

## Endocrine-Disrupting Activity of Hydraulic Fracturing Chemicals and Adverse Health Outcomes After Prenatal Exposure in Male Mice

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Oil and natural gas operations have been shown to contaminate surface and ground water with endocrine-disrupting chemicals. In the current study, we fill several gaps in our understanding of the potential environmental impacts related to this process. We measured the endocrine-disrupting activities of 24 chemicals used and/or produced by oil and gas operations for five nuclear receptors using a reporter gene assay in human endometrial cancer cells. We also quantified the concentration of 16 of these chemicals in oil and gas wastewater samples. Finally, we assessed reproductive and developmental outcomes in male C57BL/6J mice after the prenatal exposure to a mixture of these chemicals. We found that 23 commonly used oil and natural gas operation chemicals can activate or inhibit the estrogen, androgen, glucocorticoid, progesterone, and/or thyroid receptors, and mixtures of these chemicals can behave synergistically, additively, or antagonistically in vitro. Prenatal exposure to a mixture of 23 oil and gas operation chemicals at 3, 30, and 300  $\mu\text{g}/\text{kg}\cdot\text{d}$  caused decreased sperm counts and increased testes, body, heart, and thymus weights and increased serum T in male mice, suggesting multiple organ system impacts. Our results suggest possible adverse developmental and reproductive health outcomes in humans and animals exposed to potential environmentally relevant levels of oil and gas operation chemicals. (*Endocrinology* 156: 0000–0000, 2015)

**W**e recently reported that chemicals used in and/or produced by unconventional oil and natural gas operations could act as endocrine-disrupting chemicals (EDCs) (1, 2). EDCs are defined as any exogenous chemical or mixture of chemicals that can interfere with any aspect of hormone action (3). As many as 1000 EDCs have been identified (4), both synthetic and naturally occurring, that can directly interact with hormone receptors (5, 6) or indirectly interact with hormone receptors via enhance-

ment or suppression of response to endogenous hormones (7, 8), modulation of endogenous hormone levels (8, 9), or other mechanisms (10). EDCs are often able to act at environmentally relevant concentrations (below those traditionally examined in toxicological risk assessments), exhibit nonmonotonic dose-response curves (resulting in quantitatively and qualitatively different outcomes at low vs high concentrations), and routinely exert greater effects during critical windows of development when exposure

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Abbreviations: BPA, bisphenol A; DEX, dexamethasone; E2, 17 $\beta$ -estradiol; EDC, endocrine-disrupting chemical; GC-MS, gas chromatography-mass spectrometry; P4, progesterone; 9-mx, nine-chemical equimolar mixture; 23-mx, 23-chemical equimolar mixture; 24-mx, 24-chemical equimolar mixture; PND, postnatal day; SPME, solid-phase microextraction; VOC, volatile organic chemical; WW, wastewater.

can alter normal development and lead to adult disease (11–14).

Hydraulic fracturing involves the high-pressure underground injection of several million gallons of water mixed with chemicals and suspended solids to fracture the shale or coal bed layer and release trapped natural gas and/or oil (15, 16). Chemicals are added to increase the viscosity, prevent salt build-up and corrosion, and serve as antibacterial agents, among other uses described previously (17, 18). A small percentage of injected fluids are immediately recovered as flowback, whereas produced water is then generated over the life of the producing well (19). Both of these wastewaters can be heavily laden with naturally occurring radioactive compounds, heavy metals from the shale layer, and chemicals used in fracturing operations and are routinely injected into disposal wells, reused in future fracturing operations, and/or pumped into open evaporation pits for disposal (16, 19, 20). Whereas less than 20 chemicals are typically used for the hydraulic fracturing of a single well, industry has reported approximately 1000 different chemicals used throughout the entire process (15, 21); of these, more than 100 are known or suspected EDCs (1, 15, 17). Due to differences in fracturing mixtures between locations and companies, there is a ubiquitous lack of uniformity between chemicals and concentrations in wastewaters (16, 19, 22).

Oil and natural gas operations have been shown capable of contaminating surface and ground water with chemicals and other contaminants (23–28) through a variety of mechanisms: spills of chemicals during transport to and from the extraction site, the production processes, improper treatment and disposal of wastewater, failures of well casings, and structural issues surrounding abandoned wells (25–27). Previous work in our laboratory reported estrogen and androgen receptor activities for twelve chemicals used in oil and natural gas operations as well as similar activities in surface water from a drilling-dense region of Colorado, suggesting that oil and natural gas operations may be increasing EDC activities in local water sources (1). However, conclusively linking oil and natural gas operations to changes in local water qualities is uncommon because most regions have not performed baseline environmental analyses (21, 29).

Health outcomes from exposure to chemicals used in and produced by oil and natural gas operations are poorly understood (30). Many of these chemicals are associated with adverse reproductive outcomes such as miscarriage, preterm birth, and decreased fertility (2). Exposure to hydraulic fracturing fluids specifically has been shown to result in respiratory, gastrointestinal, dermatological, neurological, immunological, endocrine, reproductive, and other adverse health outcomes in humans and wildlife

(31, 32). A literature review of adverse health effects associated with 353 of the chemicals used in oil and natural gas operations found that 75% could impact sensory organs, respiratory, and gastrointestinal systems; 37% were known or suspected endocrine disruptors; and 25% were human carcinogens (17). McKenzie et al (33) recently reported an association between congenital heart defects and neural tube defects in children born to mothers who lived within 10 miles of natural gas operations during pregnancy. These outcomes have been associated with gestational EDC exposure in both humans and animals (34–37).

The goals of this study were to address three major data gaps regarding chemicals associated with oil and natural gas operations. First, we measured the estrogen, androgen, glucocorticoid, progesterone (P4), and thyroid receptor activities of 24 suspected or known EDCs used in oil and natural gas operations (Table 1). Second, we screened several wastewater samples from oil and natural gas operations in Colorado for the presence of these chemicals. Third, we assessed potential adverse reproductive and developmental outcomes from prenatal exposure to a laboratory-created mixture of these chemicals in male mice. We hypothesized the following: 1) chemicals used in oil and natural gas operations would exhibit antagonist activities for nuclear hormone receptors and may act additively in combination, and 2) a laboratory-created mixture of these chemicals would impact hormone-sensitive endpoints in prenatally exposed C57BL/6J mice.

