

Intergenerational response to the endocrine disruptor vinclozolin is influenced by maternal genotype and crossing scheme

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ABSTRACT

In utero exposure to vinclozolin (VIN), an antiandrogenic fungicide, is linked to multigenerational phenotypic and epigenetic effects. Mechanisms remain unclear. We assessed the role of antiandrogenic activity and DNA sequence context by comparing effects of VIN vs. M2 (metabolite with greater antiandrogenic activity) and wild-type C57BL/6 (B6) mice vs. mice carrying mutations at the previously reported VIN-responsive *H19/Igf2* locus. First generation offspring from VIN-treated 8nrCG mutant dams exhibited increased body weight and decreased sperm *ICR* methylation. Second generation pups sired by affected males exhibited decreased neonatal body weight but only when dam was unexposed. Offspring from M2 treatments, B6 dams, 8nrCG sires or additional mutant lines were not similarly affected. Therefore, pup response to VIN over two generations detected here was an 8nrCG-specific maternal effect, independent of antiandrogenic activity. These findings demonstrate that maternal effects and crossing scheme play a major role in multigenerational response to *in utero* exposures.

1. Introduction

Endocrine-disrupting compounds (EDCs) are environmental compounds that interfere with the homeostasis of the endocrine system. EDC exposure is of growing concern because it is linked to increased prevalence of human diseases such as cancer, diabetes, obesity, asthma, neurodegenerative disorders, and reproductive disorders [1]. Furthermore, experimental models show that expo-

sure to EDCs *in utero* can negatively influence offspring health outcomes over multiple generations [2]. Vinclozolin (VIN) is a dicarboximide fungicide that is still used commercially but in limited application in the United States (US) due to adverse effects on male reproduction detected in experimental models [3–5]. In rodents, VIN exposure *in utero* disrupts male reproduction causing reduced anogenital distance (AGD) and sperm count, cleft phallus, hypospadias, ectopic testes, vaginal pouches, and epididymal granulomas [6–8].

The mechanism by which VIN perturbs male reproduction is not fully understood. However, VIN and two of its metabolites (M1 & M2) have been shown to compete with dihydrotestosterone (DHT) for binding the androgen receptor (AR) and therefore may act in part by disrupting genomic activity of AR [9,10]. Compared to VIN and M1, M2 seemingly has greater potential to interfere with AR activity. M2 was previously reported to have a greater ability to compete with androgen for binding to AR (DHT ($K_i = 100$ nM) > hydroxyflutamide ($K_i = 175$ nM) > testosterone ($K_i = 220$ nM) > M2 ($K_i = 9.65$ μ M) > M1 ($K_i = 92$ μ M) > VIN ($K_i > 700$ μ M)) [11,12]. M2 was also shown to

Abbreviations: VIN, Vinclozolin, or 3-(3,5-dichlorophenyl)-5-methyl-5-vinylloxazolidine-2,4-dione; EDC, Endocrine disrupting compound; M1, 2-[[[(3,5-dichlorophenyl)-carbamoyl]oxy]-2-methyl-3-butenic acid; M2, 3',5'-dichloro-2-hydroxy-2-methylbut-3-enamide; AR, Androgen Receptor; ER, Estrogen Receptor; PR, Progesterone Receptor; GD, Gestational Day; PND, Postnatal day; AGD, Anogenital distance; DHT, Dihydrotestosterone; SRY, Sex determining region of the Y chromosome gene; ICR, Imprinting Control Region; BWS, Beckwith-Wiedemann syndrome; DMR, Differentially methylated region; G₀, Generation zero, parental generation; G₁, First generation; G₂, Second generation.

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be a more potent antagonist of AR transcriptional activity, acting at a dose that was more similar to hydroxyflutamide (a similarly structured known antiandrogenic compound) [9]. VIN and its metabolites have also been reported to interact with other steroid receptors. Here also, M2 was shown to be a more potent antagonist of transcriptional activity of progesterone receptor (PR) and mineralocorticoid receptor (MR) and a more potent agonist of AR and estrogen receptors (ER) α and β [13–15].

VIN exposure *in utero* was the first reported model of transgenerational epigenetic inheritance. Male rats exposed to VIN *in utero* exhibited adverse reproductive outcomes and altered DNA methylation in sperm over 3 generations [8,16]. A separate study also reported transgenerational DNA methylation changes at imprinted loci including the *H19/Igf2* imprinting control region (ICR) in mature sperm [17]. Genetic differences have been shown to influence heritable responses to VIN in reports showing that transgenerational germline epimutations could be detected in outbred CD-1 mice but not inbred 129 mice [18]. Exact sequences responsible were not investigated. The mechanism of persistence of VIN-induced phenotypic and epigenetic changes are unclear. However, recent studies show that VIN-induced methylation changes do not persist through stages of reprogramming during male germ cell development [19] suggesting an alternate mechanism of transmission.

Here, we used the VIN mouse exposure model to investigate factors that may contribute to differences in sensitivity to endocrine disruptors. We used a two-stage study design to examine the role of M2 metabolite availability (compared to VIN) and DNA sequence context (wildtype vs. mutations at the *H19/Igf2* ICR) in determining the extent and heritability of epigenetic and phenotypic outcomes in response to VIN. The mutant lines used, 8nrCG, $\Delta 2,3$ and ΔIVS , carry targeted mutations at the *H19/Igf2* ICR that mimic genetic mutations found at the epigenetically perturbed human locus in patients with the overgrowth disorder Beckwith-Wiedemann syndrome (BWS) [20–26]. Aberrant *H19/Igf2* expression is also associated with metabolic disorders such as diabetes and obesity, Wilms tumor of the kidney, adrenocortical tumors, and various other forms of cancer [27–31]. The cause of *H19/Igf2* epimutations remains unknown, however, studies suggest the genetic mutations may act by disrupting epigenetic stability at the locus [22–26]. While these mutations alone in the mouse do not perturb epigenetic status [20,21], this study will investigate whether the presence of these mutations increase sensitivity to VIN-induced epimutation.

2. Materials and methods

2.1. Animals – genetic line descriptions, sources, and housing

Animal handling was performed in accordance with the *Guide for the Care and Use of Laboratory Animals* under the corresponding animal use protocol at the University of North Carolina at Chapel Hill and housed at the David H. Murdock Institute Vivarium in Kannapolis, NC. Five mouse lines were used in the study including: (1) wild-type fully inbred C57BL/6 (B6) mice; (2) wild-type C7 mice homozygous for CAST/Eij across chromosome 7 and mixed CAST/Eij-B6 genetic background [32]; (3) mutant 8nrCG mice [20] carrying targeted mutation of 8 base pairs at 8 CpGs at the *H19/Igf2* locus; (4) mutant ΔIVS mice [20] carrying a targeted deletion of 0.9 kb at the *H19/Igf2* imprinting control region; and (5) mutant $\Delta 2,3$ mice [21] carrying a targeted deletion of 1.3 kb at the *H19/Igf2* imprinting control region. All mutant lines were previously backcrossed 6–10 generations into B6 to result in mostly (>98%) B6 genetic background as confirmed by genotyping. B6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and used directly (study 1) or purchased separately

and bred in-house to generate animals for study 2. B6-Cast7 (C7), 8nrCG, $\Delta 2,3$, and ΔIVS were derived in Dr. Marisa Bartolomei's lab at the University of Pennsylvania [32,20,21], then transferred and bred in-house at the David H. Murdock Institute Vivarium in Kannapolis to generate animals for study 1 and study 2. Sterilized water and rodent chow were fed *ad libitum*. All mice were euthanized in accordance with current recommendations by the American Veterinary Medical Association (AVMA) guidelines. All tissues were collected and flash frozen in liquid nitrogen following euthanasia.

