

**Cocoa extract exerts sex-specific effects in an aggressive hyperglycemia model: A pilot study**

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**MATERIALS AND METHODS**

*Cocoa extract production and characterization* [1–5].

*Folin-Ciocalteu colorimetric assay.* Cocoa extract ( $n=3$ ) was diluted with 40% EtOH to a final concentration of 0.2 mg/mL. A standard curve of gallic acid was prepared at 0.0-1.0 mg/mL. Each sample and standard was diluted 10X with ddH<sub>2</sub>O, followed by addition of 2N Folin-Ciocalteu reagent (1:2.5 sample solution:Folin). Sodium carbonate solution (7.5%, v/v) was added to all samples and standards. Samples and standards were incubated for 2 h at room temperature and then read at 765 nm. Total polyphenol concentration is expressed as mg Gallic Acid Equivalents (GAE).

*4-dimethylaminocinnamaldehyde (DMAC) colorimetric assay.* DMAC solution was prepared by combining stock HCl with EtOH (1:10 HCL:EtOH) and chilling at 4°C for 15 min. DMAC powder was added to the chilled solution at 0.001 g/mL. Cocoa extracts were diluted with EtOH to a final concentration of 100 ppm. A standard curve of procyanidin B2 (PCB2) was prepared at 0-100 ppm. DMAC solution was added to each sample and standard (1:5 sample:DMAC), mixed thoroughly, and read at 640 nm. Total flavanol concentration is expressed as mg PCB2 equivalents.

*Thiolysis.* Cocoa extract was diluted with MeOH to 0.5 mg/mL and then mixed (50  $\mu$ L) with 50  $\mu$ L HCl (3.3%, water) and 100  $\mu$ L benzyl mercaptan (5%, MeOH). Samples were placed in a 90°C water bath for 5 min and then cooled on ice for 5 min. Unthiolized controls were prepared with cocoa extract and MeOH without heating in the water bath. Each thiolized sample (100  $\mu$ L) was combined with 900  $\mu$ L of 0.1% formic acid in water and 0.1% formic acid in ACN (95:5 v/v). Samples were analyzed on a Waters Acquity H-Class separations module with an Acquity UPLC HSS T3 column (2.1 mm  $\times$  100 mm, 1.8  $\mu$ m) at 40°C. Binary gradient elution was performed using 0.1% formic acid in water (Phase A)

and 0.1% formic acid in ACN (Phase B). Solvent flow rate was 0.6 mL/min and the linear gradient elution was followed: 95% A (0-0.5 min), 65% A (6.5 min), 20% A (7.5-8.6 min), 95% A (8.7-10.5). (-)-electrospray ionization (ESI) together with tandem mass spectrometry (MS/MS) was used to analyze UPLC effluent on a Waters Acquity triple quadrupole (TQD) MS. (-) mode electrospray ionization (ESI) was performed with capillary, cone, and extractor voltages of -4.24 kV, 30.0 V, and 3.0 V respectively. Source temperature was 150°C and desolvation temperature was 400°C. Cone gas flows at a rate of 75 L/h and desolvation gas at 900 L/h. Argon (0.25 mL/min) was used as the collision gas in MS/MS. Multi-reaction monitoring (MRM) with a mass span of 0.2 Da was performed on parent ions and collision-induced dissociation (CID) on daughter ions. Inter-channel delays and interscan time was 1.0 s each. Additional calculations are done to account for the native monomers and are reported as DP of total flavanols. mDP oligomers and polymers and mDP of total flavanols are calculated as follows:

$$mDP (O + P) = \frac{\text{net number of monomers} + \text{net number of thiolytic derivatives}}{\text{net number of monomers}}$$

$$mDP (\text{total flavanols}) = \frac{\text{total monomers} + \text{net number of thiolytic derivatives}}{\text{total monomers}}$$