## Materials and Methods

### Chemical and mixture preparation

17 $\beta$ -Estradiol (E2; estrogen agonist, 98% pure), ICI 162780 (estrogen antagonist, 98% pure), DHT (androgen agonist,  $\geq 97.5\%$  pure), flutamide (androgen antagonist, 100% pure), T<sub>3</sub> (thyroid agonist,  $\geq 95\%$  pure), P4 (P4 agonist,  $\geq 99\%$  pure), mifepristone (glucocorticoid/P4 antagonists,  $\geq 98\%$  pure), dexamethasone (DEX; glucocorticoid agonist, 99.5% pure), and all drilling chemicals were purchased from Sigma-Aldrich Co. 1–850 (thyroid antagonist,  $\geq 95\%$  pure) was purchased from EMD Millipore. Stock solutions of all chemicals were prepared at 10 mM in HPLC-grade methanol and stored at 4°C, except T<sub>3</sub> and 1–850 (prepared in dimethylsulfoxide). The 24 oil and natural gas operation chemicals that were selected (Table 1) were chosen from lists of all known chemicals used in oil and natural gas operations (15, 17), narrowed to chemicals that were known or suspected EDCs (17), with preference given to those used in multiple chemical products (15, 17). Eight mixtures of these 24 chemicals were created and tested in vitro: a nine-chemical equimolar mixture (9-mix), a 24-chemical equimolar mixture (24-mix), a 23-chemical equimolar mixture (23-mix) with equimolar concentrations of all chemicals except bisphenol A (BPA), and five receptor-specific mixtures that were prepared at

**Table 1.** Description of Oil and Gas Operation Chemicals Used in Each Mixture Tested (Micromoles)

Chemical Name	Case Number	Oil and Gas Operation Use	Common Use	Naturally Occurring	9-Mix <sup>a</sup>	23-Mix <sup>b</sup>	ER Mix <sup>b</sup>	AR Mix <sup>b</sup>	PR Mix <sup>b</sup>	TR Mix <sup>b</sup>	GR Mix <sup>b</sup>
1,2,4-Trimethylbenzene	95-63-6	Surfactant	Dyes, resins, perfumes, gasoline constituent	X	10	10					
2-(2-Methoxyethoxy) ethanol	111-77-3	Biocide, surfactant	Industrial solvent, icing inhibitor			10	0.1	2.5			4.0
2-Ethylhexanol	104-76-7	Defoamer, breaker	Plasticizer precursor, sunscreens		10	10	0.2	1.0	10.0		2.8
2-Methyl-4-isothiazolin-3-one Acrylamide	2682-20-4	Biocide	Personal care products, preservative			10	7.5	22.5			3.0
	79-06-1	Scale control, friction reducer	Water treatment, personal care products			10		77.5			
Benzene	71-43-2	Paraffin inhibitor, surfactant	Chemical precursor, resins, rubbers, plastics	X	10	10	9.0	6.0			
BPA	80-05-7	Resins, equipment sealings	Plastics, resins, thermal receipt developer					1.3	0.7	19.0	17.5
Bronopol	52-51-7	Biocide	Personal care products, antimicrobials			10	2.3	85.0	30.0		0.3
Cumene	98-82-8	Paraffin Inhibitor	Chemical precursor, paints	X	10	10	0.9	0.6	30.0		
Drethanolamine	111-42-2	Friction reducer, corrosion inhibitor	Personal care products, chemical precursor			10	0.3	2.3	35.0		
Ethoxylated nonylphenol	9016-45-9	Surfactant, corrosion inhibitor	Detergents, dust control, icing inhibitor			10	0.2	3	0.2	1.1	0.4
Ethoxylated octylphenol	9036-19-5	Surfactant, corrosion inhibitor	Cleaning agents, paints, coatings			10	0.1	1.3	1.5	1.8	3.5
Ethylbenzene	100-41-4	Nonemulsifier, paraffin inhibitor	Styrene precursor, gasoline	X	10	10	55.0	15			
Ethylene glycol	107-21-1	Cross-linker, friction reducer	Antifreeze, coolant, polyester fiber			10	0.2	0.4	30.0	1.5	5.0
Ethylene glycol monobutyl ether	111-76-2	Surfactant, foamer	Corexit 9527, personal care products			10	0.1	0.6		8.0	
Naphthalene	91-20-3	Surfactant, acid inhibitor	Mothballs, fumigant, chemical precursor	X	10	10	2.7	2.5	20.0	1.7	0.4
N,N-dimethylformamide	68-12-2	Corrosion inhibitor	Plastics, pesticides, adhesives			10	0.3	4	40.0		
Phenol	108-95-2	Resin coating for proppants	Plasticizer precursor, resins	X		10	15.0	31	50.0		
Propylene glycol	57-55-6	Gellant, breaker	Personal care products, resins			10	27.5				
Sodium tetraborate decahydrate	1303-96-4	Cross-linker	Borax, personal care products, detergents	X		10	0.3	5			
Styrene	100-42-5	Proppant	Plastics, rubber, insulation	X	10	10	0.3			4.5	4.5
Toluene	108-88-3	Nonemulsifier, paraffin inhibitor	Chemical precursor, paints	X	10	10	80.0	70.0			
Triethylene glycol	112-27-6	Biocide, dehydration	Plasticizer precursor, air sanitizers, disinfectants			10	36.6	32.5			
Xylenes	1330-20-7	Nonemulsifier, breaker	Plasticizer precursor, polyester production	X	10	10	4.5	63.3	21.0		

Abbreviations: AR, androgen receptor; ER, estrogen receptor; GR, glucocorticoid receptor; PR, P4 receptor; TR, thyroid receptor. The table includes a list of oil and gas operation chemicals tested in this study and information on their uses in and outside industry. Descriptive columns define the chemicals present and their concentrations (in micromoles) in the various mixtures tested in this study.

<sup>a</sup> Listed concentration (in micromoles) is highest tested as well as 10-, 100-, and 1000-fold lower.

<sup>b</sup> Listed concentration (in micromoles; IC<sub>10</sub> concentrations for each component) is highest tested as well as 10-fold higher and 10- and 100-fold lower.

equipotent concentrations (all antagonists for a given receptor combined at their IC<sub>10</sub> concentrations; Table 1).

### Plasmids

For estrogen receptor testing, cells were transfected with the estrogen response element linked to the firefly luciferase gene,

3XERETKLuc (38), and CMV-β-Gal (39). For androgen receptor testing, cells were transfected with androgen receptor, CMV-AR1 (40), the androgen response element linked to the firefly luciferase gene, PSA-Enh F4TATA-luc (41), and CMV-β-Gal. For P4 receptor testing, cells were transfected with P4 receptor B, pcDNA3 PRB (42), P4 response element linked to the firefly

luciferase gene, 2XPRES-TK-Luc (43), and CMV- $\beta$ -Gal. For thyroid receptor testing, cells were transfected with thyroid receptor, pSG5-hTR $\beta$ 1 (44), thyroid response element linked to the firefly luciferase gene, pGL4-TK-2X taDR4 (45) (thymidine kinase gene minimal promoter sequence: <http://microbiology.ucdavis.edu/privalsky/pGL4CP.html>), and CMV- $\beta$ -Gal. For glucocorticoid receptor testing, cells were transfected with glucocorticoid receptor, pRST7-GR (46), the glucocorticoid response element linked to the firefly luciferase gene, MMTV-luc (47), and CMV- $\beta$ -Gal.

