SUPPLEMENTAL MATERIAL

Superior metabolic improvement of polycystic ovary syndrome traits after GLP1-based multi-agonist therapy

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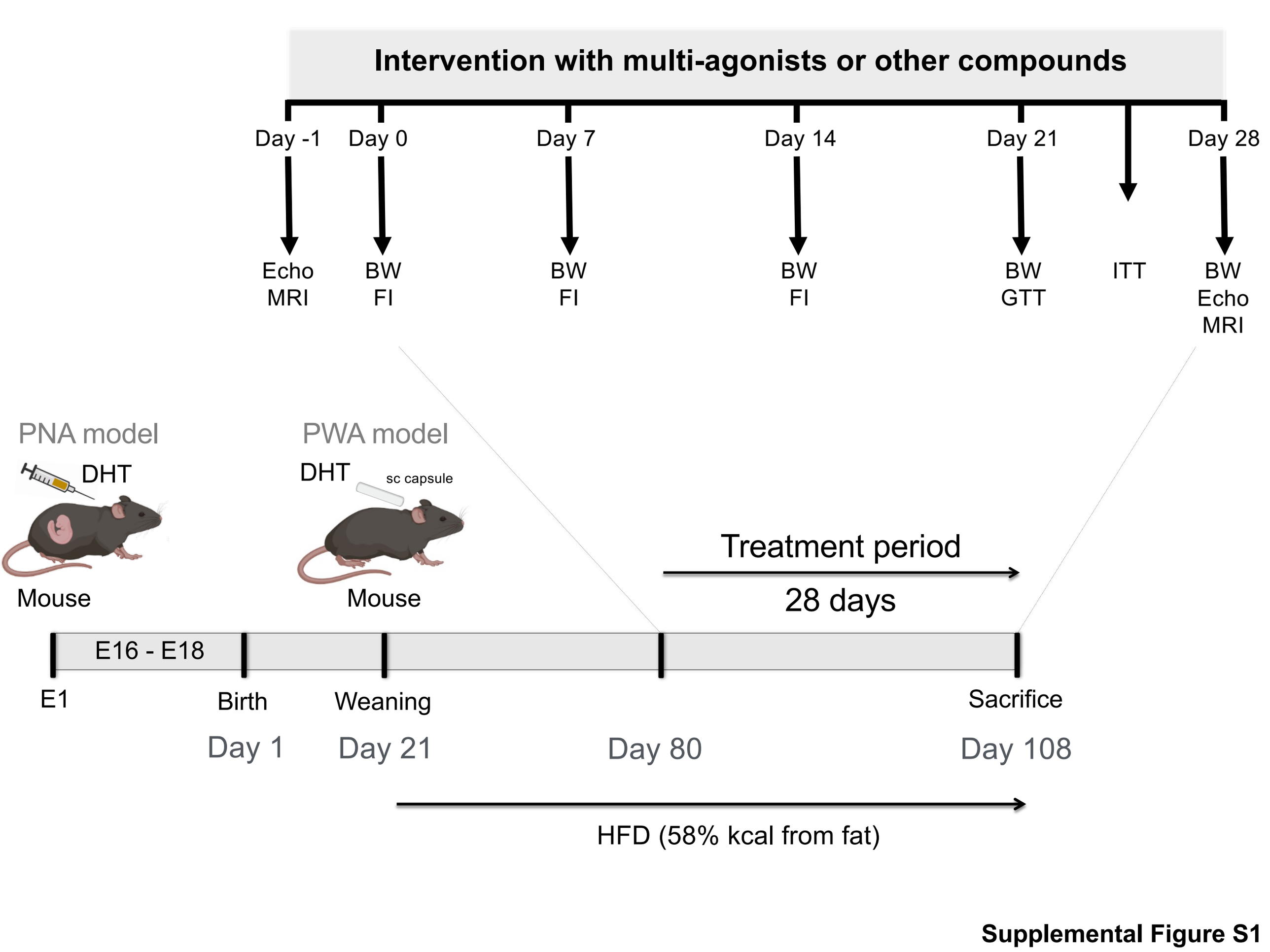
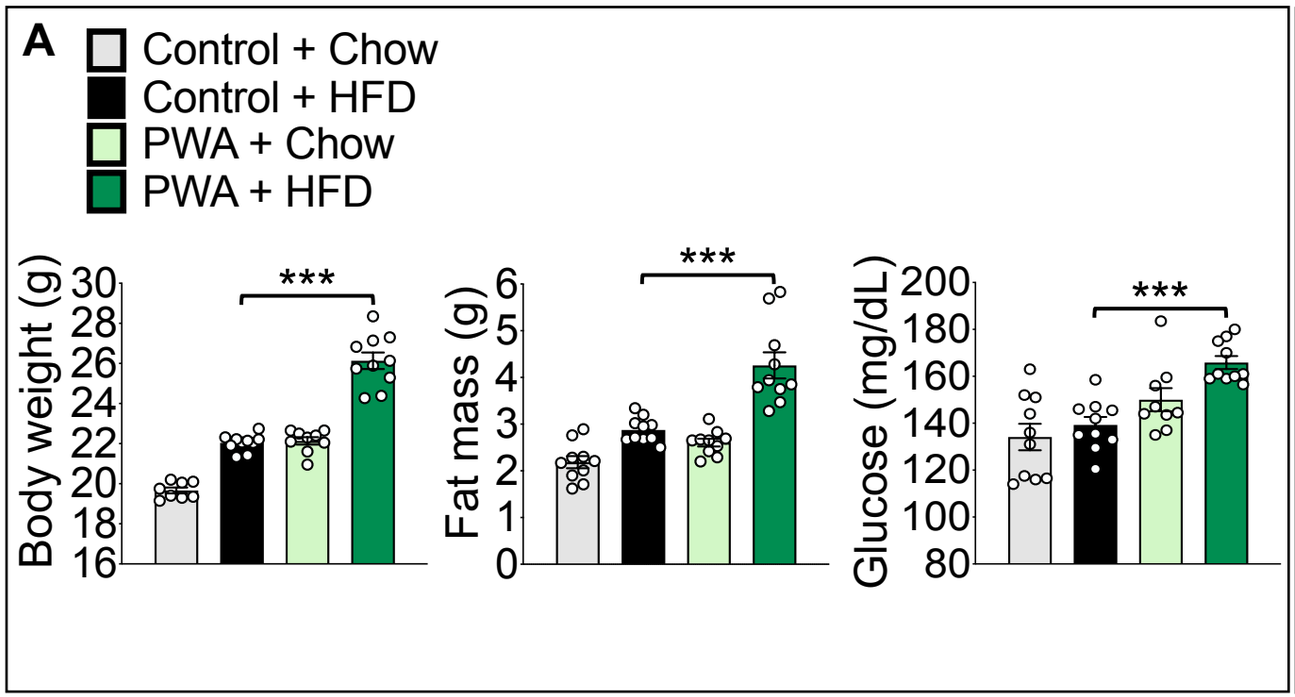
Legend to Supplemental Figures