*UPLC-MS/MS.* A Waters Acquity H-class separation module equipped with a Waters Acquity UPLC HSS T3 column (2.1 mm × 100 mm, 1.8 μm, 40°C) and VanGuard HHS T3 precolumn (1.8 μm). Samples were maintained at 10°C. Binary gradient elution was performed with 0.1% (v/v) aqueous formic acid (phase A) and 0.1% (v/v) formic acid in acetonitrile (phase B). Solvent flow rate was 0.6 mL/min and the linear gradient elution was carried out as followed: 95% A (0-0.5 min), 65% A (6.5 min), 20% A (7.5-8.75 min), 95% A (8.85-10 min). An injection volume of 10 μL was used for all samples and standards. MS/MS analysis of column effluent was performed by (-)-ESI on a Waters Acquity TQD mass spectrometer equipped with a Z-spray electrospray interface. Ionization settings are as follows: -4.25 kV ESI capillary voltage, 150°C source temperature, 400°C desolvation temperature. N<sub>2</sub> was used for cone and desolvation gasses with flow rates of 75 L/h and 900 L/h, respectively. Ar was used as a collision gas. A standard curve of (-)-epicatechin, catechin, procyanidin B2, procyanidin C1, and cinnamtannin A2 (CinA2) was prepared and flavanol concentrations (procyanidin tetramer – decamer) are expressed as CinA2 equivalents.

TABLES AND FIGURES

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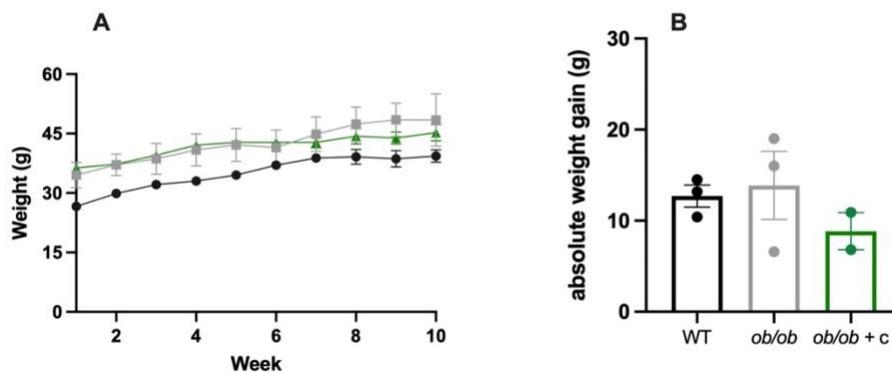
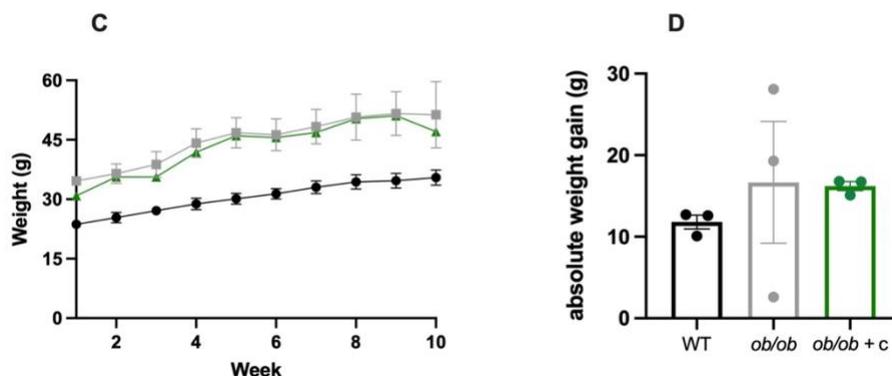
**Supplementary Table 1. Mouse genotype results**

<b>Sample ID<sup>a</sup></b>	<b>Detected <i>ob/ob</i> mutation</b>
Male	
WT (A)	--
WT (B)	--
WT ©	--
<i>ob/ob</i> (A) <sup>b</sup>	NA
<i>ob/ob</i> (B)	++
<i>ob/ob</i> (C)	++
<i>ob/ob + c</i> (A)	++
<i>ob/ob + c</i> (B)	++
<i>ob/ob + c</i> (C)	--
Female	
WT (A)	--
WT (B)	--
WT ©	--
<i>ob/ob</i> (A)	++
<i>ob/ob</i> (B)	++
<i>ob/ob</i> (C)	++
<i>ob/ob + c</i> (A)	++
<i>ob/ob + c</i> (B)	++
<i>ob/ob + c</i> (C)	++

<sup>a</sup>A, B and C designate individual animals within each group

<sup>b</sup>unable to genotype, mouse died at week 10 prior to euthanasia

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**Male****Female**

**Supplementary Figure 1.** Absolute weights during the 10-week experiment for male (A, B) and female (C, D) mice. Male mice: weight gain over time (A), total absolute weight gain (B). Female mice: weight gain over time (C), weight gain over time, total absolute weight gain (D). Dots represent individual animals; colored bars and error bars represent mean  $\pm$  SEM. For bar graphs, data were analyzed by 1-way ANOVA; due to lack of observed overall treatment effect for any graph, no post hoc tests were performed to compare treatment means. Error bars and statistical analyses are not shown for food intake, as each value is from  $n=1$  cage.