2.2. Breeding and treatments (see Fig. 1 for specific cross descriptions and timelines)

Supplemental Table 1 lists the number of pups, males, females, litters (dams), and average number of males per litter assessed in each experimental group. For generating G_1 animals, G_0 sires and dams (virgin females) were group housed after weaning/purchasing and set up to breed at approximately 8–10 weeks of age. Use of G_0 C7 males allowed for consistency in paternal contribution between lineages and also allowed for allelic expression or methylation analyses should the data have shown significant changes in the latter. G_0 dams were bred to stud males (8–24 weeks of age) and checked for vaginal plugs every morning. The first morning of a successful plug was considered GD1 and plugged females were immediately separated from stud males and placed into treatment cages. Group housed females were separated before parturition to enable identification of each litter separately. G_1 pups were counted on the morning of birth to measure litter size, sexed at PND5 to measure the number of males and females, weaned at PND28, and euthanized at PND84. For deriving the second generation of animals (G_2), 10 wk old G_1 males and females treated *in utero* were intercrossed (avoiding brother sister mating) or simultaneously outcrossed to untreated inbred B6 females. G_2 pups were counted on the morning of birth, sexed at PND5, and euthanized at PND5.

All G_0 dams were placed on a low-phytoestrogen purified diet (Teklad, TD.95092) one-week prior to mating and throughout breeding and gestation until PND5 to standardize nutrient intake and minimize potential xenoestrogenic properties of soy based diet [33]. Pregnant females were group-housed based on treatment group and plug date and treated daily with either VIN (VIN) (analytical grade >99% purity, Chem Service Inc, PA), M2 (analytical grade >99% purity, Cayman Chemical, MI), or pure corn oil (CON) (Sigma) by intraperitoneal injection. VIN and M2 were dissolved in corn oil at room temperature, prepared fresh weekly, and injected at a dose of 50 milligrams/kilogram of body weight/day (mg/kg/d) at a volume of 5 μ l per gram of body weight. To reduce potential of lineage effects confounding the treatment effects, we avoided placing sibling females in the same treatment group and stud males were used across treatment groups. Mice were treated daily from GD9.5 to GD18.5. At PND5 for G_1 pups, dams and pups and were transferred to standard rodent chow (Teklad 8604) where they remained throughout subsequent breeding until the end of the study.

We performed two separate rounds of study. For both rounds of study, all methods of treatment and breeding schemes are maintained except where described (Fig. 1). Study 1 compared the effects of VIN and M2 to CON between two genetic lineages (crossed dam \times sire): B6 \times C7 and 8nrCG \times C7. G_0 dams for these crosses were either homozygous wild-type (B6) or homozygous mutant 8nrCG mice. Study 2 compared the effects of VIN to CON among 5 genetic crosses (dam \times sire): B6 \times C7, 8nrCG \times C7, C7 \times 8nrCG, ΔIVS \times C7, and $\Delta 2,3$ \times C7. G_0 dams for these crosses were either homozygous wild-type (B6 and C7) or heterozygous mutant 8nrCG, $\Delta 2,3$, ΔIVS mice. See Supplemental Table 1 for litter and pup numbers.

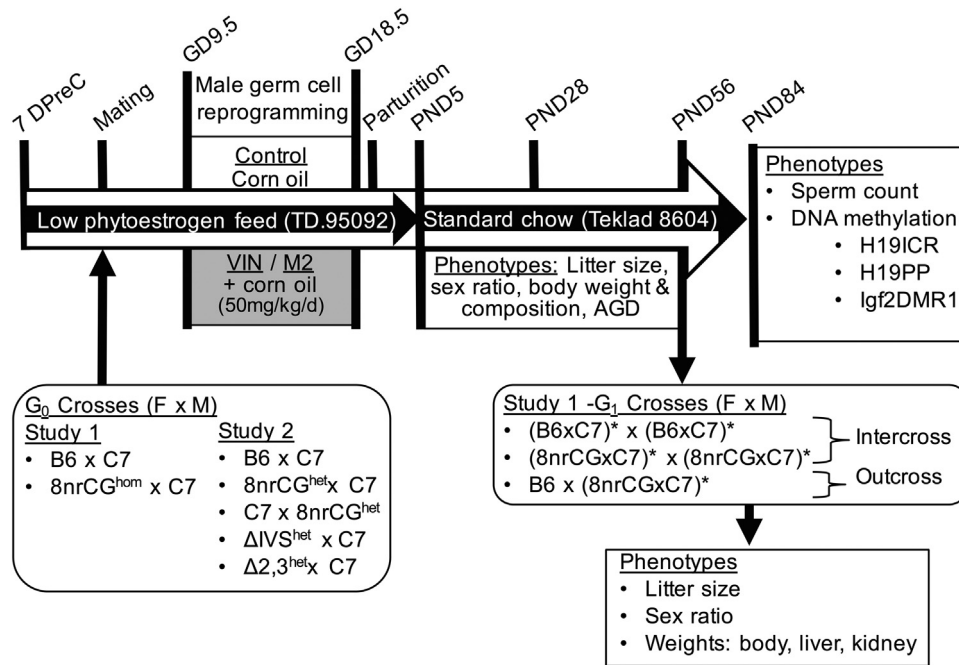


Fig. 1. Treatment Scheme. Illustrates timing of crosses, special diets, experimental treatments, and phenotypes measured. Cross descriptions are shown in order female × male. Homozygous mutant animals are indicated with “hom”, heterozygous mutant animals indicated with “het”. Asterisks (*) indicate which parental G₁ animals were exposed to treatments *in utero*. F, female; M, male; DPreC, days precoitus.

2.3. Body weight, body composition and anogenital distance measurements

Body weight at PND5, PND28, and PND56 was measured by analytical balance. Body composition was measured by magnetic resonance imaging (EchoMRI-100) at PND28 and PND56 in the UNC Nutrition Obesity Research Center Animal Metabolism Phenotyping Core (UNC NORC AMP). Percent fat and lean mass were calculated using the ratio of fat or lean mass/(total body mass – free water mass) × 100. AGD was measured using calipers. Mice were weighed at AGD measurement and changes in AGD due to body weight changes were adjusted for using a ratio of AGD/body weight.