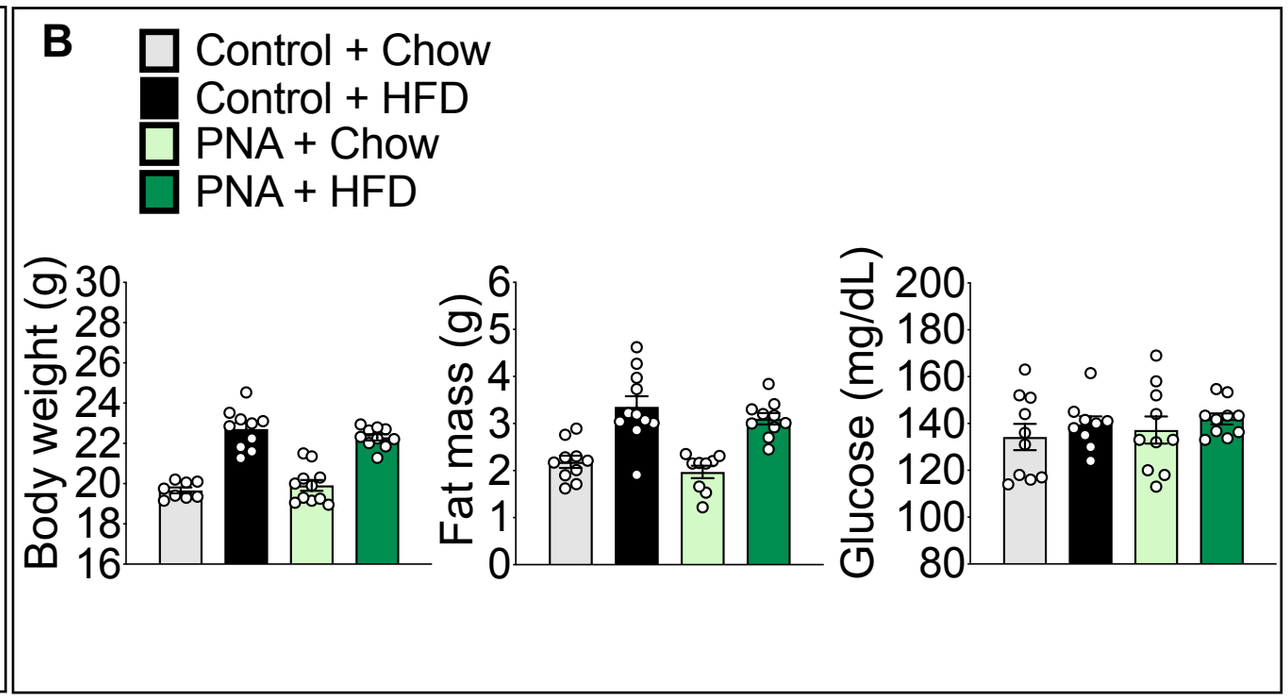
Suppl. Fig. S1. Schematic of the generation of the murine models of PCOS and the experimental design. As illustrated, the prenatal androgenization model (PNA) of PCOS was generated by administration of a single daily dose of dihydrotestosterone (DHT, 250 µg/day) to pregnant dams during gestational days 16-18, whereas the post-weaning androgenization model (PWA) was generated by implantation of s.c. capsules containing DHT (10 mg) after weaning (day 21); allowing chronic exposure to androgens until adulthood. Application of these different androgenization protocols is sufficient to induce PCOS-related traits in adult mice; with PNA mice showing modest metabolic dysregulation and ovarian irregularities, and PWA mice exhibiting chronic hyperandrogenism and salient metabolic and gonadal perturbations. From weaning onwards, mice from all experimental groups were fed a high-fat diet with 58% kcal from fat. Mice were daily injected with the different compounds from day 80 to 108 of life (28 days), and various metabolic parameters were monitored along the treatment period, including body composition (Echo MRI), body weight (BW) and food intake (FI). In addition, functional tests were conducted during the last week of treatment to assess the effects of the multi-functional compounds on glucose tolerance (GTT) and insulin sensitivity (ITT). After treatments, animals were euthanized, and blood and tissue samples were collected, processed and properly stored until analyzed. Graphic elements created in BioRender®. Tena-Sempere, M. (2024) BioRender.com/q16x877

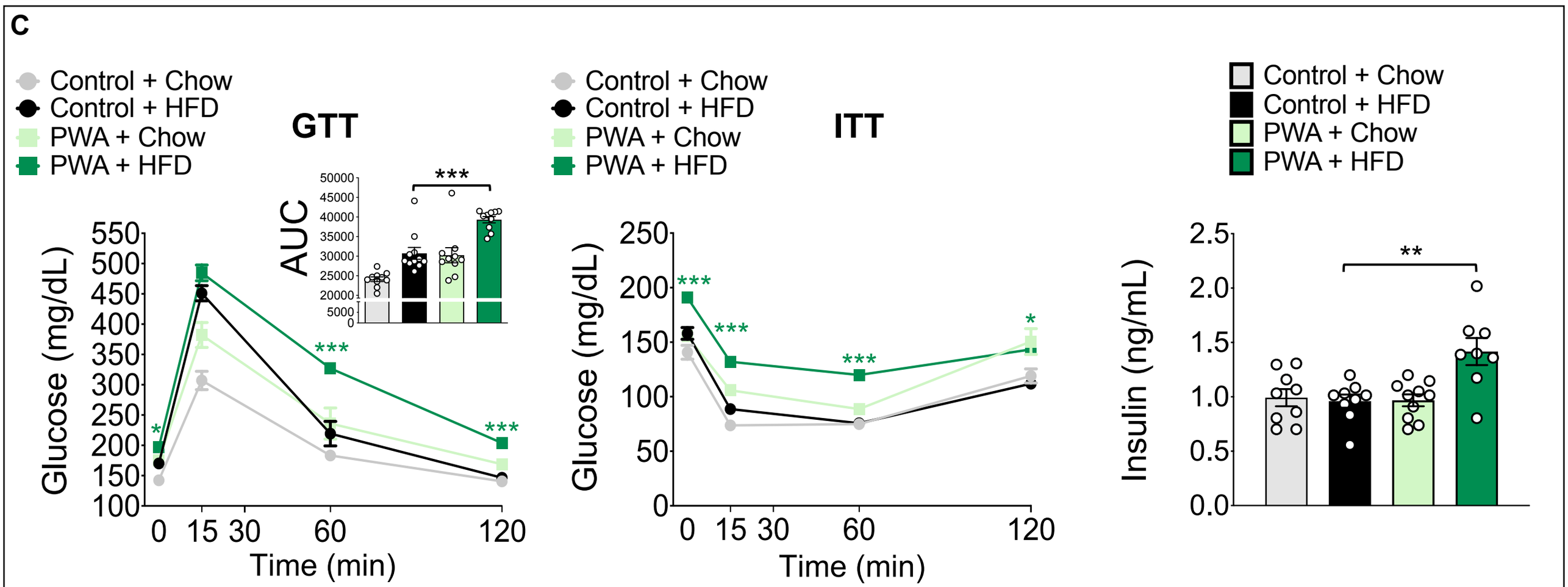


Supplemental Figure S1

Suppl. Fig. S2. Metabolic effects of a HFD in PWA and PNA mice. (**A**-**B**) Body weight, fat mass and circulating glucose levels in PWA (**A**) and PNA mice (**B**) submitted to HFD (90 days) after weaning. (**C**) Detailed characterization of the effects of HFD on glucose tolerance (GTT), insulin sensitivity (ITT) and circulating insulin levels in control and PWA mice. In order to calculate integral glucose levels in the glucose tolerance test, the area under the curve (AUC) was calculated using the trapezoidal rule. Data are presented as mean ± SEM. Group codes: Control+Chow (C+Ch); Control+HFD (C+H); PWA+Chow (P+Ch); PWA+HFD (P+H); PNA+Chow (P'+Ch); PNA+HFD (P'+HFD). For each panel, sample sizes (n) were as follows: Panel **A**: (Body weight) C+Ch=8; C+H=10; P+Ch=9; P+H=10; (Fat mass) C+Ch=10; C+H=10; P+Ch=10; P+H=10; (Glucose) C+Ch=10; C+H=10; P+Ch=9; P+H=10; Panel **B**: (Body weight) C+Ch=8; C+H=10; P'+Ch=11; P'+H=10; (Fat mass) C+Ch=10; C+H=11; P'+Ch=9; P'+H=10; (Glucose) C+Ch=10; C+H=9; P'+Ch=10; P'+H=10; Panel **C**: (GTT) C+Ch=10; C+H=11; P+Ch=10; P+H=10; (ITT) C+Ch=7; C+H=11; P+Ch=10; P+H=9; (Insulin) C+Ch=9, C+H=9; P+Ch=10; P+H=8. Statistically significant differences were assessed by one-way ANOVA followed by Tukey's multiple comparison tests to analyze the effects of HFD in PWA or PNA mice vs. its effects in non-androgenized control mice. Asterisks indicate statistical significance *P < 0.05, **P < 0.01, ***P < 0.001. Source data are provided as a Source Excel Data file (see Data Availability).