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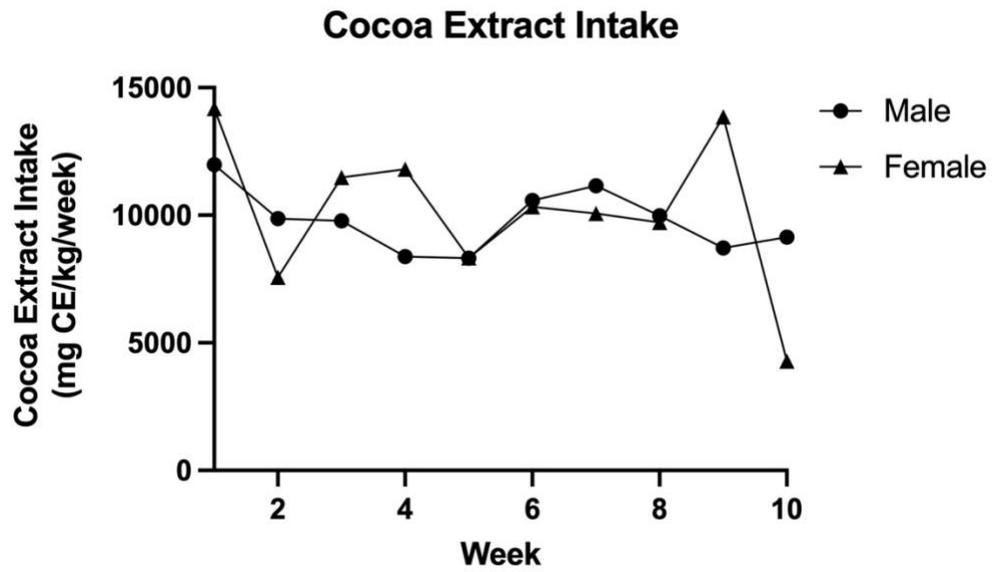
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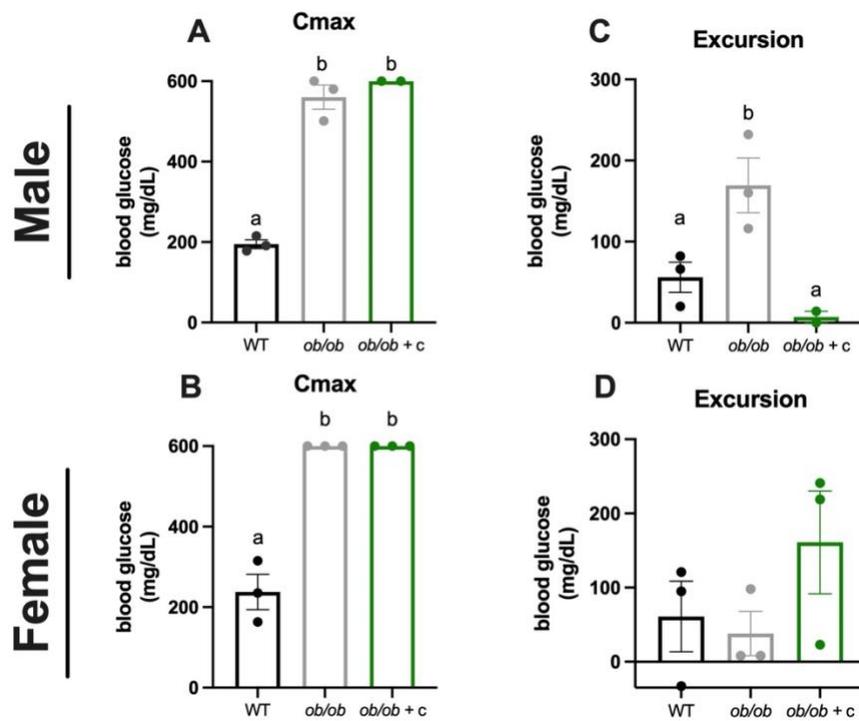
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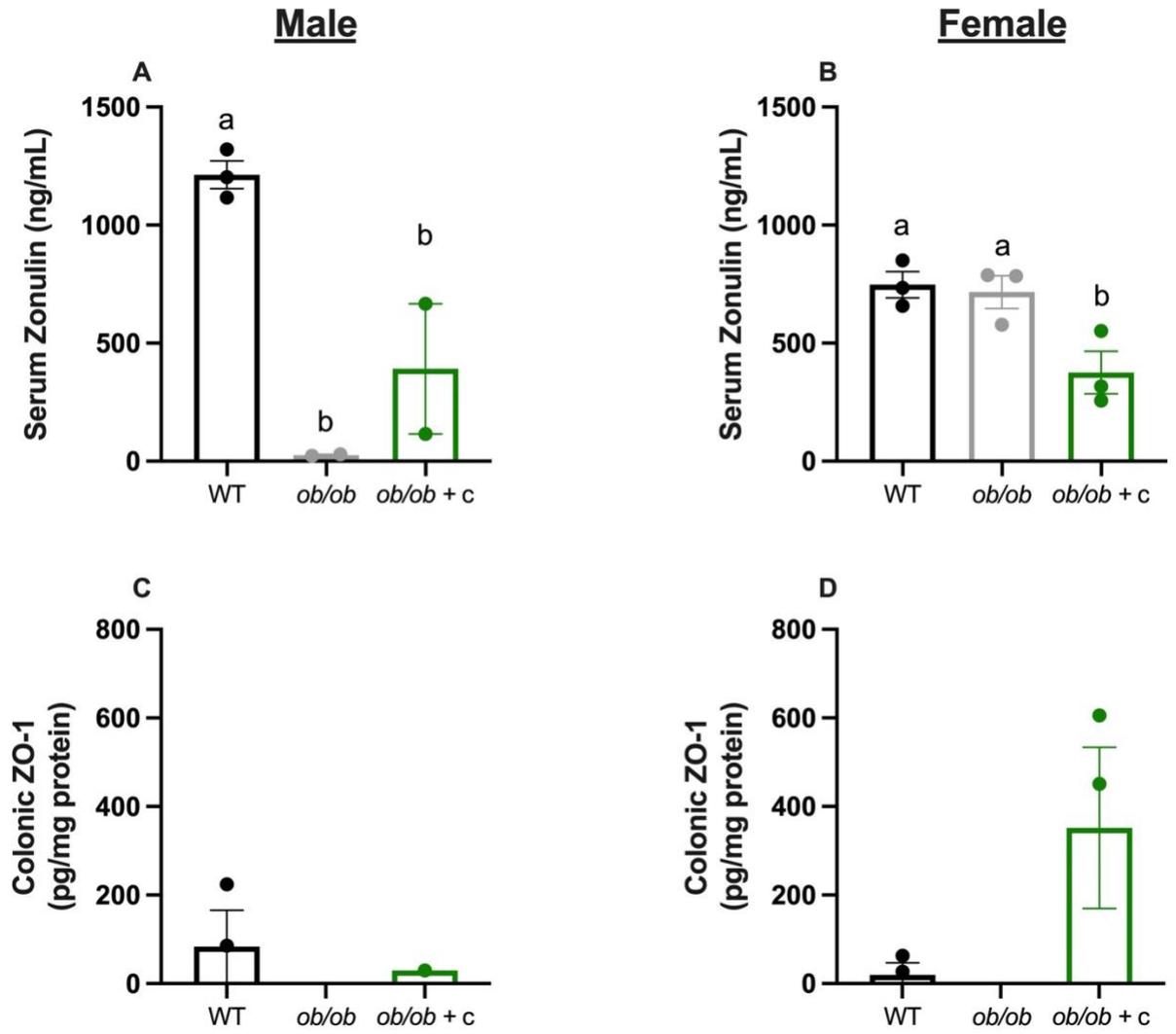
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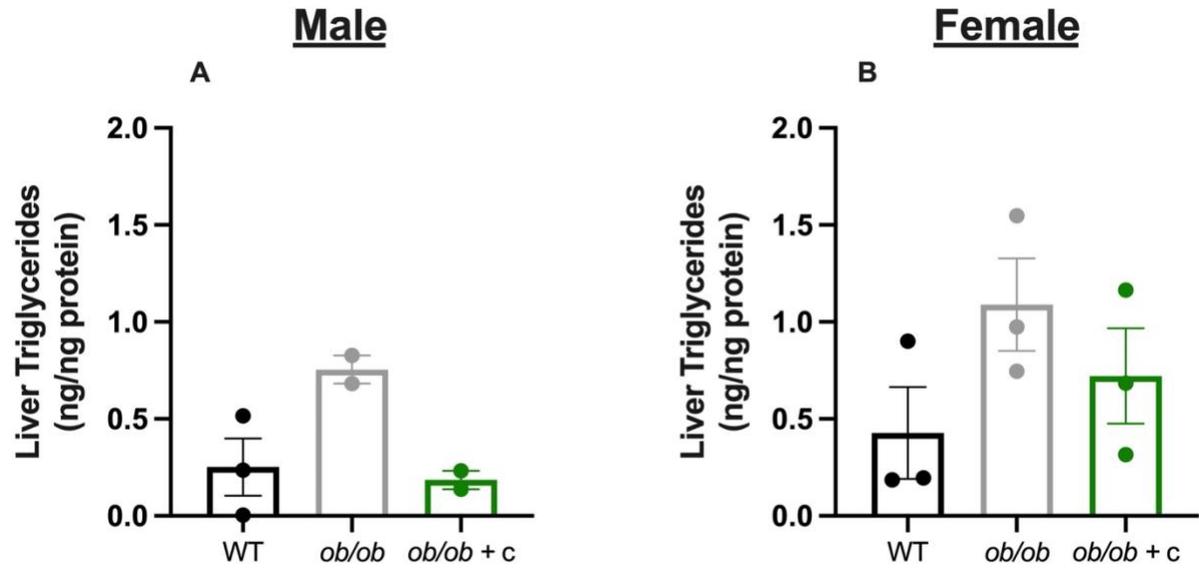
*Supplementary Figure 2. Cocoa extract intake for both male and female mice. Male and female treatments without cocoa extract supplementation are not shown. Error bars and statistical analyses are not shown for extract intake, as each value is from n=1 cage..*