### Hormone receptor reporter gene assays

Ishikawa cells (Sigma catalog number 99040201) and HepG-2 cells (American Type Culture Collection; number HB-8065) were maintained and transiently transfected for hormone receptor assays as described previously (1, 48). Cells were induced with dilution series of the positive controls (Supplemental Figure 1) or of the selected subset of chemicals (10  $\mu$ M to 10 nM), diluted in medium using a 1% methanol vehicle.

### Sample toxicity

The CellTiter 96 nonradioactive cell proliferation assay (Promega; catalog number G4000) was used to assess cell toxicity in Ishikawa cells, according to slightly amended manufacturer's instructions (49). The Ishikawa cells were seeded into 96-well tissue culture plates at approximately 30 000 cells/well and allowed to settle. Cells were induced as described above using a 1% methanol vehicle and incubated for 18–20 hours before addition of dye solution. Plates were then incubated for 1 hour, solubilization/stop solution added, incubated for another hour, and absorbance read at 570 nm. Toxicity was determined by a significant decrease in the absorbance exceeding at least 15% of the baseline levels. Toxicity was exhibited by 100  $\mu$ M of methyl-4-isothiazolin, bisphenol A, bronopol, ethoxylated octylphenol, and ethoxylated nonylphenol as well as the 9-mix, 23-mix, and 24-mix mixtures. Toxicity was confirmed using the  $\beta$ -galactosidase vector described previously (1).

### Calculation of hormone receptor agonist and antagonist activities

All receptor activities were first compared with 1% methanol or 0.1% dimethylsulfoxide vehicle controls, depending on the vehicle used. Chemical response was set as a fold induction relative to this vehicle control response, prior to calculating relative responses to positive control agonists and/or antagonists. Agonist activities were then calculated as a percentage activity relative to the maximal positive control responses of 2 nM E2, 30 nM DHT, 3 nM P4, 1 nM T<sub>3</sub>, and 200 nM DEX, for estrogen, androgen, P4, thyroid, and glucocorticoid receptor assays, respectively. Antagonist activities were calculated as a percentage suppression or enhancement of the positive controls at their EC<sub>50</sub>s: 20 pM E2, 300 pM DHT, 100 pM P4, 2 nM T<sub>3</sub>, and 50 nM DEX for the estrogen, androgen, P4, thyroid, and glucocorticoid receptor assays.

### Method of isoboles mixture activity assessment

Assessment of the mixture interactions was performed using the method of isoboles, as described previously (50). Briefly, activity exhibited by mixtures was compared with single chemical activities using the following equation:  $d_A/D_A + d_B/D_B +$

$d_C/D_C + \dots = 1$ , where  $d_{A/B/C}$  denotes the concentration of chemicals A, B, and C in a mixture that produces a specific effect, and  $D_{A/B/C}$  denotes the concentration of the individual chemicals required to exhibit the same effect. Values of 1 denote additivity, values above 1 denote antagonism, and those below 1 denote synergism. Mixtures were assessed only at a concentration in which data on all chemicals was present to avoid extrapolation.

### Analysis of oil and gas wastewater samples

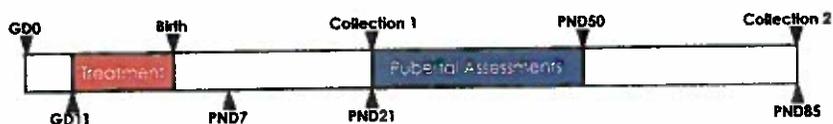
Three oil and gas wastewater (WW) samples were collected in Garfield County, Colorado, either directly from the source or from immediate spills of these fluids into the environment. Quantitation using authentic standards was performed for 16 of the 24 industry-reported oil and gas operation chemicals examined in this study. WW1 was collected from a ruptured industry WW pipeline that was actively leaking. This sample separated into organic and aqueous layers after collection, and these phases were analyzed separately. WW2 and WW3 were collected from recovered fluids stored in collection tanks. WW2 was collected from an open WW storage tank, and WW3 was collected from a closed WW storage tank. WW samples were shipped in glass containers on ice to our laboratory and stored at 4°C until processing. Aliquots for analytical investigation were stored in volatile organic chemical (VOC)-certified vials at -20°C.

### Analysis of EDCs by solid-phase microextraction (SPME) followed by gas chromatography-mass spectrometry (GC-MS)

VOCs in water were quantified according to previously published methods (51, 52). Briefly, a headspace SPME process, using an 85- $\mu$ m carboxen/polydimethylsiloxane fiber, was used to extract VOCs. Analysis was performed with an Agilent 6890N gas chromatograph coupled with an Agilent 5973N quadrupole mass spectrometer (GC-MS; Agilent Technologies). The VOCs were separated by a Hewlett Packard cross-linked methylsiloxane DB-5 capillary column (30 m  $\times$  0.25 mm). The mass spectra of each peak was characterized by comparison with reference standards and mass spectral libraries supplied by the National Institute for Standard and Technology (National Institute for Standard and Technology/Environmental Protection Agency/National Institutes of Health Mass Spectral Library version 2.0.f, 2009). Selection of diagnostic and quantitative ions was optimized and the calibration equations were developed following the procedure described previously (51, 52).

### Analysis of EDCs by liquid-liquid extraction followed by GC-MS

The nonvolatile EDCs were extracted by a 1:1 water-dichloromethane liquid-liquid procedure described previously (51, 52). The identification and quantification of EDCs were performed using a Varian 3400cx GC with a Hewlett Packard cross-linked methylsiloxane DB-5 capillary column (30 m  $\times$  0.25 mm) coupled with a Varian Saturn 2000 ion-trap mass selective detector (Varian Inc). The quantitative ions were selected by injecting a standard, and selection of diagnostic and quantitative ions was optimized by a procedure described previously (51). The most predominant product ions were recorded and background spectra subtraction was performed to optimize the selectivity and sensitivity. The ions providing the highest signal to



**Figure 1.** Mouse exposure and collection time line. Time line of oil and gas operation chemical exposure to pregnant dams and assessment of developmentally exposed pups. Colored bars represent ongoing treatment or measurements and black arrows represent set collection or measurement points. GD, gestational day.

noise ratios were selected and calibration curves were developed using seven concentrations of the standards.