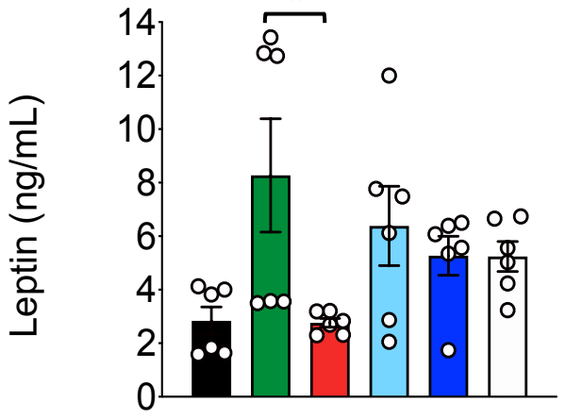


Supplemental Figure S2

Suppl. Fig. S3. Hormonal and reproductive effects of high doses of different GLP1-based multi-agonist in PWA mice. (**A**-**G**) Effects on circulating leptin (**A**), adiponectin (**B**) and FGF21 levels (**C**), ovarian and uterus weight (**D-E**), serum LH concentrations (**F**) and representative images of ovarian histology (**G**) in PWA mice chronically treated with vehicle, GLP1/E (100 nmol/kg), GLP1/GIP (10 nmol/kg), GLP1/GIP/Glucagon triagonist (10 nmol/kg) or metformin (300mg/kg) during 28 days. Data are presented as mean ± SEM. Group codes: Control+Veh (C); PWA+Veh (P); PWA+GLP1/E (G/E); PWA+GLP1/GIP (G/G); PWA+Triagonist (T); PWA+Metformin (M). For each panel, sample sizes (n) were as follows: Panel **A**: C=6; P=6; G/E=6; G/G=6; T=6; M=6; Panel **B**: C=10; P=9; G/E=11; G/G=11; T=12; M=9; Panel **C**: C=7; P=7; G/E=7; G/G=9; T=8; M=6; Panels **D-E**: C=10; P=10; G/E=11; G/G=11; T=12; M=9; **F**: C=10; P=9; G/E=11; G/G=10; T=8; M=5; Panel **G**: C=5; P=5; G/E=5; G/G=5; T=5; M=5. Statistically significant differences were assessed by one-way ANOVA followed by Tukey's multiple comparison tests to analyze the effects of compound intervention vs. vehicle administration in PWA mice. For reference purposes, control non-androgenized mice are included (black bars). Asterisks indicate statistical significance *P < 0.05, **P < 0.01, ***P < 0.001. CL: corpus luteum, F: follicle. Scale bars correspond to 200 µm. Source data are provided as a Source Excel Data file (see Data Availability).

Control + Veh
PWA + Veh
PWA + GLP1/E2 100 nmol/kg
PWA + GLP1/GIP 10 nmol/kg
PWA + Triagonist 10 nmol/kg
PWA + Metformin 300 mg/kg

A



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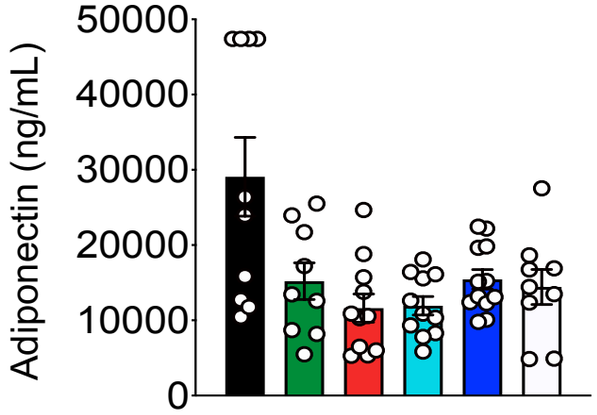

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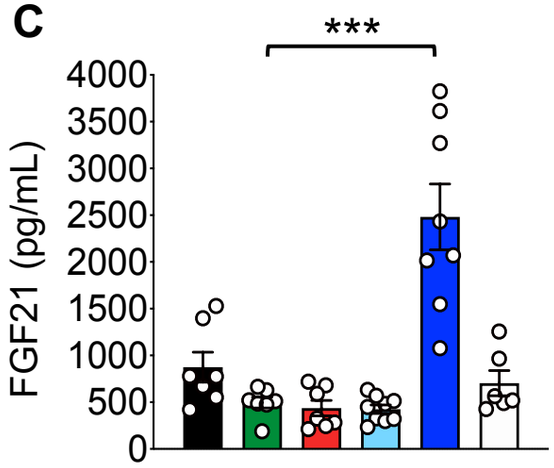

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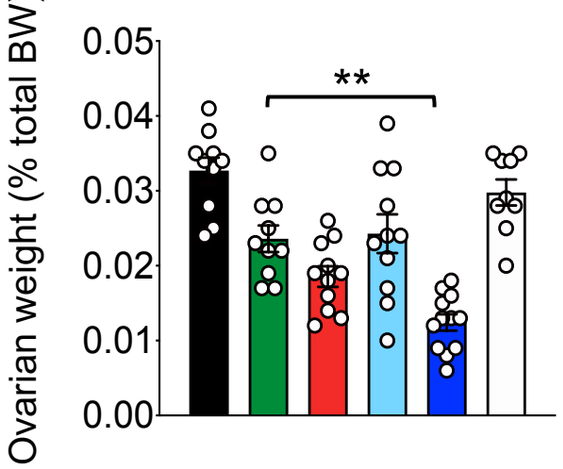

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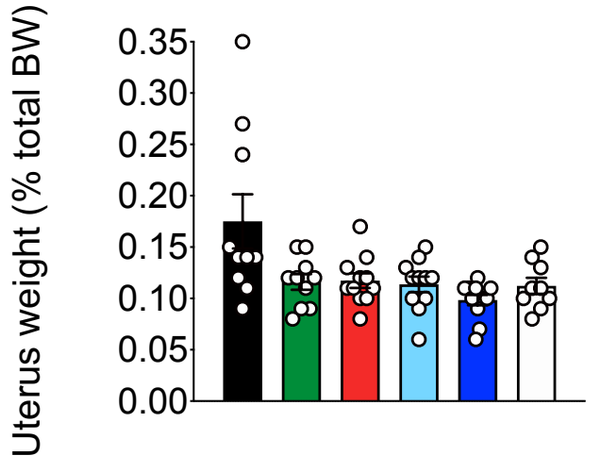

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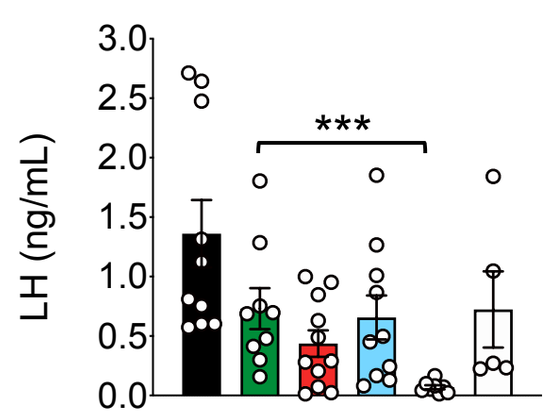