2.4. Sperm isolation and count

Intact cauda epididymis and vas deferens were isolated from PND84 males immediately after euthanasia in 1 ml of HTF buffer (pH7.5, 100 mM NaCl, 5 mM KCl, 0.368 mM KH₂PO₄, 0.2 mM MgSO₄, 2 mM CaCl₂ and 5 mg/ml BSA) at 37 °C and scored in 4-well petri dishes under a dissection microscope. Scored tissues were incubated for 10 min at 37 °C for sperm swim out. Sperm in supernatant was transferred to fresh 1.5 ml microcentrifuge tubes and 3 ul were used in a 1:50 dilution of phosphate buffered saline (PBS) while remaining cells were pelleted by centrifugation, flash frozen in liquid nitrogen and stored at –80 °C. Sperm counts and purity was measured in the 1:50 dilution using a hemocytometer and inverted light microscope.

2.5. Methylation detection & genotyping

Genomic DNA from sperm, tongue, and abdominal fat was isolated using phenol-chloroform extraction [34] and from liver using the Anaprep 12, an automated magnetic-bead based nucleic acid extraction system (BioChain Institute Inc, CA). DNA quality was assessed by NanoDrop 2000 spectrometer (Thermo Scientific, DE) and double stranded DNA concentrations measured by Quant-it PicoGreen dsDNA assay (#P7589, Life technologies, NY). Genomic

DNA was bisulfite-converted using the EZ DNA methylation Gold kit according to the manufacturer’s protocol (#D5006, Zymo research, CA). All pyrosequencing PCR and sequencing primers and sequenced regions were confirmed to be devoid of SNPs or deletions. Pyrosequencing was performed using Pyromark Q96MD instrument according to the manufacturers’ instructions (Qiagen, MD). Assays for *Igf2DMR1* and *H19PP* were previously described [21]. *H19ICR.Rp1* assays methylated at the *H19/Igf2* ICR using primers forward 5’- GAGGTATAAGAATTTTGTAAAGGAGATTATG-3’ and biotinylated reverse 5’- ACTAAACTTAAATAACCCACAACATTAC-3’ and pyrosequenced using primer 5’- ATTAGTTGTGGGGTT-3’. PCRs for each sample and locus were performed in duplicate and CpG methylation was determined by pyrosequencing. The average methylation of all CpGs combined within the assayed region are presented as mean methylation (%) and used in all downstream analysis. The number of CpGs assayed for each region was 3 (*Igf2DMR1*), 6 (*H19Rp1*), and 3 (*H19PP*).

Sex of G₁ offspring was confirmed by *SRY* amplification using primers forward 5’- TTGCTAGAGAGCATGGAGGGCCATGTCAA-3’ and reverse 5’-CCACTCCTCTGTGACACTTTAGCCCTCCGA-3’ [35].

2.6. Statistical analyses

Least squares linear regression was performed separately for each genetic line with adjustment for sex and genotype where applicable using the JMP Pro software version 13.0 (SAS, NC) to compare among treatment groups for phenotypes in study 1 G₁ phenotypes (litter size, % male, litter survival, PND56 AGD/body weight, PND5 body weight, PND28 body weight, PND28% fat mass, PND28% lean mass, liver *H19ICR.Rp1*, *Igf2DMR1* and *H19PP* methylation, and abdominal fat and tongue *H19ICR.Rp1* methylation); and G₂ phenotypes (litter size, % male, PND5 body weight, PND5 kidney weight/body weight, and PND5 liver weight/body weight); and phenotypes in study 2 (adult body weight and sperm methylation at *H19/Igf2*). Treatment effects with p < 0.1 were assessed further using Dunnett’s test to compare CON to either VIN or M2. Correlations between sperm *H19ICR.Rp1* methylation and body

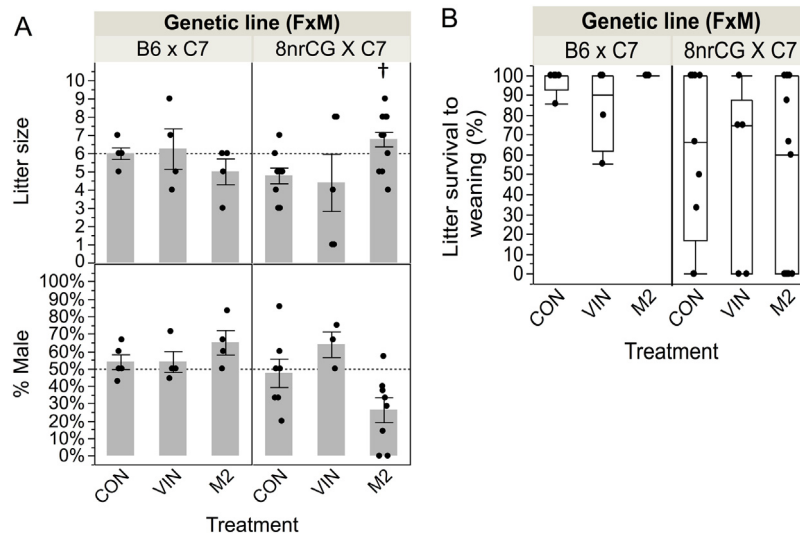


Fig. 2. G_0 cross reproductive outcomes (Study 1). (A) Bar graph of average litter size at birth (top panel) and average percent of males in each litter (% male, bottom panel). Each dot represents data for one litter. Dashed horizontal lines represent expected litter size and expected proportion of males for B6 litters. (B) Box and whiskers plot of percent of pups in each litter that survived to weaning. Error bars indicate standard error of the mean (SEM). Treatment group(s) with a significant difference from CON is denoted by (*). Sample sizes for each treatment group are listed from left to right as shown in each graph (N = number of litters): litter size at birth N = 5, 4, 4, 9, 5, 13; % male at PND5 N = 5, 4, 4, 7, 3, 8; litter survival to weaning (N = 5, 4, 4, 9, 5, 13). Reduction in N between litter size and % male reflects death of whole litter before pups were sexed at PND5.

weight were determined separately for each genetic line by Spearman correlation using the JMP Pro software version 13.0 (SAS, NC). Statistical analyses to compare between treatment groups for study 1 G_1 phenotypes (PND56 body weight, PND56 percent fat mass, PND56 percent lean mass, sperm count, liver and sperm methylation levels at *H19/Igf2* ICR) were performed using linear mixed effects models with fixed effects for treatments and random effect for litter using the nlme package in R [36,37]. For all models, we adjusted for sex where applicable and compared the difference between CON and either VIN or M2 for each line. Benjamini-Hochberg (BH) correction [38] was used to adjust p-values (adj.p) for multiple comparisons within each dataset and a false discovery rate of 0.1 was used as the significance threshold.

3. Results

3.1. Phenotypic effects of in utero VIN exposure are genetic line-dependent

To determine whether the underlying genetic sequence at the site of an epimutation influences phenotypic and epigenetic response to VIN, we first compared the effects of *in utero* VIN exposure in offspring from wild-type inbred B6 females to offspring from homozygous mutant 8nrCG females (both crossed to wild-type inbred C7 sires, Fig. 1). Pregnant mice were treated in three separate groups with either corn oil (CON), VIN dissolved in corn oil (VIN) or M2 dissolved in corn oil (M2), from GD9.5 to GD18.5 (Fig. 1). This is a critical period in which the fetus undergoes gonadal sex determination, genome wide epigenetic reprogramming, imprint establishment at ICRs, and widespread developmental changes [39–41].