### Analysis of polar EDCs by liquid chromatography and mass spectrometry

The concentrations of 2-methyl-4-isothiazolin-3-one, acrylamide, dimethylformamide, and bronopol were determined using Waters Alliance 2695 HPLC system coupled with Waters Acquity TQ triple quadrupole mass spectrometer (HPLC-liquid chromatography and tandem mass spectrometry). Methyl-isothiazolin, acrylamide, and dimethylformamide were separated by a Phenomenex Kinetex C<sub>18</sub> (100 mm × 4.6 mm; 2.6 μm particle size) reverse-phase column using electrospray ionization in the positive ion mode (ES+) with a capillary voltage of 1.5 kV. For the analysis of bronopol, the tandem mass spectrometry system was operated in the negative ion mode (ES-). The molecular parent ions were screened and the product ions used for the quantifications were determined from the spectra of analytical standard solutions. Analytical data were processed using Waters Empower software (Waters Corp).

### Animal work

C57BL/6J mice were housed in sterile polysulfone cages under temperature- and light-controlled (12 h light, 12 h dark) conditions in a barrier animal facility. All experimental procedures were performed according to an approved University of Missouri Animal Care and Use Committee protocol and were in accordance with the National Research Council's *Guide for the Care and Use of Laboratory Animals*. All experimental mice were fed LabDiet 5053 and received acidified water ad libitum from glass bottles.

Ten-week-old mice were time mated and denoted as gestational day 0 on the day of vaginal plug (Figure 1). On gestational day 11, dams were provided with experimental treatments in their drinking water. Test concentrations included a 0.2% ethanol vehicle, 166.67 μg/ml flutamide control (androgen antagonist; estimated exposure 50 mg/kg · d), and four concentrations of a mixture of 23 oil and gas operation chemicals, with each individual chemical present at 0.01, 0.10, 1.0, and 10 μg/mL (3, 30, 300, 3000 μg/kg · d of each chemical estimated exposure). The mixture included all chemicals tested *in vitro* except for BPA, which was excluded due to being the only chemical exhibiting estrogenic activity and the failure to detect BPA in our WW samples, despite the industry use of resins that may contribute BPA to WW (15). Experimental doses were provided until birth and dams were then reverted to standard acidified water. Water intake was monitored by weighing the drinking water bottle every day of the experiment, and consumption (based on water weight loss) was not found to significantly differ in experimental groups relative to the vehicle control (data not shown). Food intake was not monitored.

Anogenital distances were assessed with calipers on postnatal day (PND) 7 and fully retained nipples (areolae could not be observed) on PND 13, and body weights were measured through PND21 for all male pups (Figure 1). One randomly selected male pup from each litter was necropsied on PND21. All animals were euthanized by carbon dioxide asphyxiation and cardiac puncture, and tissues were excised. Tissues were weighed, formalin fixed for 24 hours for histological evaluation, homogenized in lysis binding solution (Ambion RNAqueous) for RNA isolation, or snap frozen in liquid nitrogen for later analysis. After the PND21 collection, all remaining males were assessed for age of preputial separation. A second collection of one male from each litter was performed on approximately PND85 in the same manner as described above with the addition of sperm assessments.

### Serum T measurements

Blood was collected from mice via cardiac puncture at time of necropsy and stored on ice. Blood was then spun at 12 000 rpm for 15 minutes to separate, serum removed, transferred to separate vial, and stored at -80°C until analysis. Serum T measurements were performed by an enzyme immunoassay according to the manufacturer's specifications (Cayman Chemical; catalog number 582701). Briefly, serum samples (50 μL) were diluted in ultrapure water (950 μL), extracted with diethyl ether (5 mL), frozen on dry ice, supernatant ether layer removed and dried under a gentle stream of nitrogen gas, and samples reconstituted in enzyme immunoassay buffer for measurement.

### Sperm assessment

Sperm quantitation, motility, and morphology assessments were performed as follows and as described in the Supplemental Materials. Both cauda epididymides were excised and placed into 600 μL DMEM (Gibco; catalog number 11885). Tissues were macerated with a needle and sperm allowed to swim out into medium for 10 minutes at 37°C and 5% CO<sub>2</sub> conditions. After 10 minutes, the tissue was removed with forceps, and the sperm concentrations were assessed using a hemacytometer after dilution with a 2% paraformaldehyde solution.

### Statistical analysis

Linear models were used to analyze the results from all single data-point-per-litter data sets (sex ratio, litter size, organ weights, body weights after weaning, sperm assessments, liver gene expression). Linear mixed models were used to analyze the results from all multiple data-point-per-litter data sets (anogenital distance, body weights before weaning, pubertal development, cardiac myocyte diameter), and incorporated random effects to account for dependence among repeated litter measurements. Fixed effects considered included treatment, litter identification, sex ratio, litter size, body weight, birth weight (PND7), and/or date of measurement or collection when appropriate for the different analyses performed. Variables were transformed to achieve normal distributions when necessary and adjusted means backtransformed for data display. Least-squares means were used for planned contrasts and to determine 95% confidence intervals for differences to the vehicle control. Diag-

nostic plots were used to assess the fit of the model and to check distributional assumptions. Proc GLM and GLIMMIX in SAS 9.4 (SAS Inc.) were used for the data analysis.

## Results

### EDC activities of chemicals used in oil and natural gas operations

We tested 24 chemicals for antagonist and agonist activity for five nuclear receptors. Antagonist activity was exhibited by 21, 21, 12, 7, and 10 chemicals for the estrogen, androgen, P4, thyroid, and glucocorticoid receptors, respectively (Figure 2). Agonist activity was exhibited by one, one, and two chemicals for the estrogen, P4, and thyroid receptors, respectively. At least one type of receptor activity was observed for all chemicals except 1,2,4-trimethylbenzene. Antagonist activities were the most potent for the estrogen receptor (Figure 2A), with two chemicals exhibiting an  $IC_{50}$  below 10  $\mu$ M and eight chemicals reaching an  $IC_{50}$ . Antagonist activities for the androgen and P4 receptors also included two chemicals exhibiting an  $IC_{50}$  below 10  $\mu$ M, with six and four chemicals reaching an  $IC_{50}$ , respectively (Figures 2B and 1C). Antagonist activities for the thyroid and glucocorticoid receptors were the least potent, with three chemicals reaching an  $IC_{50}$  for both receptors (Figure 2, D and E) and one chemical exhibiting an  $IC_{50}$  below 10  $\mu$ M for the glucocorticoid receptor (Figure 2E). Androgen receptor activities only were further tested in HepG-2 liver cancer cells to assess reproducibility across cell lines (Supplemental Figure 2). Many of these chemicals exhibited similar activities, with 17 active antagonists compared with 21 in Ishikawa cells (Figure 2). However, some chemicals displayed significantly greater (acrylamide, phenol, and diethylene glycol methyl ether) or lesser (bronopol, dimethylformamide, ethylbenzene, ethylene glycol, and methyl-4-isothiazolin) antagonism, likely due to the hepatic metabolism and production of differentially active metabolites (Supplemental Figure 2).