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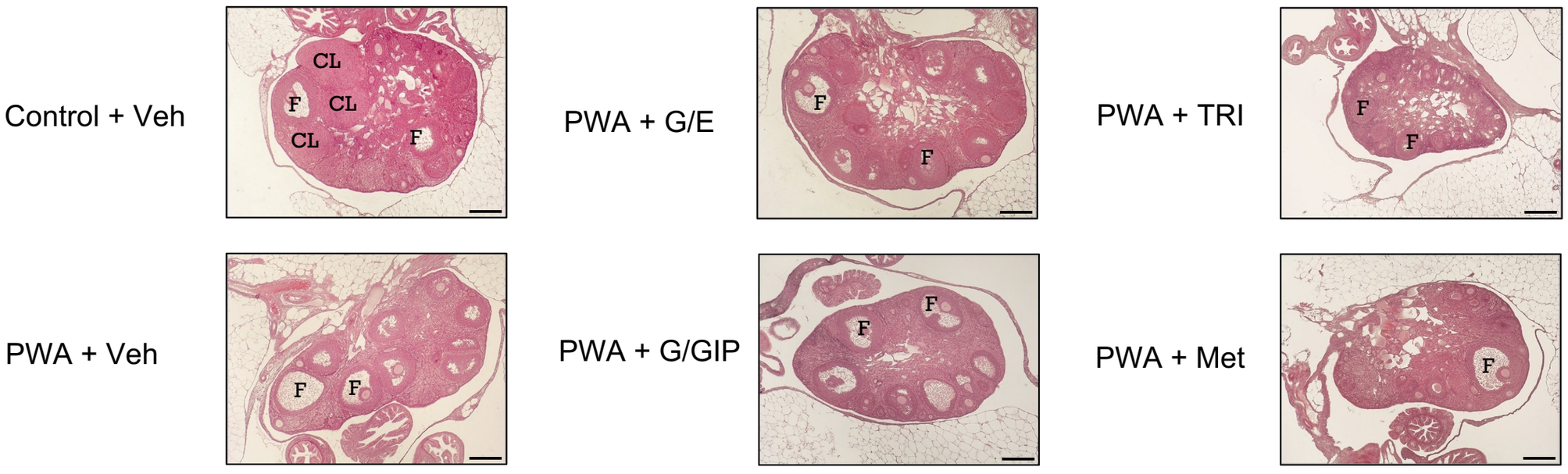
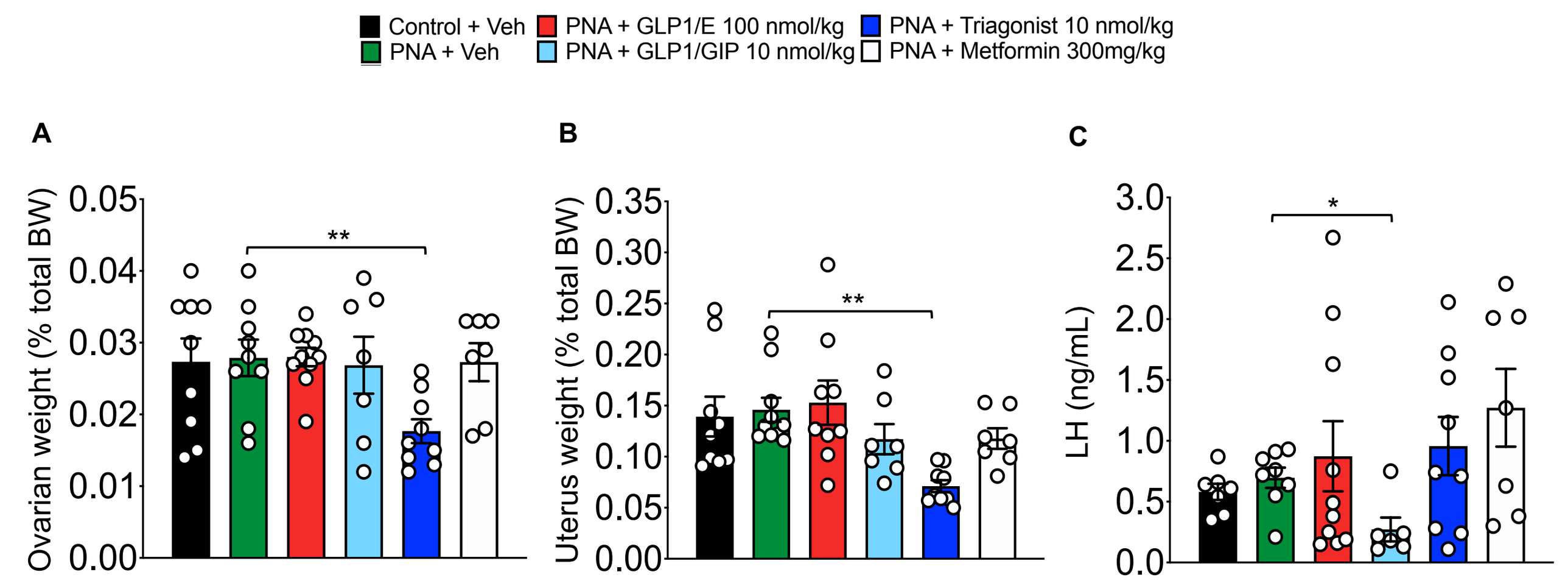