3.1.1. Cross reproductive outcomes

To assess the effect of treatment on reproductive outcomes, we measured litter size, sex ratio and pup survival to weaning. For B6 mice, litter size, sex ratios and pup survival to weaning were not significantly affected by treatment with either VIN nor M2 (Fig. 2A and B). VIN treatment in 8nrCG mice did not significantly affect litter size or sex ratio (Fig. 2A). However, M2 treatment

in 8nrCG mice was linked to a significant increase in litter size (42%, $p=0.044$, adj.p = 0.099) (Fig. 2A). Interestingly, M2 had the lowest average in percent of males per litter (26.4% compared to 47.5% for CON) but this was not statistically significant ($p=0.105$, adj.p = 0.189). We assessed further whether males were disproportionately dying perinatally or inaccurately sexed due to feminized features. We found no direct correlation between litter size and proportion of males in the 8nrCG M2 group and sex misdetermination was ruled out by SRY genotyping. Compared to B6, 8nrCG crosses exhibited a substantial difference in survival to weaning ($p=0.010$, adj.p = 0.084) with a high rate (9/28 litters) of whole litter mortality in the 8nrCG crosses (Fig. 2B). There was no significant difference in gestational weight at GD18.5 or weight gain from GD9.5-GD18.5 between B6 and 8nrCG mice ($p=0.412$, $p=0.810$, respectively) making additional prenatal lethality unlikely. Partial litter mortality in the 8nrCG mice resulted in the death of an additional 18 pups, 15 of which died before the animals were sexed at PND5. Postnatal mortality in the 8nrCG mice was similar across all treatment groups (Fig. 2B), and was not correlated with male/female ratios ($p=0.148$) indicating that male and female offspring exhibited similar levels of postnatal mortality.

3.1.2. G_1 body weight & body composition outcomes

To assess the effect of *in utero* treatment on adult phenotypes, we measured adult (PND56) body weight and body composition as growth/developmental endpoints. B6 mice showed no significant difference in PND56 body weight for VIN or M2 treatment groups (Fig. 3A). However, VIN-treated 8nrCG mice had significantly higher PND56 body weight (3.6 g, 18% increase on average, $p=0.0008$, adj.p = 0.024), while M2-treated mice were unaffected ($p=0.718$, adj.p = 0.886) (Fig. 3A). Male and female adult offspring were affected similarly (Fig. 3A). The increase in VIN-treated 8nrCG bodyweight was present as early as PND28 (1.8 g, 12%, $p=0.0018$, adj.p = 0.0273) but not detected at PND5 (Supplemental Fig. 1A & B).

To determine whether the increase in body weight in the 8nrCG line was related to a change in body composition (disproportionate increase in fat or lean mass), we compared percent fat and lean mass (tissue mass/(body mass – free water mass)) between treatments

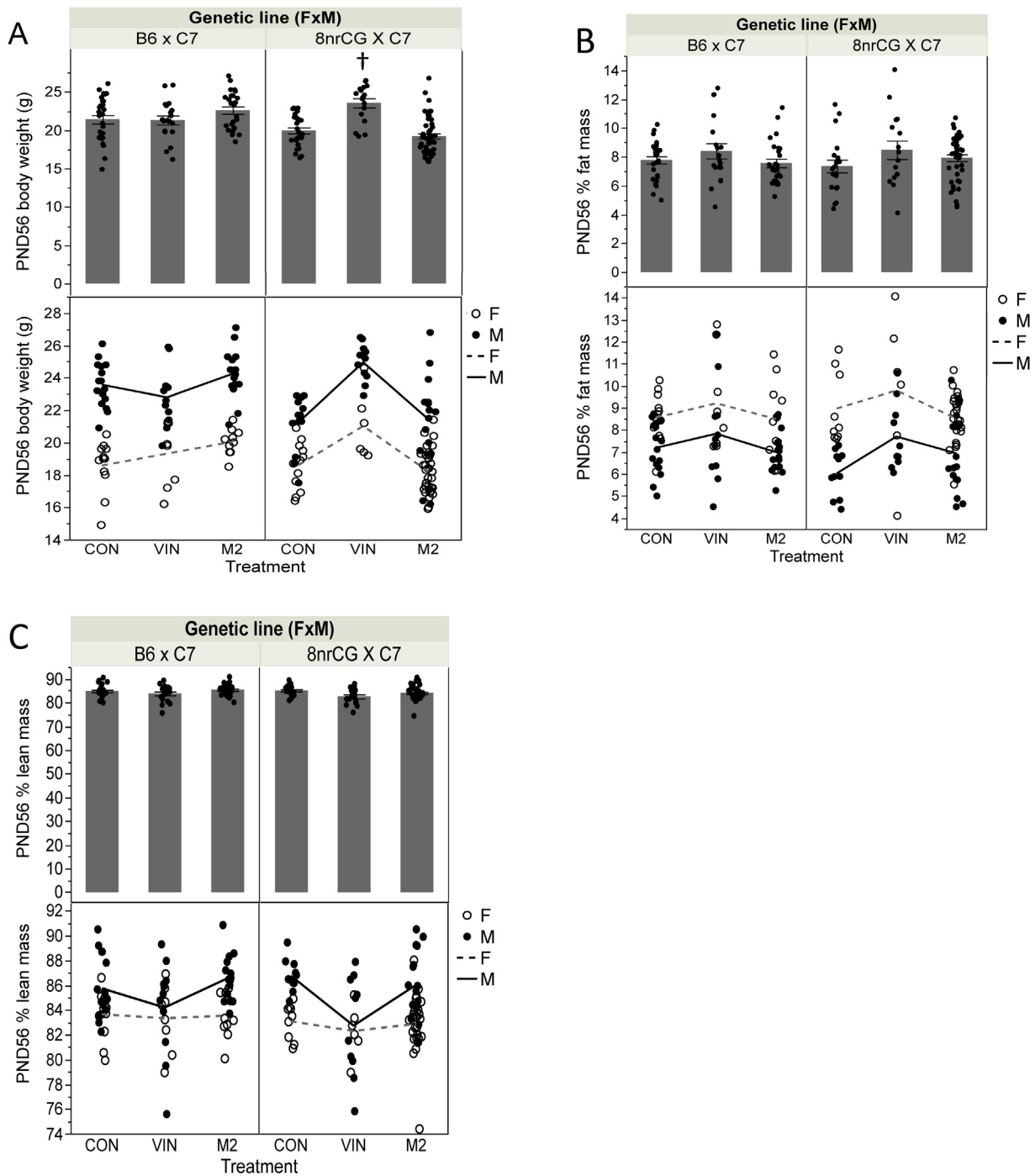


Fig. 3. G_1 body weight and body composition outcomes (Study 1). PND56 (A) body weight; (B) percent fat mass; (C) percent lean mass. Top panels show bar graph of average body weight for both sexes combined. Bottom panel is enlarged to only show portion y-axis covered by data points. Individual (dots) weights are separated by sex, male (filled dots), female (unfilled dots); lines connect means between groups for males (solid line) or females (dashed line). Error bars indicate standard error of the mean (SEM). F, female; M, male. Treatment group(s) with a significant difference from CON is denoted by (†). Sample sizes for each treatment group are listed from left to right as shown in each graph (n = number of mice, both sexes combined; N = number of litters): PND56 bodyweight n = 28, 19, 26, 27, 17, 50; N = 5, 4, 5, 7, 3, 7; PND56% fat mass n = 28, 19, 26, 20, 16, 43; N = 5, 4, 5, 5, 3, 6; PND56% lean mass n = 28, 19, 26, 20, 16, 43; N = 5, 4, 5, 5, 3, 6.