The alkylphenol ethoxylates (ethoxylated octylphenol and nonylphenol) exhibited the most potent antagonism for all five receptor systems, although their activity varied between them. The highest potency of inhibition was observed for the P4 and estrogen receptors, with approximately 10-fold less potent activity for the androgen and thyroid receptors (Figure 2). The glycol ethers (ethylene glycol, ethylene glycol butyl ether, diethylene glycol methyl ether, triethylene glycol, and propylene glycol) displayed potent activity for the estrogen and androgen receptors, with little activity exhibited for the other receptor

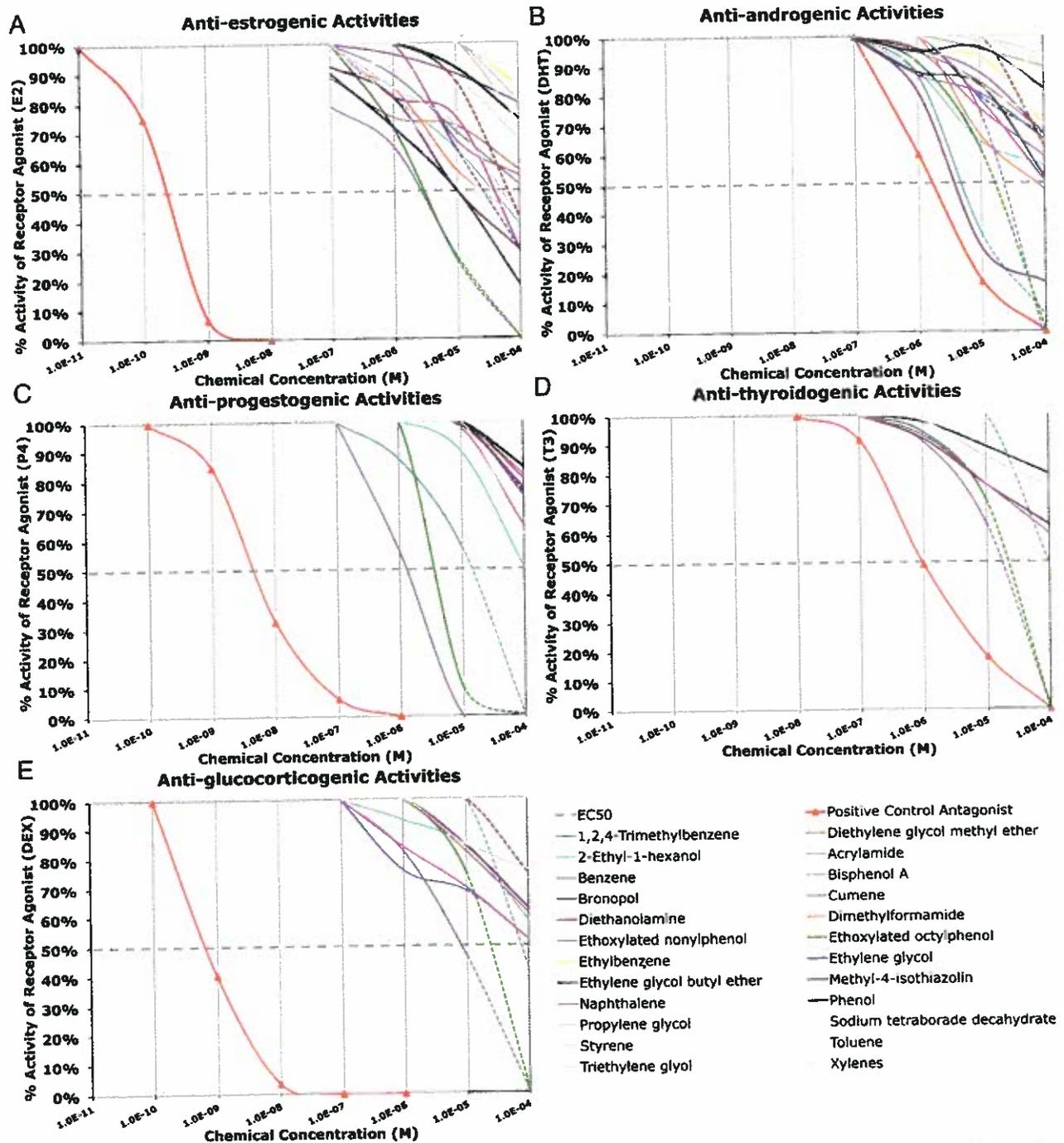
systems. To our knowledge this is the first report of direct receptor activities for many of these chemicals.