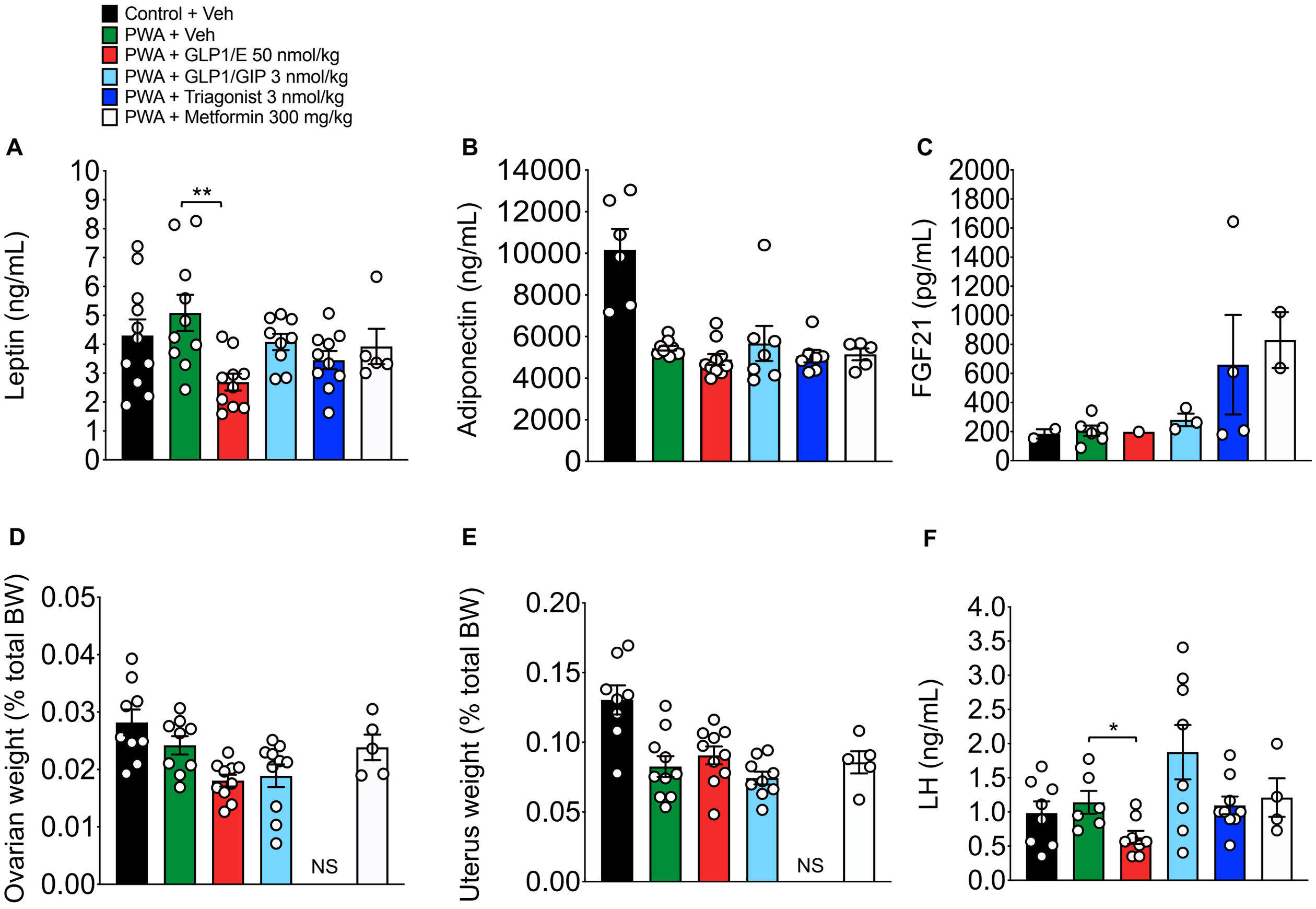
Supplemental Figure S3

Suppl. Fig. S4. Hormonal and reproductive effects of high doses of different GLP1-based multi-agonist in PNA mice. (**A**-**D**) Effects on ovarian weight (**A**), uterus weight (**B**), circulating LH levels (**C**) and representative images of ovarian histology (**D**) in PNA mice chronically treated with vehicle, GLP1/E (100 nmol/kg), GLP1/GIP (10 nmol/kg), GLP1/GIP/ Glucagon triagonist (10 nmol/kg) or metformin (300mg/kg) during 28 days. Data are presented as mean ± SEM. Group codes: Control+Veh (C); PNA+Veh (P'); PNA+GLP1/E (G/E); PNA+GLP1/GIP (G/G); PNA+ Triagonist (T); PNA+Metformin (M). For each panel, sample sizes (n) were as follows: Panel **A**: C=9; P'=9; G/E=10; G/G=7; T=9; M=7; Panel **B**: C=9; P'=9; G/E=9; G/G=7; T=9; M=7; Panel **C**: C=7; P'=8; G/E=10; G/G=6; T=9; M=7; Panel **D**: C=5; P'=5; G/E=5; G/G=5; T=5; M=5. Statistically significant differences were assessed by one-way ANOVA followed by Tukey's multiple comparison tests to analyze the effects of compound intervention vs. vehicle administration in PNA mice. For reference purposes, control non-androgenized mice are included (black bars). Asterisks indicate statistical significance *P < 0.05, **P < 0.01, ***P < 0.001. CL: corpus luteum, F: follicle. Scale bars correspond to 200 µm. Source data are provided as a Source Excel Data file (see Data Availability).



Supplemental Figure S4

Suppl. Fig. S5. Hormonal and reproductive effects of lower doses of different GLP1-based multi-agonist in PWA mice. (**A**-**F**). Effects on serum leptin (**A**), adiponectin (**B**) and FGF21 levels (**C**), ovarian and uterus weight (**D-E**), and serum LH concentrations (**F**) in PWA female mice daily administered with vehicle (saline), GLP1/E (50 nmol/kg), GLP1/GIP (3 nmol/kg), GLP1/GIP/Glucagon triagonist (3 nmol/kg) or metformin (300 mg/kg) during 28 days. Note that ovarian and uterus weight data form triagonist-treated PWA mice could not be recorded due to a technical problem during sampling and are not shown (NS). Data are presented as mean ± SEM. Group codes: Control+Veh (C); PWA+Veh (P); PWA+GLP1/E (G/E); PWA+GLP1/GIP (G/G); PWA+Triagonist (T); PWA+Metformin (M). For each panel, sample sizes (n) were as follows: Panel **A**: C=11; P=10; G/E=10; G/G=9; T=10; M=5; Panel **B**: C=6; P=10; G/E=10; G/G=7; T=7; M=5; Panel **C**: C=2; P=6; G/E=1; G/G=3; T=4; M=2; Panel **D**: C=9; P=9; G/E=10; G/G=10; M=5; Panel **E**: C=8; P=10; G/E=10; G/G=9; M=5; Panel **F**: C=8; P=6; G/E=8; G/G=8; T=9; M=4. Statistically significant differences were assessed by one-way ANOVA followed by Tukey's multiple comparison tests to analyze the effects of compound intervention vs. vehicle administration in PWA mice. For reference purposes, control non-androgenized mice are included (black bars). Asterisks indicate statistical significance *P < 0.05, **P < 0.01, ***P < 0.001. Source data are provided as a Source Excel Data file (see Data Availability).

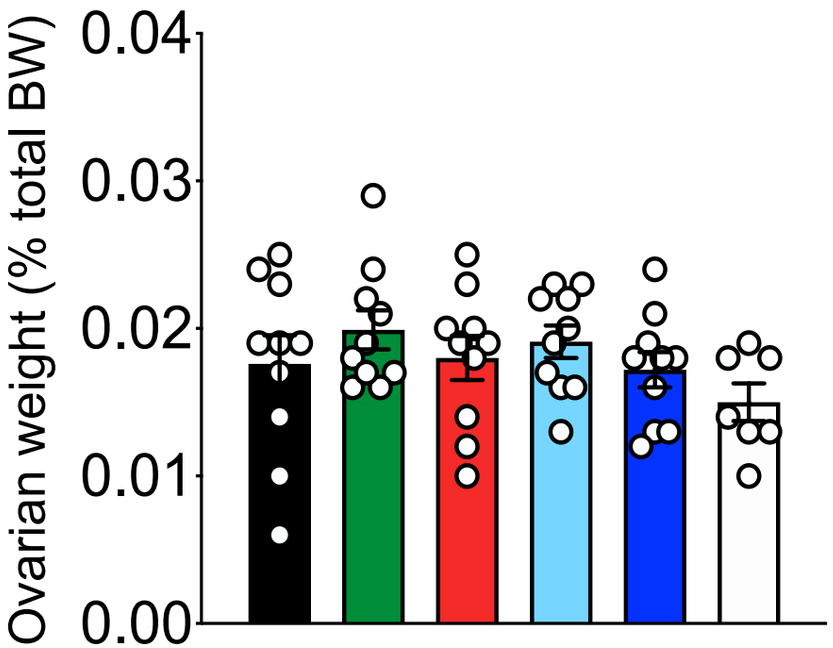


Supplemental Figure S5

Suppl. Fig. S6. Hormonal and reproductive effects of low doses of different GLP1-based multi-agonist in PNA mice. (**A**-**C**) Effects on ovarian weight (**A**), uterus weight (**B**) and circulating LH levels (**C**) in PNA mice chronically treated with vehicle, GLP1/E (50 nmol/kg), GLP1/GIP (3 nmol/kg), GLP1/GIP/ Glucagon triagonist (3 nmol/kg) or metformin (300mg/kg) during 28 days. Data are presented as mean ± SEM. Group codes: Control+Veh (C); PNA+Veh (P'); PNA+GLP1/E (G/E); PNA+GLP1/GIP (G/G); PNA+ Triagonist (T); PNA+Metformin (M). For each panel, sample sizes (n) were as follows: Panel **A**: C=10; P'=10; G/E=10; G/G=10; T=10; M=7; Panel **B**: C=11; P'=10; G/E=10; G/G=11; T=10; M=7; Panel **C**: C=10; P'=10; G/E=9; G/G=10; T=8; M=8. Statistically significant differences were assessed by one-way ANOVA followed by Tukey's multiple comparison tests to analyze the effects of compound intervention vs. vehicle administration in PNA mice. For reference purposes, control non-androgenized mice are included (black bars). Asterisks indicate statistical significance *P < 0.05, **P < 0.01, ***P < 0.001. Source data are provided as a Source Excel Data file (see Data Availability).

Control + Veh
PNA + Veh
PNA + GLP1/E 50 nmol/kg
PNA + GLP1/GIP 3 nmol/kg
PNA + Triagonist 3 nmol/kg
PNA + Metformin 300 mg/kg