and genetic lines. Percent fat mass was significantly increased at PND28 for VIN-treated 8nrCG mice (15% increase, $p=0.019$, $\text{adj.}p=0.0988$) (Supplemental Fig. 1C). However, by PND56, percent fat mass was not significantly increased for any treatment group in either B6 or 8nrCG lineage ($p=0.248$, $\text{adj.}p=0.623$ and $p=0.083$, $\text{adj.}p=0.313$, respectively) (Fig. 3B). Percent lean mass was not significantly changed for any treatment group for either lineage either at PND28 or PND56 (Fig. 3C & Supplemental Fig. 1D). Taken together, these data suggest that the increase in adult body

weight is likely due to developmental phenotype that persisted into adulthood and though changes in adiposity may play a role, this is not the main driver of this phenotype. Since these effects were only observed in VIN-treated mice, and not M2, they likely occur independent of antiandrogenic activity.

3.1.3. G_1 reproductive development outcomes

To determine whether the antiandrogenic activity of VIN and M2 reported previously, is sufficient to alter reproductive devel-

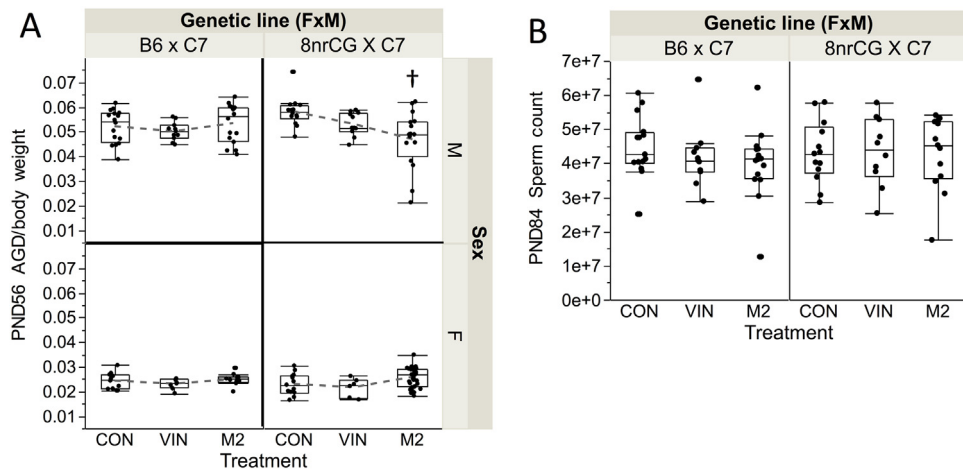


Fig. 4. G₁ reproductive development outcomes (Study 1). Box and whiskers plot shown for PND56 (A) male AGD (normalized to body weight, top panel) and female AGD (normalized to body weight, bottom panel); (B) PND84 total sperm count. Error bars indicate standard error of the mean (SEM). Treatment group(s) with a significant difference from CON is denoted by (†). Sample sizes for each treatment group are listed from left to right as shown in each graph (n = number of mice, both sexes combined; N = number of litters): PND56 male AGD/bodyweight n = 16, 11, 16, 14, 11, 16; N = 5, 4, 5, 6, 3, 6; PND56 female AGD/bodyweight n = 12, 8, 10, 13, 6, 34; N = 5, 4, 5, 5, 3, 7; PND84 sperm count (n = 15, 11, 16, 13, 10, 13; N = 5, 4, 5, 5, 3, 5).

opment and male fertility in this model, we compared adult AGD and sperm count between genetic lineages and treatment groups. After normalization to body weight (AGD/body weight), B6 males and females did not exhibit any significant differences in PND56 AGD in either VIN or M2 treatment groups (Fig. 4A). On the other hand, 8nrCG males exhibited a significant decrease in PND56 AGD in the M2 treatment group (19% decrease, $p = 0.0014$, $\text{adj.}p = 0.024$) (Fig. 4A), while females were not significantly affected ($p = 0.070$, $\text{adj.}p = 0.298$) (Fig. 4A). There were no statistically significant changes in sperm count for either genetic lineage or treatment group (Fig. 4B). These data show that in this model of VIN and M2 exposure, antiandrogenic effects on reproductive development were minimal and unlikely to affect fertility. M2 exposure had a greater effect on reproductive development compared to VIN (as expected based on previously reported antiandrogenic activity),

and 8nrCG mice were more susceptible than B6. Importantly, it confirms that body weight changes detected in the VIN treatment group are not linked to antiandrogenic activity.

3.2. VIN-induced body weight increase is independent of methylation state at *H19/Igf2*

The VIN dose used in this study (50 mg/kg/d) was previously reported to alter DNA methylation state at the *H19/Igf2* locus in sperm of FVB/N mice [17]. To test whether the body weight sensitivity of 8nrCG mice to VIN exposure is due to methylation perturbation at the mutated *H19/Igf2* locus, we compared methylation state at 3 loci across the *H19/Igf2* imprinted domain between B6 and 8nrCG lineages treated with VIN or M2. These loci were selected due to proximity to the 8nrCG mutation at the *H19/Igf2* ICR