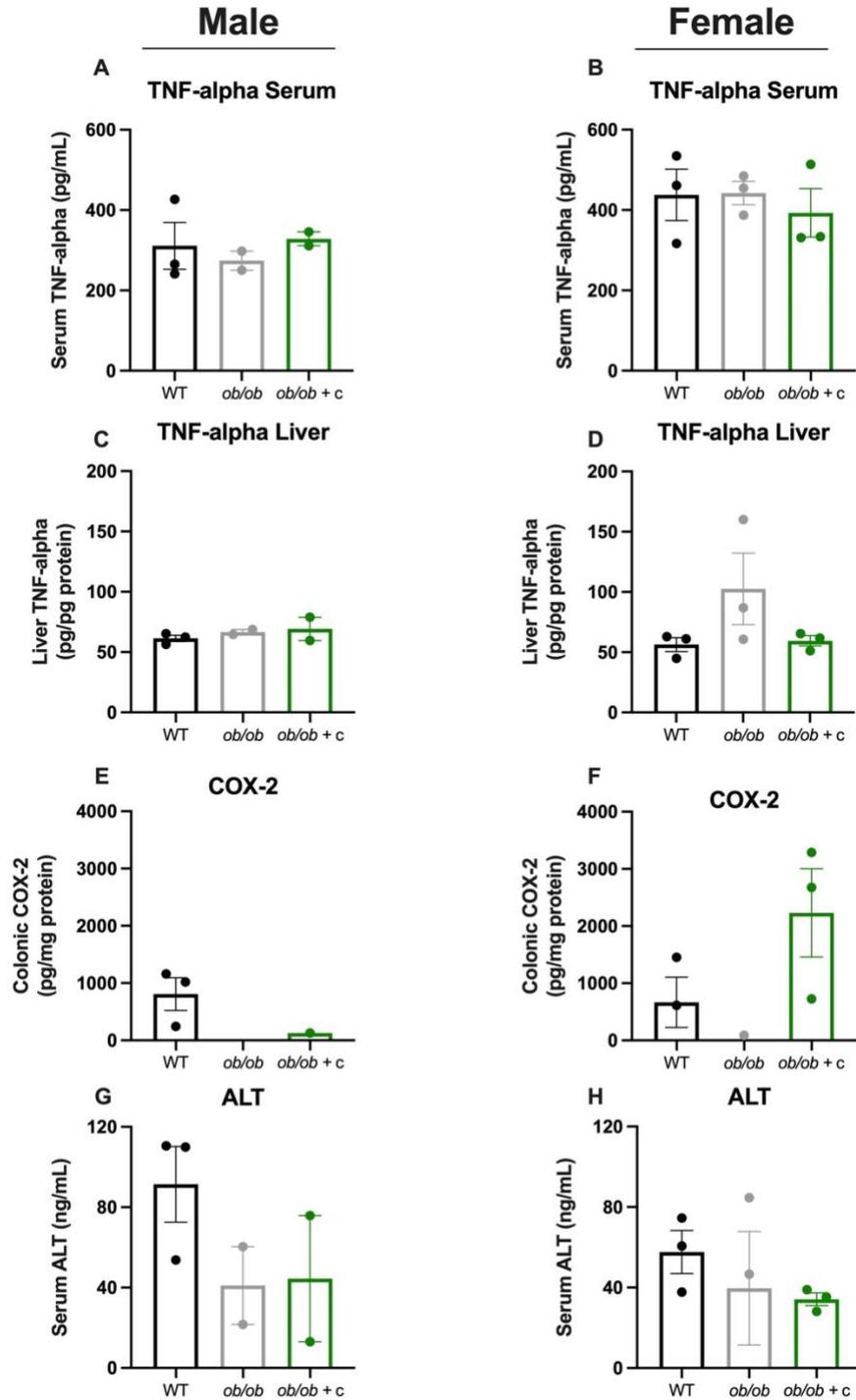
**Supplementary Figure 3.** Glucose tolerance test measures. Maximal blood glucose values (Cmax, A, B), blood glucose excursion from baseline (C, D) for week 5 GTT. Values are presented as mean  $\pm$  SEM. Data were analyzed by 1-way ANOVA. If a significant treatment effect was detected, Tukey's post hoc test was performed to compare treatment means. Bars not sharing a common superscript letter are significantly different ( $P < 0.05$ ). It must be noted that the maximum glucometer reading was 600 mg/dL and therefore in some cases the true glucose reading could not be determined. The apparent lack of variability among high readings is not necessarily representative of a lack of biological variability, but rather a limitation in the instrumentation utilized.