A



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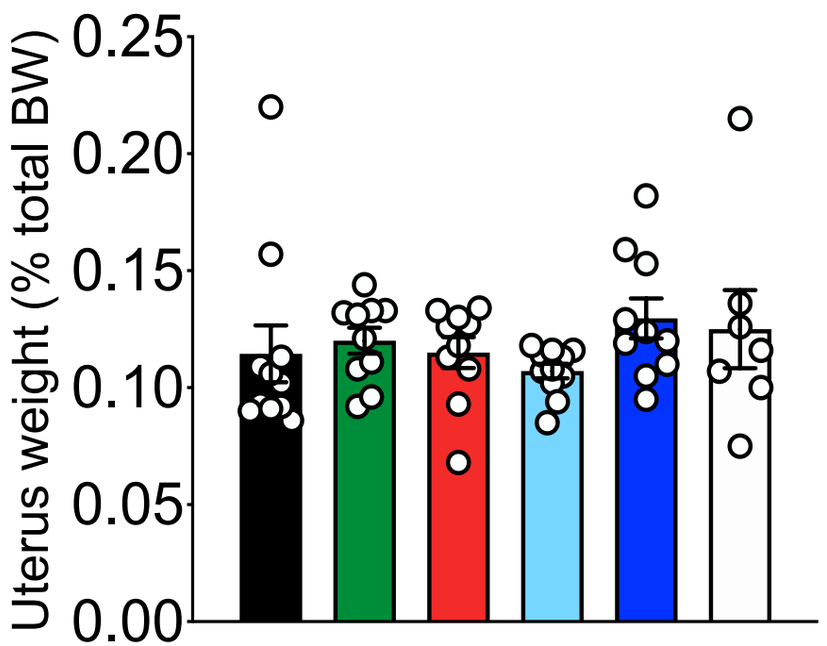
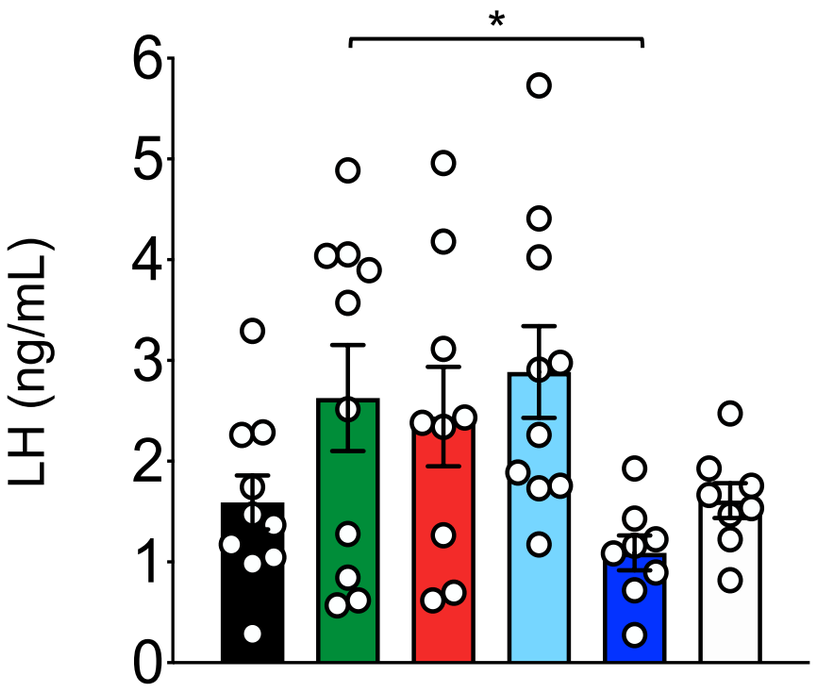
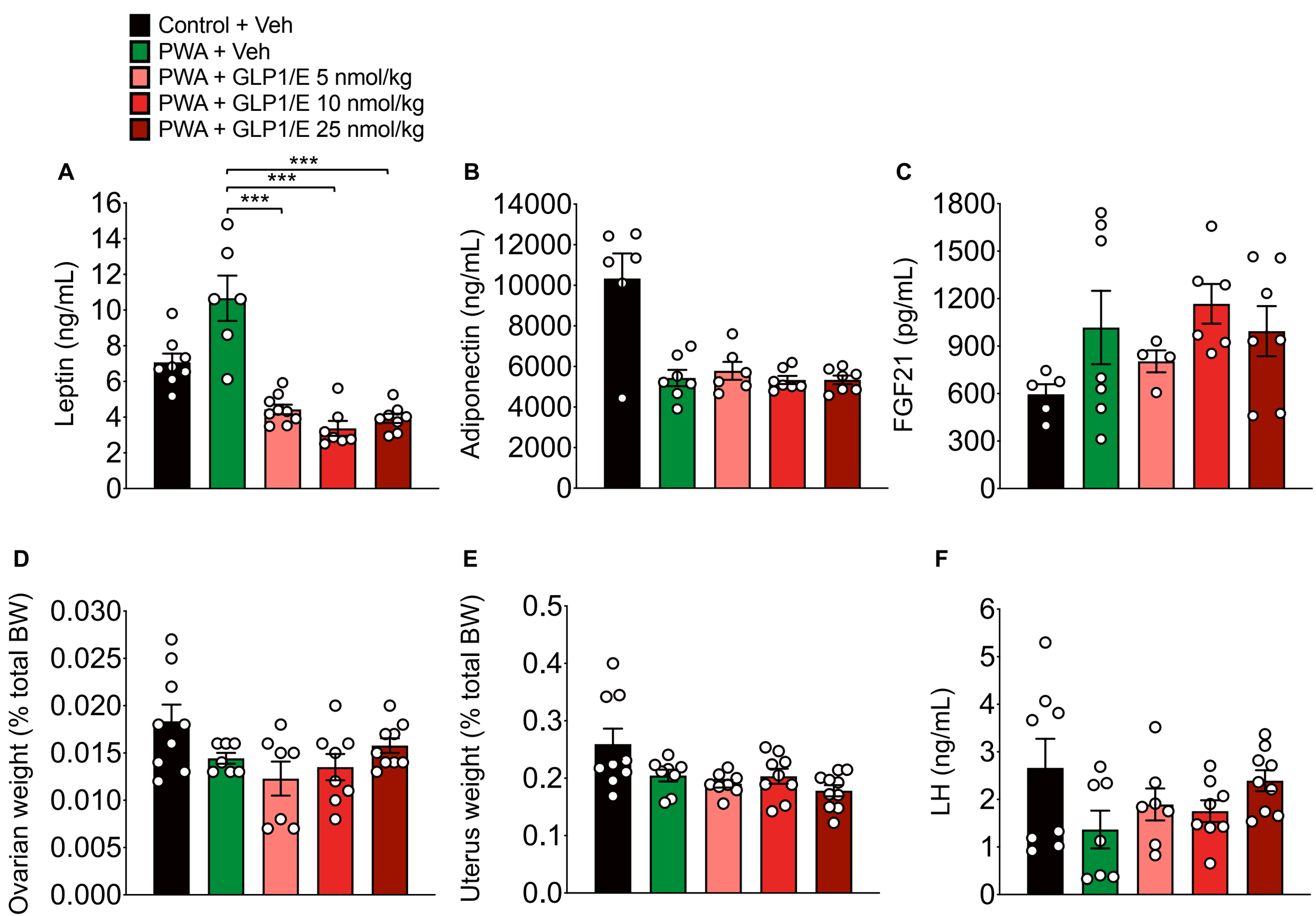