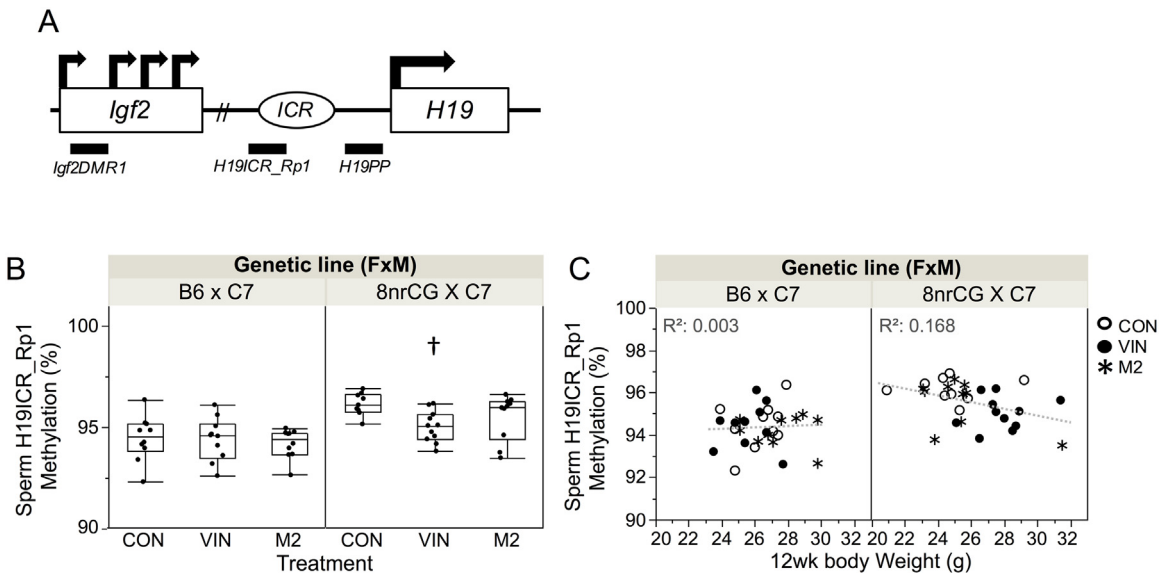


Fig. 5. G₁ methylation outcomes at *H19/Igf2* locus (Study 1). (A) Schematic of *H19/Igf2* imprinted domain and loci assayed for methylation changes: *Igf2DMR1*, *H19ICR_Rp1*, and *H19PP* (locations indicated by horizontal bars below the domain). (B) Box and whiskers plots of percent methylation at *H19ICR_Rp1* in G₁ sperm samples; each dot represents the mean methylation of 6 CpGs across the region analyzed for each individual sample. Error bars indicate standard error of the mean (SEM). (C) Correlation between G₁ sperm *H19ICR_Rp1* methylation levels and 12wk body weight. Treatment group(s) with a significant difference from CON is denoted by (†). Sample sizes for each treatment group are listed from left to right as shown in each graph (n = number of mice, both sexes combined; N = number of litters): Sperm *H19ICR_Rp1* Methylation n = 10, 10, 10, 9, 11, 10; N = 5, 4, 5, 4, 3, 5.

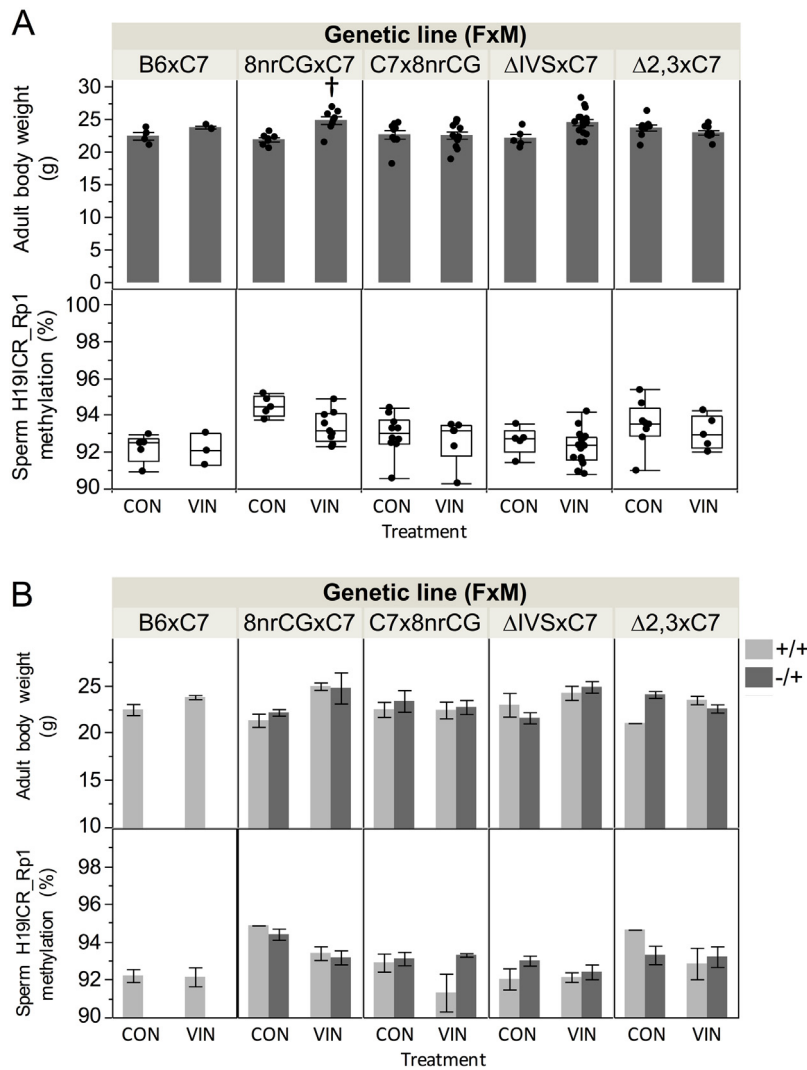


Fig. 6. G₁ male adult body weight and methylation outcomes across 5 genetic lines screened for VIN sensitivity (Study 2). (A) Bar graph of average adult (8–9 wks) body weight (top panel) and box and whiskers plots of percent methylation at *H19ICR.Rp1* in G₁ sperm samples (bottom panel); (B) Bar graph of same data shown in (A) but figure is enlarged to show differences/similarities between genotypes. Error bars indicate standard error of the mean (SEM). Treatment group(s) with a significant difference from CON is denoted by (†). Sample sizes for each treatment group are listed from left to right as shown in each graph (n = number of mice, genotypes combined; N = number of litters): Adult body weight n = 4, 3, 7, 8, 9, 12, 5, 16, 9, 9; N = 2, 2, 3, 3, 3, 4, 2, 4, 4, 4; Sperm *H19ICR.Rp1* methylation n = 5, 3, 5, 9, 10, 6, 5, 16, 8, 5; N = 2, 2, 2, 3, 3, 3, 2, 4, 4, 3.

(*H19ICR.Rp1*) and proximity to differentially methylated regions (DMR) linked to expression of *Igf2* (*Igf2DMR1*) and *H19* (*H19PP*). We found that methylation levels in adult male liver were not affected by genetic lineage or treatment groups for any of the 3 loci tested (Supplemental Fig. 2A). A small but statistically significant decrease in methylation was detected in 8nrCG VIN sperm at the *H19ICR.Rp1* (1%, $p = 0.012$, adj. $p = 0.099$) (Fig. 5B). Sperm methylation at *H19ICR.Rp1* was seemingly related to body weight in 8nrCG and not B6 mice, but this relationship was not statistically significant after correction for multiple testing ($p = 0.025$, adj. $p = 0.139$) (Fig. 5C). Methylation at *H19ICR.Rp1* in tongue (muscle) or abdominal fat in 8nrCG mice was not affected by treatment (Supplemental Fig. 2B). In sum, these data suggest that individuals may have shared susceptibility to VIN-induced body weight and sperm methylation changes. However, body weight differences are likely not directly driven by methylation differences at the *H19/Igf2* locus in 8nrCG mice, thus another mechanism is at play.

