the current study may be due to the fact that all of our animals were exercising. In fact, while exercise was shown to increase skeletal muscle PGC-1α in KD fed mice, KD feeding alone did not increase PGC-1α vs SC (Hyatt et al. 2016). The observed increases in hepatic PGC-1α content occurred despite lower hepatic SIRT1 content in KD compared with SC and WD groups. SIRT1 is a major cytosolic deacetylase and a primary function is to deacetylate PGC-1α, thus increasing its transcriptional activity (Amat et al. 2009). Others also report increases in PGC-1α transcription independent of SIRT1 and also in the presence of exercise (Cantó and Auwerx 2009; Terada et al. 2002). Future studies should explore the acetylation status of hepatic PGC-1α and its transcriptional activity in response to KD and exercise, as well as in sedentary conditions, to fully elucidate the impacts of a KD and exercise on hepatic mitochondrial biogenesis.

Mitochondrial processes of autophagy, termed mitophagy, is the clearance of damaged or poorly functioning mitochondria. Mitophagy and biogenesis are intricately linked in an effort to maintain mitochondrial homeostasis (Kim et al. 2007). With increases in markers of hepatic mitochondrial mass/content and mitochondrial biogenesis, we suspected that mitophagy may be blunted in exercising WD and KD fed animals. Indeed, a marker of mitophagy, LC3II/LC3I ratio, was decreased with WD and KD feeding, along with elevated p62, indicative of impaired mitophagic activity (Yoshii and Mizushima 2017). As these are surrogate measures of mitophagy, future research should investigate the impact of a KD on mitophagic flux in hepatic tissue.

Female rats displayed a higher hepatic LC3II/LC3I ratio, as well as increased TFAM and ATG12:5, key players in mitochondrial biogenesis and mitophagy. Female rats also exhibited greater increases in the content of markers related to hepatic lipid import (CD36) and decreases in de novo lipogenesis (inactivation of ACC through increased pACC) vs males, findings likely linked to greater increases in pAMPK/AMPK ratio. These data suggest that females may have a greater propensity for
increased hepatic fatty acid oxidation, potentially serving as a protective mechanism against NAFLD, which has been shown by others previously (Pramfalk et al. 2015). These data are in agreement with previous studies from our lab and others, in which females exhibited greater capacity for hepatic mitophagy (Cunningham et al. 2018; Sheldon et al. 2016; Von Schulze et al. 2018). Although speculative, this may be due in part to elevated levels of circulating sex hormones, which have been shown to be protective against increased hepatic steatosis (Gutierrez-Grobe et al. 2010; Kamada et al. 2011; Lonardo et al. 2006).

In summary, very high fat, low carbohydrate KD feeding in conjunction with wheel running exercise exerts a number of beneficial effects on hepatic health in male and female rats. Specifically, KD feeding favorably changed serum markers of lipids, glucose and insulin and reduced markers of hepatic oxidative stress, inflammation and lipogenesis. Furthermore, KD feeding increased select markers of hepatic mitochondrial content and mitochondrial biogenesis. However, compared with low fat SC, KD feeding also increased hepatic TAGs and downregulated hepatic proteins involved in electron transport chain despite rats undergoing voluntary physical activity. KD are prescribed with increasing frequency to treat metabolic disease including obesity, T2D and NAFLD. This study provides novel insight into the impact of changes in dietary macronutrient content in conjunction with exercise on liver health in both male and female rats. The metabolic effects of KD are not yet completely understood and warrant further investigation.
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The authors have no conflicts of interest to report.

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Table 1. Macronutrient content of each respective diet

<table>
<thead>
<tr>
<th>Diet</th>
<th>SC</th>
<th>WD</th>
<th>KD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calorie (per gram)</td>
<td>3.2</td>
<td>4.5</td>
<td>6.7</td>
</tr>
<tr>
<td>Carbohydrate (% of kcal)</td>
<td>56.5</td>
<td>42.7</td>
<td>0.3</td>
</tr>
<tr>
<td>Protein (% of kcal)</td>
<td>26.5</td>
<td>15.2</td>
<td>9.2</td>
</tr>
<tr>
<td>Fat (% of kcal)</td>
<td>17.0</td>
<td>42.0</td>
<td>90.5</td>
</tr>
<tr>
<td>Fatty acid profile (% of fat)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saturated fat</td>
<td>26.3</td>
<td>61.8</td>
<td>25.0</td>
</tr>
<tr>
<td>Monounsaturated fat</td>
<td>29.4</td>
<td>27.3</td>
<td>20.8</td>
</tr>
<tr>
<td>Polyunsaturated fat</td>
<td>22.1</td>
<td>4.7</td>
<td>50.0</td>
</tr>
<tr>
<td>Unknown</td>
<td>22.2</td>
<td>6.2</td>
<td>4.2</td>
</tr>
<tr>
<td>Cholesterol (% by weight)</td>
<td>trace</td>
<td>0.2</td>
<td>-</td>
</tr>
<tr>
<td>Biomarker</td>
<td>SC</td>
<td>Male WD</td>
<td>KD</td>
</tr>
<tr>
<td>---------------------------------</td>
<td>--------</td>
<td>---------------</td>
<td>-------</td>
</tr>
<tr>
<td>Glucose, mg/dL</td>
<td>286.9±33.7</td>
<td>446.8±83.9*</td>
<td>234.2±28.0*</td>
</tr>
<tr>
<td>Insulin, ng/dL</td>
<td>4.5±0.4</td>
<td>5.2±0.7</td>
<td>3.5±0.5*</td>
</tr>
<tr>
<td>Ketone Bodies, mmol/L</td>
<td>0.98±0.05</td>
<td>0.94±0.06</td>
<td>2.77±0.24*</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>112.6±17.1</td>
<td>182.3±35.1*</td>
<td>117.1±23.5#</td>
</tr>
<tr>
<td>Total Cholesterol, mmol/L</td>
<td>0.75±0.02</td>
<td>0.96±0.10*</td>
<td>0.79±0.05*</td>
</tr>
<tr>
<td>Non-esterified Fatty Acids, mg/dL</td>
<td>0.55±0.13</td>
<td>0.34±0.13</td>
<td>0.49±0.20</td>
</tr>
<tr>
<td>ALT, U/L</td>
<td>50.0±14.8</td>
<td>35.0±2.5</td>
<td>58.0±3.9</td>
</tr>
<tr>
<td>AST, U/L</td>
<td>155.6±40.7</td>
<td>159.1±28.0</td>
<td>212.1±43.1</td>
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<tr>
<td>Total Antioxidant Capacity, mM</td>
<td>1.35±0.2</td>
<td>1.64±0.2</td>
<td>1.45±0.2</td>
</tr>
<tr>
<td>TBARS, µm</td>
<td>7.4±0.6</td>
<td>7.7±0.8</td>
<td>5.2±0.7</td>
</tr>
</tbody>
</table>