C



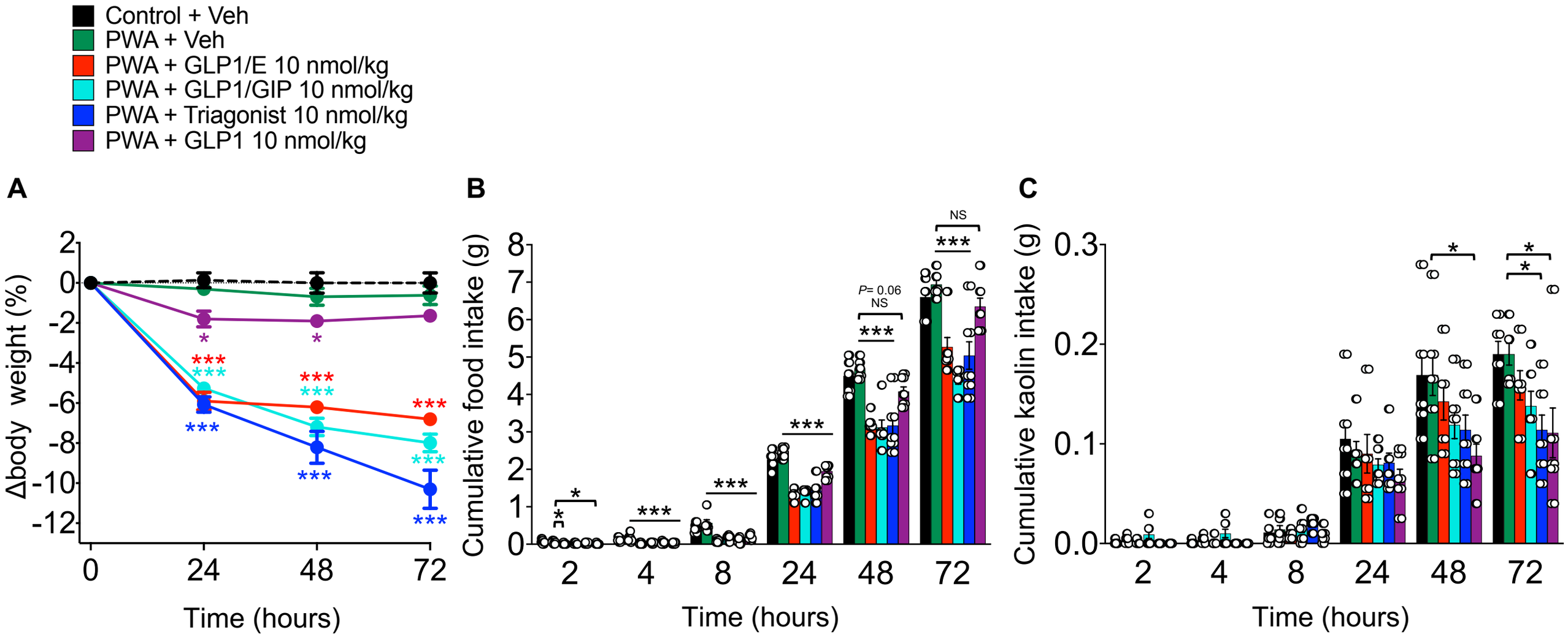
Supplemental Figure S6

Suppl. Fig. S7. Hormonal and reproductive effects of very low doses of GLP1/E in PWA mice. (**A-F**) Effects on circulating leptin (**A**), adiponectin (**B**) and FGF21 levels (**C**), ovarian and uterus weight (**D-E**), and serum LH concentrations (**F**) in PWA mice daily administered with vehicle (saline) or GLP1/E (5, 10 and 25 nmol/kg) during 28 days. Data are presented as mean ± SEM. Group codes: Control+Veh (C); PWA+Veh (P); PWA+GLP1/E 5 nmol/kg (G/E5); PWA+ GLP1/E 10 nmol/kg (G/E10); PWA+ GLP1/E 25 nmol/kg (G/E25). For each panel, sample sizes (n) were as follows: Panel **A**: C=8; P=6; G/E5=9; G/E10=7; G/E25=8; Panel **B**: C=6; P=7; G/E5=6; G/E10=7; G/E25=7; Panel **C**: C=5; P=7; G/E5=4; G/E10=6; G/E25=7; Panel **D**: C=9; P=7; G/E5=7; G/E10=8; G/E25=9; Panel **E**: C=9; P=8; G/E5=8; G/E10=9; G/E25=10; Panel **F**: C=8; P=7; G/E5=7; G/E10=8; G/E25=9. Statistically significant differences were assessed by one-way ANOVA followed by Tukey's multiple comparison tests to analyze the effects of compound intervention vs. vehicle administration in PWA mice. For reference purposes, control non-androgenized mice are included (black bars). Asterisks indicate statistical significance *P < 0.05, **P < 0.01, ***P < 0.001. Source data are provided as a Source Excel Data file (see Data Availability).



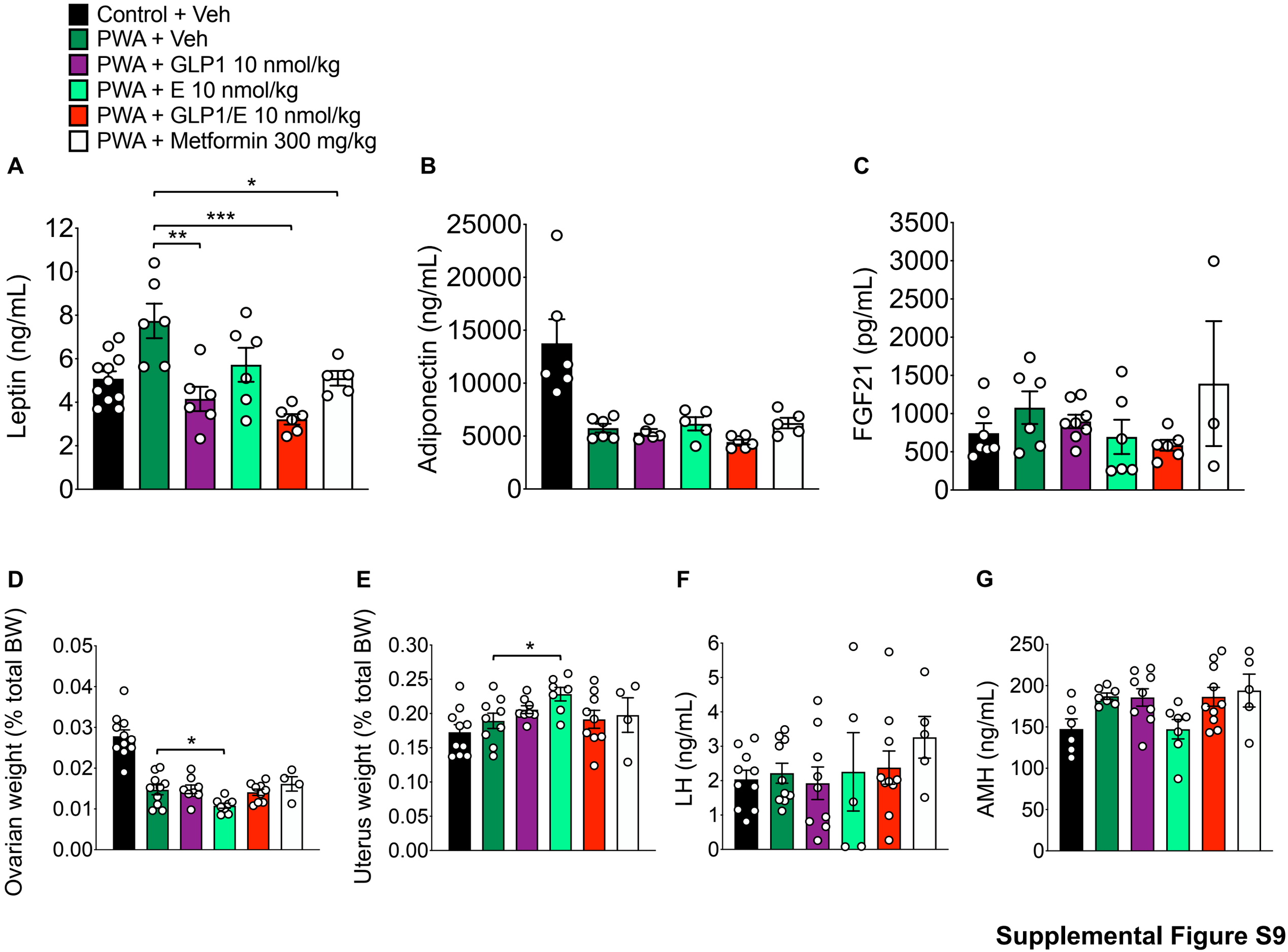
Supplemental Figure S7

Suppl. Fig. S8. Gastrointestinal effects of equivalent doses of GLP1-based multi-agonists or GLP1 in PWA mice. (**A**-**C**) Effects on body weight change (**A**), food intake (**B**) and kaolin intake (**C)** in PWA female mice daily treated with vehicle, or 10 nmol/kg doses of GLP1/E, GLP1/GIP, triagonist or GLP1 during 3 days. Data are presented as mean ± SEM. Group codes: Control+Veh (C); PWA+Veh (P); PWA+GLP1/E (G/E); PWA+GLP1/GIP (G/G); PWA+Triagonist (T); PWA+GLP1 (G). For each panel, sample sizes (n) were as follows: Panels **A-B**: C=10; P=10; G/E=10; G/G=10; T=10; G=10; Panel **C**: C=10; P=10; G/E=8; G/G=10; T=10; G=10. Statistically significant differences were assessed by one-way ANOVA followed by Tukey's multiple comparison tests to analyze the effects of compound intervention vs. vehicle administration in PWA mice. For reference purposes, control non-androgenized mice are included (black bars and dashed lines). Asterisks indicate statistical significance *P < 0.05, **P < 0.01, ***P < 0.001. Source data are provided as a Source Excel Data file (see Data Availability).