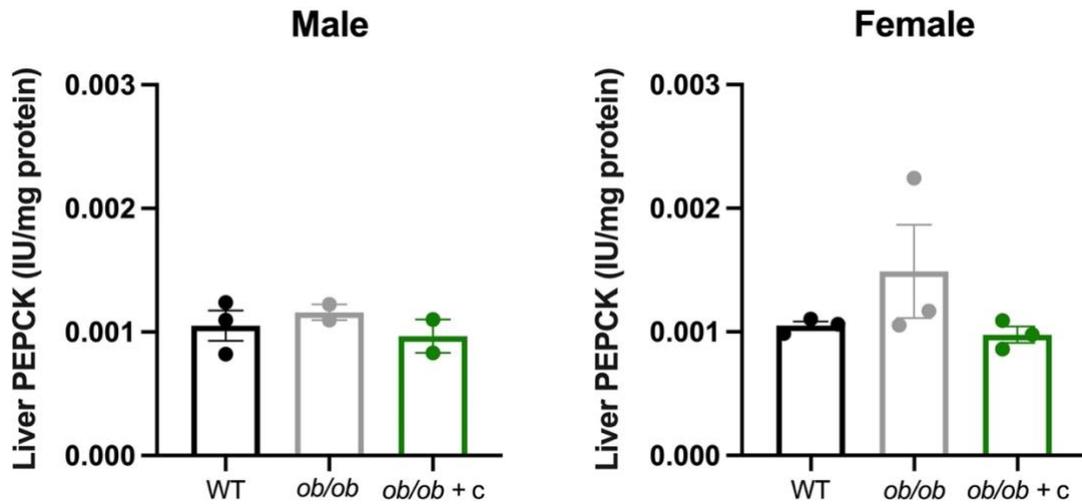
**Supplementary Figure 4.** Serum zonulin protein levels (A-B) and colonic zona occludens-1 (ZO-1) protein levels (C-D) following 10 weeks of treatment. Values are presented as mean  $\pm$  SEM. Data were analyzed by 1-way ANOVA. If a significant treatment effect was detected, Tukey's post hoc test was performed to compare treatment means. Bars not sharing a common superscript letter are significantly different ( $P < 0.05$ ).