(* significant difference (p≤ 0.05) from SC. # significant difference (p≤ 0.05) between WD and KD. $ significant difference (p ≤ 0.05) for male vs. female within diet. Values are presented as mean ± SE. n = 7-12/group).
Figures

**Figure 1 - Effects of diet and sex on animal characteristics:** Body mass changes over time (A), mean Change in Body Mass (B), End Body Fat Percentage (C), Food Intake (grams/day) (D), Energy Intake (kcals/day) (E), Distance ran/night (weekly) (F), Average distance ran/night (overall) (G). Main effects, interactions and p values reported in text above graphs. * significant difference (p ≤ 0.05) from SC. # significant difference (p ≤ 0.05) between WD and KD. $ significant difference (p ≤ 0.05) for male vs. female within diet. Values are presented as mean ± SE (n = 7-14/group).

**Figure 2 - Effects of diet and sex on liver triglycerides hepatic oxidative stress and inflammation:** Liver triglycerides (A), Hepatic GSH (B), Hepatic GSSG (C), Hepatic GPX1 (D), Hepatic oxidative stress (E), Hepatic Inflammation (F), Representative western blots (G). Main effects, interactions and p values reported in text above graphs. * significant difference (p ≤ 0.05) from SC. # significant difference (p ≤ 0.05) between WD and KD. $ significant difference (p ≤ 0.05) for male vs. female within diet. Values are presented as mean ± SE (n = 7-14/group).

**Figure 3 - Effects of diet and sex on hepatic lipid regulation:** Hepatic AMPK content (A), Hepatic de novo lipogenesis (B), Hepatic CD36 (C), Representative western blots (D). Main effects, interactions and p values reported in text above graphs. * significant difference (p ≤ 0.05) from SC. # significant difference (p ≤ 0.05) between WD and KD. $ significant difference (p ≤ 0.05) for male vs. female within diet. Values are presented as mean ± SE (n = 7-14/group).

**Figure 4 - Effects of diet and sex on hepatic mitochondrial content:** Hepatic citrate synthase activity (A), Hepatic cytochrome c protein content (B), Hepatic mitochondrial content (C), Representative western blots (D). Main effects, interactions and p values reported in text above graphs. * significant difference (p ≤ 0.05) from SC. # significant difference (p ≤ 0.05) between WD and KD. $ significant difference (p ≤ 0.05) for male vs. female within diet. Values are presented as mean ± SE (n = 7-14/group).

**Figure 5 - Effects of diet and sex on hepatic mitochondrial biogenesis and hepatic autophagy/mitophagy:** Hepatic mitochondrial biogenesis proteins (A), Hepatic mitochondrial autophagy/mitophagy protein content (B), Representative western blots (C). Main effects, interactions and p values reported in text above graphs. * significant difference (p ≤ 0.05) from SC. # significant difference (p ≤ 0.05) between WD and KD. $ significant difference (p ≤ 0.05) for male vs. female within diet. Values are presented as mean ± SE (n = 7-14/group).
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476x289mm (300 x 300 DPI)
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Figure 4 - Effects of diet and sex on hepatic mitochondrial content: Hepatic citrate synthase activity (A), Hepatic cytochrome c protein content (B), Hepatic mitochondrial content (C), Representative western blots (D). Main effects, interactions and p values reported in text above graphs. * significant difference ($p \leq 0.05$) from SC. # significant difference ($p \leq 0.05$) between WD and KD. $\$$ significant difference ($p \leq 0.05$) for male vs. female within diet. Values are presented as mean ± SE (n = 7-14/group).
Figure 5 - Effects of diet and sex on hepatic mitochondrial biogenesis and hepatic autophagy/mitophagy: Hepatic mitochondrial biogenesis proteins (A), Hepatic mitochondrial autophagy/mitophagy protein content (B), Representative western blots (C). Main effects, interactions and p values reported in text above graphs.

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385x270mm (300 x 300 DPI)