Supplemental Figure S8

Suppl. Fig. S9. Hormonal and reproductive effects of equivalent doses of GLP1, estrogen or GLP1/E in PWA mice. (**A-G**) Effects on circulating leptin (**A**), adiponectin (**B**) and FGF21 levels (**C**), ovarian and uterus weight (**D-E**), and serum LH (**F**) and anti-Mullerian hormone (AMH) concentrations (**G**) in PWA female mice daily administered with vehicle (saline), GLP1 (10 nmol/kg), estrogen (10 nmol/kg), GLP1/E (10 nmol/kg), or metformin (300 mg/kg) during 28 days. Data are presented as mean ± SEM. Group codes: Control+Veh (C); PWA+Veh (P); PWA+GLP1 (G); PWA+E (E); PWA+GLP1/E (G/E); PWA+Metformin (M). For each panel, sample sizes (n) were as follows: Panel **A**: C=11; P=6; G=6; E=6; G/E=6; M=5; Panel **B**: C=6; P=6; G=5; E=5; G/E=6; M=5; Panel **C**: C=7; P=6; G=8; E=6; G/E=6; M=3; Panel **D**: C=11; P=10; G=8; E=8; G/E=9; M=4; Panel **E**: C=10; P=9; G=8; E=7; G/E=9; M=4; Panel **F**: C=10; P=10; G=9; E=5; G/E=10; M=5; Panel **G**: C=6; P=7; G=9; E=7; G/E=10; M=5, Statistically significant differences were assessed by one-way ANOVA followed by Tukey's multiple comparison tests to analyze the effects of compound intervention vs. vehicle administration in PWA mice. For reference purposes, control non-androgenized mice are included (black bars). Asterisks indicate statistical significance *P < 0.05, **P < 0.01, ***P < 0.001. Source data are provided as a Source Excel Data file (see Data Availability).



Supplemental Figure S9

Supplemental Tables

Suppl. Table S1. Sex steroids levels in PWA and PNA mice after treatment with equivalent doses of GLP1, estrogen or GLP1/E during 28 days.

Data are presented as mean ± SEM. Group codes for **PWA**: Control+Veh (C); PWA+Veh (P); PWA+GLP1 (G); PWA+E (E); PWA+GLP1/E (G/E); PWA+Metformin (M). Sample sizes (n) for PWA groups were as follows: (**DHT**) C=14; P=8; G=7; E=7; G/E=9, M=4, (**A4**) C=16; P=10; G=9; E=7; G/E=9, M=3, (**P4**) C=12; P=10; G=9; E=8; G/E=9, M=4, (**E**) C=15; P=10; G=8, E=7; G/E=10, M=4. Group codes for **PNA**: Control+Chow (C+Ch); PNA+Veh (P'); PNA+GLP1 (P'+G); PNA+E (P'+E); PNA+GLP1/E (P'+G/E); PNA+Metformin (P'+M). Sample sizes (n) for PNA groups were as follows: (**DHT**) C+Ch=14; P'=10; P'+G=9; P'+E=10; P'+G/E=12; P'+M=7; (**A4**) C+Ch=16; P'=8; P'+G=9; P'+E=9; P'+G/E=11; P'+M=5, (**P4**) C+Ch=12; P'=9; P'+G=9; P'+E=9; P'+G/E=10; P'+M=6; (**E**) C+Ch=15; P'=8; P'+G=10; P'+E=9; P'+G/E=11; P'+M=6. Statistically significant differences were assessed by one-way ANOVA followed by Tukey's multiple comparison tests.

* Statistical differences vs vehicle-treated PWA or PNA mice ($P < 0.05$).

[a] Samples below the limit of quantification (LOQ) were assigned the minimal LOQ value for each specific analyte (DHT: 5 pg/mL).

DHT: Dihydrotestosterone; A4: Androstenedione; P4: Progesterone; E: Estradiol.

Source data are provided as a Source Excel Data file (see Data Availability).

Post-weaning Androgenization (PWA)

	Control + Chow	PWA + Veh	GLP-1	E	GLP1/E	Metformin
DHT (pg/ml)	17.05 ± 1.35	906.88 ± 67.06	758 ± 74.12	1137.1 ± 112.5	580.78 ± 38.71	858.50 ± 238.07
A4 (pg/ml)	7.98 ± 0.43	6.34 ± 0.97	11.86 ± 2.58	8.16 ± 2.69	5.14 ± 0.14	5.37 ± 0.37
P4 (ng/ml)	0.81 ± 0.18	0.58 ± 0.13	0.39 ± 0.14	0.39 ± 0.15	0.45 ± .12	0.99 ± 0.24
E2 (pg/ml)	0.99 ± 0.09	1.69 ± 0.37	2.68 ± 0.57	2.13 ± 0.50	1.41 ± 0.43	1.80 ± 0.35

Prenatal Androgenization (PNA)

	Control + Chow	PNA + Veh	GLP-1	E	GLP1/E	Metformin
DHT (pg/ml)	5 ± 0.00[a]	5 ± 0.00[a]	5 ± 0.00[a]	5 ± 0.00[a]	5 ± 0.00[a]	5 ± 0.00[a]
A4 (pg/ml)	7.98 ± 0.43	19.35 ± 2.61	9.66 ± 1.73[*]	6.00 ± 0.43[*]	12.42 ± 1.96	9.44 ± 1.95[*]
P4 (ng/ml)	0.81 ± 0.18	19.00 ± 7.50	1.65 ± 0.29[*]	0.81 ± 0.19[*]	1.65 ± 0.26[*]	1.04 ± 0.29[*]
E2 (pg/ml)	0.99 ± 0.09	1.46 ± 0.30	2.35 ± 0.45	1.69 ± 0.38	2.61 ± 0.42	1.34 ± 0.38

Suppl. Table S2. Major effects of GLP1 multi-agonists in PCOS models and comparison with (non-androgenized) female obesity. A summary of the main effects of the GLP1 multi-agonists, GLP1/E, GLP1/GIP and GLP1/GIP/Glucagon, on different metabolic and reproductive traits in the models of post-weaning (PWA) and prenatal (PNA) androgenization, is presented. Results are compared with the profiles of responses to GLP1 multi-agonists in diet-induced obese, female mice, as fragmentarily reported in previous literature (see references). Altogether, our data document a superior efficacy of GLP1/E in managing metabolic and reproductive perturbations in androgenic PCOS models, which is specific and not shared with conventional models of (non-androgenic) obesity.

	Post-weaning androgenization (current work)			Prenatal androgenization (current work)			Non Androgenized + HFD (Ref: 1,2,3)		
	GLP1/E	GLP1/GIP	Triagonist	GLP1/E	GLP1/GIP	Triagonist	GLP1/E	GLP1/GIP	Triagonist
Body weight	↓	-	↓	↓	-	-	↓	↓	↓
Fat mass	↓	-	-	↓	-	-	↓	↓	↓
Cumm. Food intake	↓	-	-	↓	-	-	↓	↓	-
Glucose tolerance	↑	↑	↑	-	-	-	NR	↑	-
Insulin sensitivity	↑	↑	↑	↑	↑	↑	NR	↑	-
Insulin	↓	-	-	-	-	-	NR	-	-
Fasting glucose	↓	↓	-	↓	↓	-	-	↓	-
Uterus weight	-	-	-	-	-	-	-	NR	NR
Ovarian cyclicity	-	-	-	↑	-	-	NR	NR	NR

NR: not reported

- : not statistically significant

References

1. Finan B, et al. Targeted estrogen delivery reverses the metabolic syndrome. Nat Med. 2012; 18:1847-56.
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3. Sachs S, et al. Plasma proteome profiles treatment efficacy of incretin dual agonism in diet-induced obese female and male mice. Diabetes Obes Metab. 2021;23:195-207