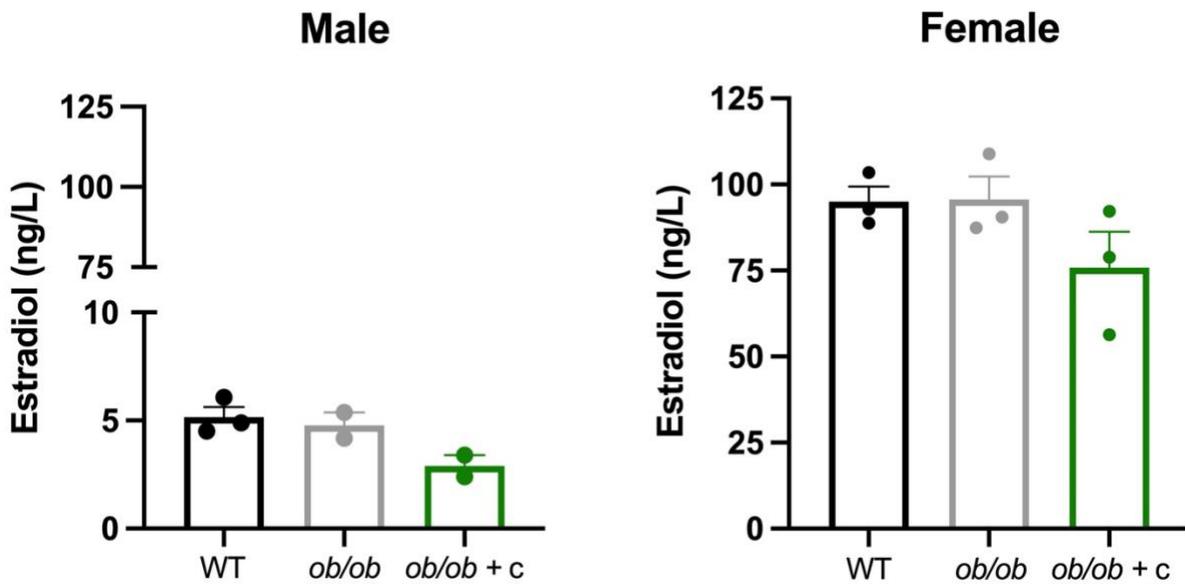
*Supplementary Figure 5. Hepatic triglyceride levels following 10 weeks of treatment. Values are presented as mean  $\pm$  SEM. Data were analyzed by 1-way ANOVA. If a significant treatment effect was detected, Tukey's post hoc test was performed to compare treatment means. Bars not sharing a common superscript letter are significantly different ( $P < 0.05$ ).*