3.3. VIN-induced outcomes are 8nrCG specific and due to a maternal effect and not pup genotype

To determine whether VIN-induced phenotypic and epigenetic outcomes observed in the 8nrCG mutant mouse line are caused by genetic mutations at the *H19/Igf2* ICR, we screened a panel including two additional *H19/Igf2* ICR mutant lines. We compared the response of adult male offspring from heterozygous 8nrCG, Δ2,3, ΔIVS, and wild-type B6 dams (all crossed to C7 males) and offspring from the reciprocal cross C7 dams × 8nrCG males (to determine parent of origin dependence) (Fig. 1).

When comparing 8–9wk old male body weights and methylation at the *H19/Igf2* ICR in sperm between VIN and CON-treated mice across the panel of mice, we reproduced the 8nrCG lineage specific VIN-induced effects observed in study 1. Similar to study 1, offspring from 8nrCG but not B6 dams exhibited a significant increase in body weight in the VIN treatment group compared to CON (3 g, 14% increase, $p = 0.002$, adj. $p = 0.020$) (Fig. 6A). However, sperm methylation at the *H19/Igf2* ICR in VIN-exposed offspring

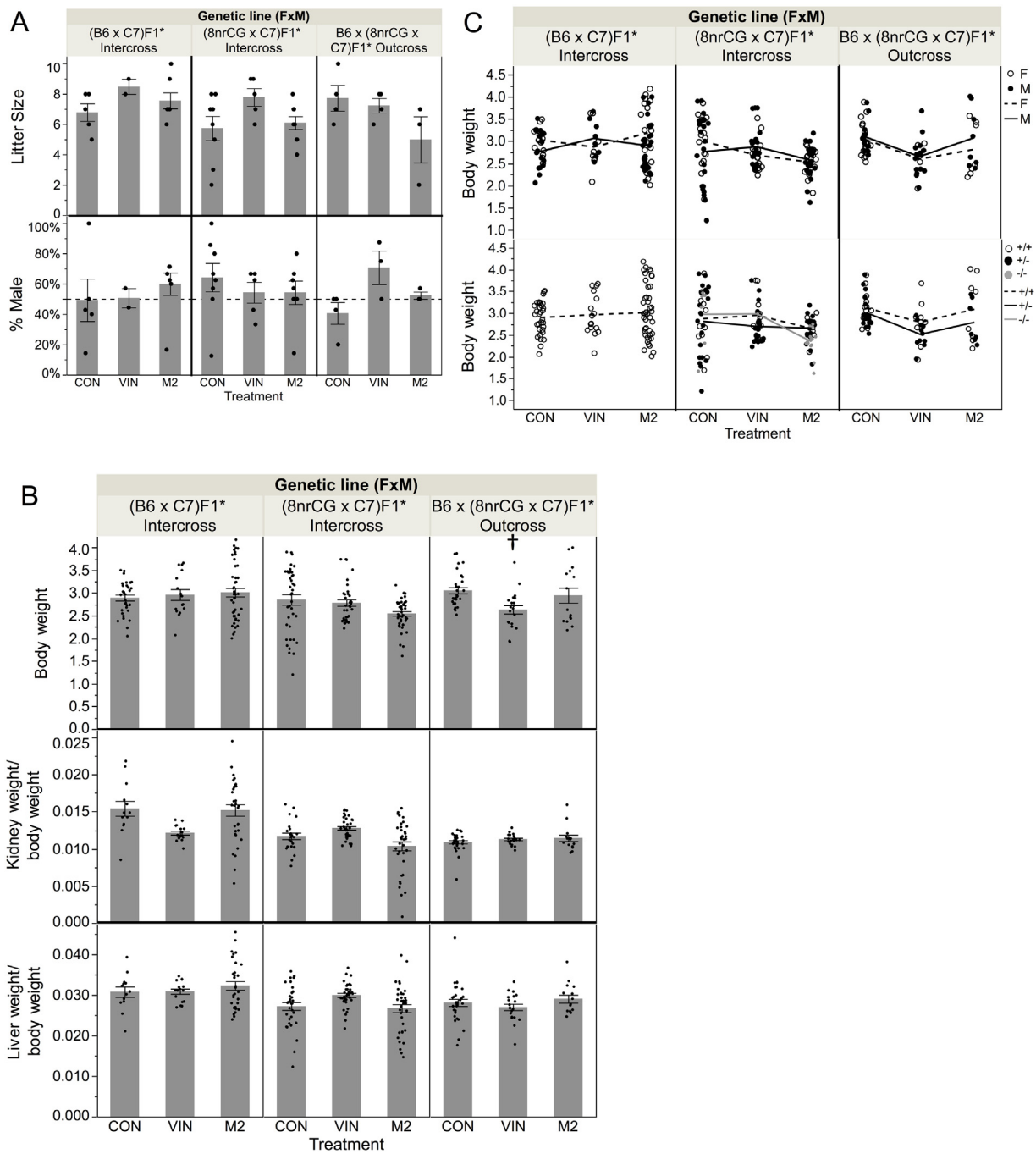


Fig. 7. G₂ offspring reproductive and birth outcomes at PND5 (Study 1). Asterisks (*) indicates treated parent(s). (A) Bar graph of average litter size (top panel) and % of litter born male (bottom panel). (B) Bar graph of average body weight (top panel); kidney weight/normalized to body weight (middle panel); and liver weight/normalized to body weight (bottom panel). (C) Samples separated by sex, male (M, filled dots) and female (F, unfilled dots) (top panel); or separated by genotype, wildtype (+/+, unfilled dots), heterozygous mutant (+/-, filled dots), and homozygous mutant (-/-, grey dots) (bottom panel); lines connect means between treatment groups for sexes (top panel) or genotypes (bottom panel). Error bars indicate standard error of the mean (SEM). Treatment group(s) with a significant difference from CON is denoted by (†). Sample sizes for each treatment group are listed from left to right as shown in each graph (n = number of mice, genotypes combined; N = number of litters): Litter size N = 5, 2, 7, 8, 5, 9, 4, 4, 3; % male N = 5, 2, 7, 8, 5, 7, 4, 3, 3; PND5 Body weight n = 32, 16, 44, 40, 36, 43, 31, 20, 15; N = 5, 2, 6, 7, 5, 7, 5, 3, 3; PND5 Kidney weight/Body weight n = 13, 16, 32, 23, 36, 36, 31, 20, 15; N = 2, 2, 4, 5, 5, 6, 5, 3, 3; PND5 Liver weight/Body weight n = 13, 16, 32, 32, 36, 37, 31, 20, 15; N = 2, 2, 4, 6, 5, 6, 5, 3, 3.

from 8nrCG dams showed a similar trend but was not statistically significant after correction for multiple testing, possibly due to the smaller sample size reducing power to detect such a small change (1%, $p=0.031$, $adj.p=0.103$). Although we could not statistically test due to small sample size, both 8nrCG offspring genotypes (+/+ and -/+) were on average affected similarly for body weight and methylation (Fig. 6B). Neither B6 nor the other mutant mice ($\Delta 2,3$

and $\Delta 1V5$) were affected by treatment (Fig. 6A). VIN-exposed offspring from 8nrCG sires (reciprocal cross, C7 x 8nrCG) were also unaffected (Fig. 6A). We conclude that the VIN-induced methylation and body weight changes are likely due to an 8nrCG lineage specific effect that was only observed when the mother carried an 8nrCG allele and was independent of pup genotype.