### EDC activity of mixtures of oil and natural gas operation chemicals

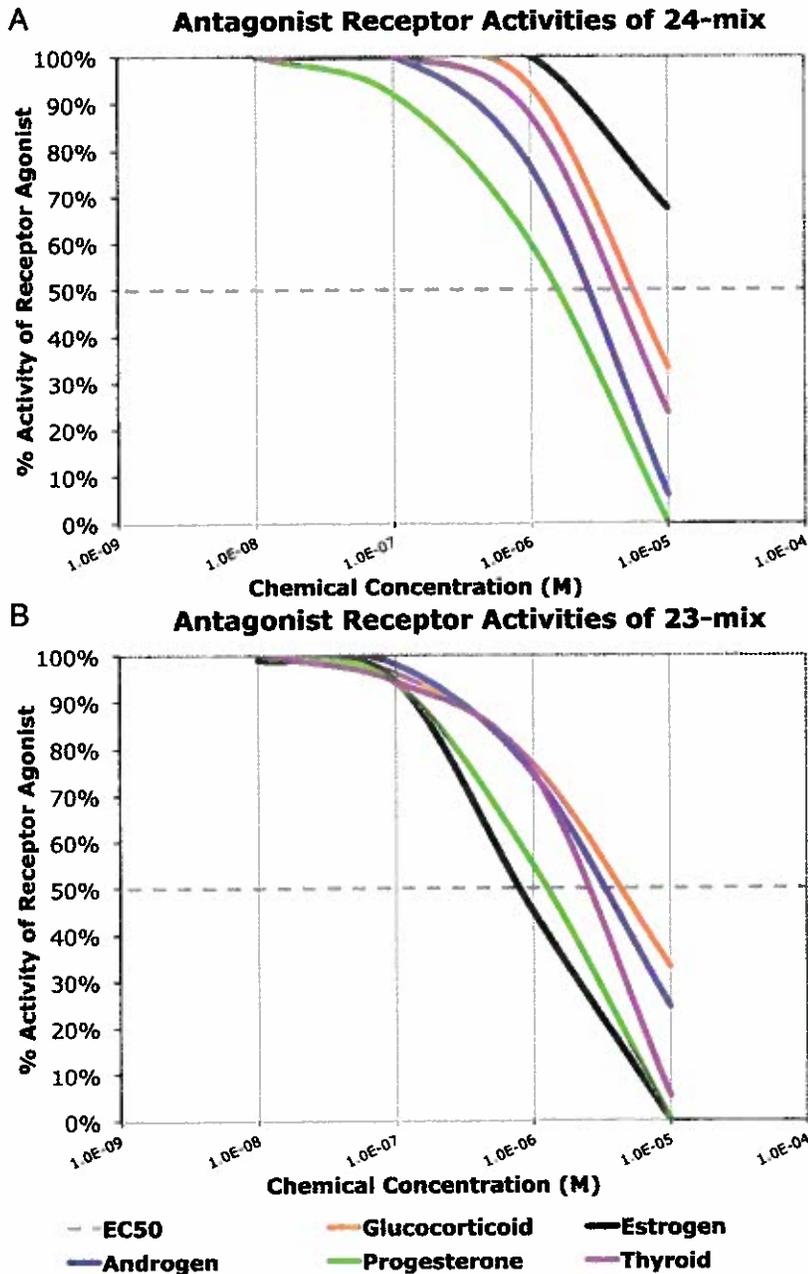
Mixtures of 9, 23, and 24 chemicals associated with oil and natural gas operations were tested *in vitro* for agonism and antagonism of five nuclear receptors, and receptor-specific mixtures were assessed for the disruption of their individual receptors (Figure 3 and Table 2). Agonist activities were not observed for any mixture other than the 24-mix for the estrogen receptor, which included BPA. Assessment of the antagonist mixture interactions was performed using the method of isoboles (additive response = 1, less than additive > 1, greater than additive < 1). Chemical mixtures exhibited mixed activities including greater than additive, additive, and less than additive responses. Apparent synergism (greater than additive response) was exhibited by the 23-mix (no BPA) for the estrogen and thyroid receptors, with scores that were 3-fold and 5-fold less than the expected response of 1 (Figure 3 and Table 2). Four mixtures exhibited apparent additivity, with scores less than 2-fold deviated from 1, and the remaining 14 mixtures exhibited less than additive responses, with scores more than 2-fold greater than 1. The receptor-specific mixtures and the 9-mix and 24-mixes behaved additively or less than additively, although they typically exhibited more potent responses than individual components (Figure 3A and Table 2), except in mixtures that included agonists such as the 24-mix for the estrogen and P4 receptors. When the estrogenic constituent of the 24-mix was excluded (BPA), the resulting combination (23-mix) then exhibited a more potent response than individual constituents (Figure 3B) and the isobole score dropped to below 1, denoting synergism (Table 2).

### Analytical identification of chemicals present in oil and gas WW samples

Sixteen of the 24 EDCs examined *in vitro* were quantified in WW samples from Garfield County, Colorado. Concentrations of these chemicals ranged from 0.01 to 79 mg/L in the aqueous WW samples and as high as 5.9 g/L in the organic fraction of WW1 (Table 3). Most of the concentrations detected were greater than the experimental concentrations used in our *in vitro* assays and *in vivo* animal study; concentrations in the aqueous samples averaged 11.81 mg/L, with some constituents at up to 8-fold greater concentrations than the average. Concentrations of chemicals in the organic phase of the fractionated WW1 sample averaged 1.16 g/L, with some constituents at up to 6-fold greater concentrations than the average.



**Figure 2.** Receptor activities of selected chemicals used in oil and gas operations. Representative dose responses of selected hydraulic fracturing chemicals tested for antiestrogenic (A), antiandrogenic (B), antiprogestogenic (C), antithyroid (D), and antiglucocorticogenic (E) activities in the Ishikawa cell line. Antagonist activities presented as the percentage suppression of 20 pM E2, 300 pM DHT, 100 pM P4, 2 nM T<sub>3</sub>, 50 pM DEX (set to 100%) for antiestrogenic, antiandrogenic, antiprogestogenic, antithyroid, and antiglucocorticogenic activities, respectively, for each chemical from 0.1 to 100  $\mu$ M. Positive control antagonists for each assay are provided in red within each pane and include the following: ICI (antiestrogen, A), flutamide (antiandrogen, B), mifepristone (antiprogestogen and antiglucocorticoid; C and E), and 1-850 (antithyroid, D). Twelve chemicals presented herein were previously assessed for antiestrogenic and antiandrogenic activities a report published elsewhere (Kassotis et al [1]). Notably, the data presented herein were assessed using new chemical stocks and in the case of antiandrogenic data, with a different cell based system.



**Figure 3.** Receptor activities of laboratory-created mixtures of chemicals used in oil and gas operations. Representative dose responses of mixtures of chemicals used in oil and gas operations tested for antiestrogenic (black), antiandrogenic (blue), antiprogestogenic (green), antithyroid (purple), and antiglucocorticogenic (orange) activities in the Ishikawa cell line. Antagonist activities presented as the percentage suppression of 20 pM E<sub>2</sub>, 300 pM DHT, 100 pM P<sub>4</sub>, 2 nM T<sub>3</sub>, 50 pM DEX (set to 100%) for antiestrogenic, antiandrogenic, antiprogestogenic, antithyroid, and antiglucocorticogenic activities, respectively, for each chemical from 0.1 to 100  $\mu$ M. Additional information on chemicals used to create each mixture is presented in Table 1.

#### Adverse health outcomes in mice exposed to mixture of 23 oil and natural gas operation chemicals

C57BL/6J dams were exposed to a laboratory-created mixture of oil and gas operation chemicals via their drink-

ing water from gestational day 11 until birth, and male offspring were then assessed at several time points. The calculated maternal doses based on consumption of treated drinking water were 3, 30, 300, and 3000  $\mu$ g/kg  $\cdot$  d for Mix3, Mix30, Mix300, and Mix3000, respectively.