**Supplementary Figure 6.** Serum TNF- $\alpha$  (A-B), hepatic TNF- $\alpha$  (C-D), colonic COX-2 (E-F), and serum ALT (G-H) levels following 10 weeks of treatment. Values are presented as mean  $\pm$  SEM. Data were analyzed by 1-way ANOVA. If a significant treatment effect was detected, Tukey's post hoc test was performed to compare treatment means. Bars not sharing a common superscript letter are significantly different ( $P < 0.05$ ). Values not shown were not detectable or not within the quantifiable range (negative).



*Supplementary Figure 7. Hepatic phosphoenolpyruvate carboxykinase (PEPCK) levels following 10 weeks of treatment. Values are presented as mean  $\pm$  SEM. Data were analyzed by 1-way ANOVA. If a significant treatment effect was detected, Tukey's post hoc test was performed to compare treatment means. Bars not sharing a common superscript letter are significantly different ( $P < 0.05$ ). Values not shown were not detectable (negative).*



*Supplementary Figure 8. Serum estradiol levels in male and female mice following 10 weeks of treatment. Values are presented as mean  $\pm$  SEM. Data were analyzed by 1-way ANOVA. If a significant treatment effect was detected, Tukey's post hoc test was performed to compare treatment means. Bars not sharing a common superscript letter are significantly different ( $P < 0.05$ ). Bars displaying no superscript letter have no significant differences ( $P < 0.05$ ).*

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