3.4. Paternal VIN exposure in utero decreases pup neonatal body weight in a cross-dependent manner

VIN exposure was previously shown to result in heritable phenotypes [8,16,18]. Therefore, we determined whether paternal (G_1) VIN exposure *in utero* alters unexposed second generation offspring body weight or epigenetic outcomes. To determine whether the exposed male germline was sufficient to transmit any VIN-induced effects, we compared offspring from study 1 G_1 males generated by intercross (to treated G_1 females) or outcross (to untreated B6 females) (Fig. 1).

3.4.1. Cross reproductive outcomes

There were no statistically significant reductions detected in litter size or sex ratio associated with treatment group or genetic lineage for either intercross or outcross groups (Fig. 7A). Therefore, in support of data described above, G_1 male and female fertility were not substantially affected by VIN or M2 treatment *in utero*.

3.4.2. G_2 growth/developmental outcomes

After adjustment for pup sex and genotype, we found that G_2 neonatal (PND5) body weight was only significantly different for pups from VIN-treated 8nrCG outcrossed sires compared to CON sires (approx. 420 mg, 14%, $p=0.0025$, $adj.p=0.035$) (Fig. 7B). Although we could not statistically test due to small sample size, both 8nrCG offspring genotypes (+/+ and -/+) and sexes were on average affected similarly for body weight (Fig. 7C). Differences in body weight for 8nrCG outcross pups is likely due to differences in growth and not adiposity since kidney and liver showed similar decreases in weight that were completely normalized after correction for body weight (Fig. 7B). Similar to our findings in the parental mice, VIN-related changes in G_2 8nrCG neonatal (PND5) body weight did not coincide with methylation changes at the *H19/Igf2* ICR (Supplemental Fig. 3) as one might expect for a loss of imprinting-related growth phenotype. PND5 body weight, kidney and liver weight (after normalization to body weight) was not significantly different from CON for any other lineages or treatments (Fig. 7B). Taken together, these data demonstrate that first generation *in utero* exposure to VIN can alter second generation offspring development when transmitted through the paternal germline in a manner seemingly independent of antiandrogenic activity but strongly influenced by genetic lineage and crossing scheme.

4. Discussion

Here, we use several genetic mouse lines carrying mutations at the *H19/Igf2* ICR to assess the effect of genetic sequence differences on phenotypic and epigenetic response to VIN exposure during fetal development. We found that compared to wild-type B6 mice, the 8nrCG mutant line was more susceptible to VIN-induced changes in body weight, body composition and *H19/Igf2* ICR methylation. Importantly, we reproduced the body weight finding and showed that these changes were seemingly independent of sex and pup genotype at the *H19/Igf2* ICR. Furthermore, assessment of reciprocal crosses showed that a maternal effect specific to the 8nrCG line was likely responsible for VIN-induced effects since they were present when offspring from 8nrCG dams were assessed but not when 8nrCG sires were used. We cannot rule out that maternal genotype at the *H19/Igf2* ICR is still responsible, but this seems unlikely since none of the other mutant lines that exhibit greater loss of imprinting at *H19/Igf2* were significantly affected [20,21]. It is possible that 129/SvJ loci in the <2% of the genome that remains after backcrossing to B6 are responsible. We also showed that *in utero* VIN treatment in one generation altered body weight in a second generation but significant changes were only detected in the 8nrCG lineage and specifically when mice were outcrossed to

an unexposed female. Thus, contrary to expectation, intercrossing schemes may actually mask intergenerational effects. Overall, these data show that even small differences in maternal genetic lineage confer significant susceptibility to phenotypic and epigenetic effects of environmental exposures *in utero* and therefore careful attention should be taken in study design to control for such features.

We were unable to replicate previous findings of sperm methylation changes at the *H19/Igf2* ICR associated with VIN exposure [17]. This could be explained by either a strain effect or an aggregate effect of other environmental factors such as diet. We did detect a very small change (~1%) in G_1 sperm methylation associated with VIN in the 8nrCG lineage. No methylation differences were detected in second generation pups. It is very unlikely that the 1% methylation change in itself is biologically relevant or causative of any of the phenotypes observed in either generation tested. However, the correlation between sperm methylation and body weight in the first generation suggests the two may be somehow physiologically linked. We propose that both sperm methylation changes and body weight differences associated with VIN are separate and indirect outcomes of perturbation of gross development that likely began *in utero* at the time of treatment. 8nrCG crosses exhibited increased perinatal lethality, and although it was not linked to treatment, it may allow for increased susceptibility to such developmental perturbation when exposed to VIN *in utero*. Further studies are required to dissect the pathways responsible.

In our assessment of the effects of VIN compared to effects of the M2 metabolite, we found that offspring exposed to M2 behaved more similarly to CON-treated animals for almost all of the phenotypes tested except those related to male reproductive development. Even then, there were only minimal antiandrogenic effects on male reproductive development. This was surprising since M2 has generally been shown to have greater antagonistic activity as a ligand for steroid receptors including AR and ER [13]. However, it does demonstrate that the effects of VIN reported here are likely independent of such activity and confirms previous studies that could not replicate VIN effects using flutamide [42]. Since our sample sizes here are somewhat modest, the lack of effect of the M2 treatment group also presents as a secondary control in this study showing that the effects we observe are likely specific to VIN and not due to random variation in the population. This is true for outcomes in both generations and genetic lineages.

5. Conclusions

These data show that VIN may alter other important developmental pathways in a manner independent of antiandrogenic function and that genetic strain sensitivity can have a significant effect on outcomes. While VIN may not be of particular concern as a toxicant due to restricted use in many countries, these data are highly relevant for similar dicarboximide fungicides such as iprodione that are still widely used in the US [43] as well as other common EDCs. Studies determining the effects of EDC exposure for safety and risk assessment purposes will benefit significantly from comparing effects on different genetic lineages.

Conflict of interest

The authors declare no conflict of interest. The authors alone are responsible for the content and writing of this article.

Author contributions

Study design, conception, and supervision of research was performed by FI. Animal husbandry, treatment, and tissue/cell col-

lection was performed by KC, EP, RK, and FI. Pyrosequencing data generation analyses were performed by EP, ME, and FI. Statistical analyses were performed by KK, ML, and FI. Manuscript was drafted and revised by EP, ME and FI with input from all authors. All authors have approved this manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.reprotox.2018.03.005>.

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