Developmental exposure to Mix3 and Mix300 increased testis weights by 10% and 6% at PND21 (Figure 4A). At PND85, 7%, 7%, and 9% increased testis weights were observed in the Mix3, Mix3000, and flutamide groups, although not significantly in the Mix3 group (Figure 4B). Decreased epididymal sperm counts were observed in the Mix30 and Mix300 groups at PND85 and were reduced 24%, 33%, 35%, 17%, and 17% in the Mix3, Mix30, Mix300, Mix3000, and flutamide antiandrogen control groups, respectively (Figure 4C). Sperm motility tended to be reduced in the Mix30 group (Figure 4D). No differences in sperm morphology, as measured by midpiece bends and cytoplasmic droplets (53), were observed between treatments. Serum T was assessed at PND85, and developmental exposure to Mix300 and Mix3000 was found to increase T concentrations by 319% and 516%, respectively, although not significantly in the Mix300 group ( $P < .1$ ; Figure 5).

In many cases but not all, effects were primarily noted in the Mix3 and Mix300 groups. Increased body weights were observed in the Mix300 group at PND21 (24% increase; Figure 6A and Supplemental Tables 1 and 2). After corrections for body weight, thymus weight was 8% higher in the Mix300 group at PND21 (Figure 6B), and heart weight was 7% higher in the Mix3 group

(Figure 6C). Spleen weight tended to be higher in the Mix3 and Mix300 groups at PND21, and kidney weight tended to be higher in the Mix300 group at PND21 and PND85 (Supplemental Table 1). Some other outcomes were suggestive of effects, eg, all Mix groups exhibited a trend for

**Table 2.** Isobolographic Calculations for In Vitro Chemical Mixture Responses

Receptor Mixture	Receptor Mix <sup>a</sup>	24-Mix <sup>b</sup>	23-Mix <sup>b</sup>	9-Mix <sup>c</sup>
Estrogen	2.13	3.96	0.78	8.50
Androgen	4.44	2.22	2.03	1.18
P4	2.04	1.71	1.17	N/A
Thyroid	1.61	2.30	0.55	N/A
Glucocorticoid	3.22	4.91 <sup>d</sup>	2.43	N/A

Abbreviation: N/A, mixtures did not exhibit significant antagonism, so isobolographic values could not be calculated. Calculated isobolographic values used to assess interactions of chemical mixtures with specific receptors. If calculated value is equal to 1.0, response is considered additive. Values less than 1.0 are considered synergistic, and values greater than 1.0 are considered antagonistic. The 23-mix contains all hydraulic fracturing chemicals examined in this study except for BPA, and the 9-mix contains only trimethylbenzene, 2-ethylhexanol, benzene, cumene, ethylbenzene, naphthalene, styrene, toluene, and xylenes.

<sup>a</sup> Only active chemicals for each given receptor combined at individual IC<sub>10</sub> values.

<sup>b</sup> All chemicals at 1  $\mu$ M concentration.

<sup>c</sup> All chemicals at 10  $\mu$ M concentration.

<sup>d</sup> All chemicals at 2  $\mu$ M concentration.

decreased anogenital distance, also reduced in the flutamide group ( $P < .1$ ; Supplemental Table 2). Significant differences in litter size, sex ratio, and age of preputial separation were not observed for any treatments (Supplemental Table 2). The flutamide control pups exhibited between one to nine fully retained nipples, whereas no vehicle or mixture animals retained any, although dark focal spots (areola) could not be assessed. Cannibalization of entire litters before PND7 occurred only in Mix groups, with one, two, and three cases observed in the Mix3, Mix30, and Mix3000 groups, respectively. Thyroid-regulated gene expression was also assessed in the liver, although no significant differences were noted in either malic or Spot14 expression between experimental groups (Supplemental Figure 3).

Persistent effects were noted for testes weight, with increased weights noted at both PND21 and PND85. Effects on the thymus, heart, kidney, and spleen weight appeared to be transient, with weights returning to normal by PND85. We further assessed hearts at PND85 and found that Mix3 mice had larger cardiac myocytes than vehicle control animals (Figure 6D and Supplemental Figure 4). Thus, despite an apparent transient effect on heart weight in the Mix3 hearts, the architecture was still impacted at PND85 (Figure 6D).

## Discussion

We report, for the first time, adverse male reproductive health outcomes in mice (decreased epididymal sperm counts, increased testis weights, increased serum T) after

prenatal exposure to a laboratory-created mixture of oil and gas operation chemicals provided via drinking water at concentrations equal to and below those detected in industry WW samples (Figures 4 and 5 and Table 3). We further report mechanistic data on the antagonist interactions of these chemicals in vitro with the estrogen, androgen, glucocorticoid, P4, and thyroid receptors (Figures 2 and 3 and Table 2). Our laboratory previously reported estrogen and androgen receptor activities of 12 chemicals used in oil and natural gas operations and elevation of these activities in surface and ground water collected near industry WW spills (1). The current work expanded the analysis to an additional 12 chemicals and three additional receptors (P4, thyroid, and glucocorticoid). This work addressed key data gaps including analytical WW analysis, nuclear receptor bioactivity of chemicals, and potential health outcomes. Importantly, we have largely used one cell line to assess the disruption of five nuclear receptor systems. These cells may lack specific coactivators required to observe all agonist and/or antagonist activities in some systems and thus could prevent complete characterization of all interactions.

Analytical identification of 16 oil and gas operation EDCs, tested in vitro and in vivo herein, was performed on three industry WW samples from Colorado. Twelve of these EDCs were measured at concentrations ranging from approximately 0.01–5900 mg/L (Table 3). Other researchers have recently reported analytical approaches for identification and quantitation of oil and gas WW samples (54–56), measuring many organic compounds at milligrams per liter to grams per liter concentrations (54, 56). WW is typically diluted into natural water sources after spills. Many of these chemicals have been reported in ground water at average concentrations from 0.1 to 1.0 mg/L near hydraulic fracturing operations (27, 57), although few studies have assessed concentrations of these chemicals in drinking water. Considering these concentrations, it is likely that environmentally realistic human exposure would be in the range of 3–30  $\mu$ g/kg  $\cdot$  d, experimental doses assessed in this study, suggesting that we have appropriately captured environmentally relevant oral exposure levels for wildlife and/or humans living in dense-drilling regions. Some of these chemicals also antagonized hormone receptors in vitro at concentrations as low as 1.0  $\mu$ M (~100  $\mu$ g/L), demonstrating that these ground water samples often contained bioactive concentrations of these chemicals as demonstrated in our reporter gene assays (Figure 2).

Importantly, we thus report for the first time adverse health outcomes in mice after the exposure to a laboratory-created mixture of commonly used oil and gas operation chemicals at likely environmentally relevant levels in

**Table 3.** Concentrations (Milligrams per Liter) of EDCs in Oil and Gas WW Samples

Chemicals	Case Number	LOD <sup>a</sup>	WW1 (Aqu)	WW1 (Org)	WW2 (Aqu)	WW3 (Aqu)
1,2,4-Trimethylbenzene	95-63-6	0.05 <sup>b</sup>	78.5	5870	0.32	2.71
2-(2-Methoxyethoxy) ethanol	111-77-3	10 800 <sup>c</sup>	<LOD	<LOD	<LOD	<LOD
2-Ethylhexanol	104-76-7	0.02 <sup>b</sup>	<LOD	<LOD	<LOD	<LOD
2-Methyl-4-isothiazolin-3-one	2682-20-4	8.8 <sup>d</sup>	9.51	7.75	0.05	0.08
Acrylamide	79-06-1	4.3 <sup>d</sup>	99.2	119	0.65	0.87
Benzene	71-43-2	0.07 <sup>b</sup>	0.90	332	1.82	4.56
Bisphenol A	80-05-7	670 <sup>c</sup>	<LOD	<LOD	<LOD	<LOD
Bronopol	52-51-7	41.7 <sup>d</sup>	<LOD	<LOD	<LOD	<LOD
Cumene	98-82-8	0.03 <sup>b</sup>	4.21	129	0.01	0.16
Ethylbenzene	100-41-4	0.05 <sup>b</sup>	7.34	1100	0.11	0.98
Ethylene glycol butyl ether	111-76-2	1840 <sup>c</sup>	<LOD	<LOD	77.5	<LOD
N,n-dimethylformamide	68-12-2	96.8 <sup>d</sup>	519	511	35.5	13.4
Naphthalene	91-20-3	0.01 <sup>b</sup>	3.54	265	0.11	0.20
Styrene	100-42-5	0.03 <sup>b</sup>	<LOD	52.0	<LOD	<LOD
Toluene	108-88-3	0.05 <sup>b</sup>	27.3	1410	4.76	11.8
M-xylene + p-xylene		0.04 <sup>b</sup>	40.1	2060	0.73	3.87
O-xylene	95-47-6	0.06 <sup>b</sup>	15.0	396	0.86	2.71

Abbreviations: Aqu, LC-MS, liquid chromatography and mass spectrometry; LOD, limits of detection; Org, organic. Concentrations are in milligrams per liter of select EDCs used in oil and gas operations and examined in this study in three industry WW samples from Garfield County, Colorado.

<sup>a</sup> LOD provided in micrograms per liter concentrations.

<sup>b</sup> LOD determined via SPME and GC-MS analysis described in *Materials and Methods*.

<sup>c</sup> LOD determined via liquid-liquid extraction and GC-MS analysis described in *Materials and Methods*.

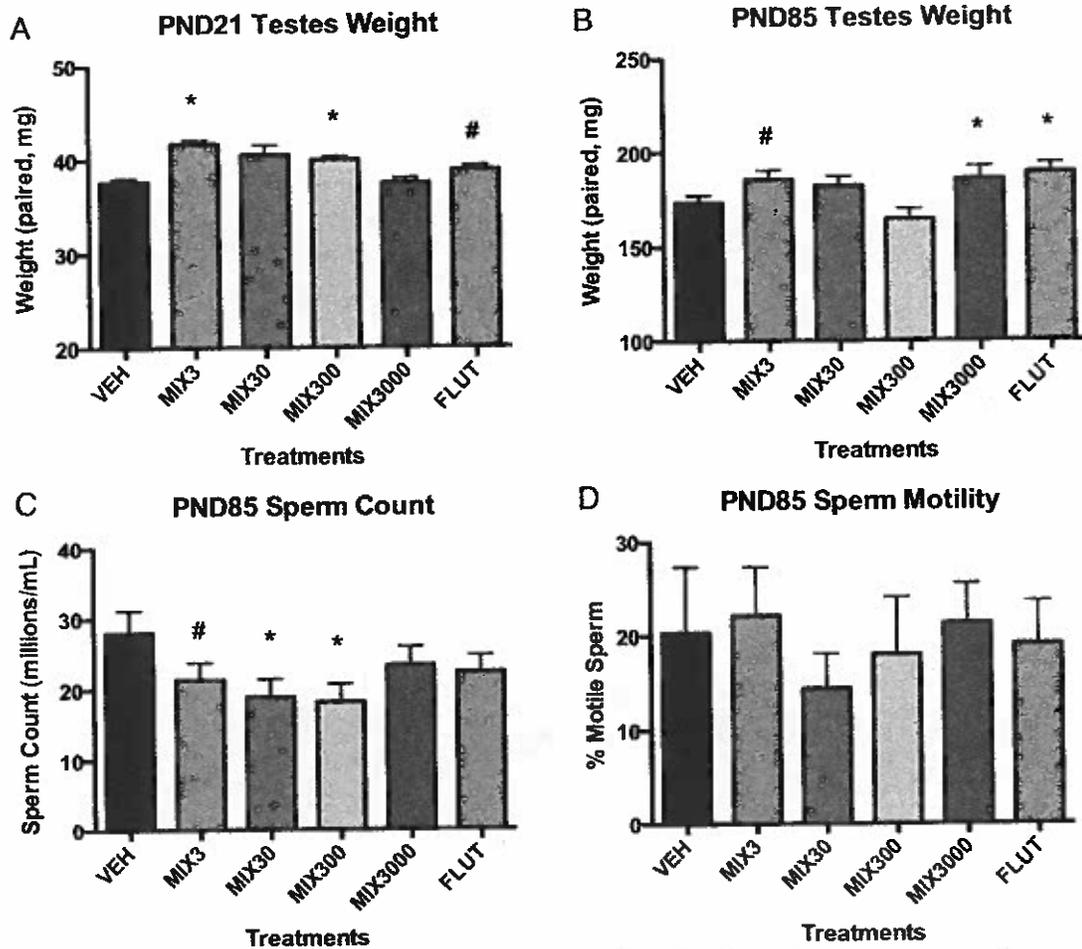
<sup>d</sup> LOD determined via reverse-phase LC-MS analysis described in *Materials and Methods*.

drinking water. Mice exhibited increased testis weights at PND21 and PND85 as well as decreased sperm count at PND85, indicating decreased spermatogenesis efficiency by testicular tissue (Figure 4). Increased testis weights and a trend for decreased sperm count were also observed in the flutamide control, although in utero exposure to flutamide is generally associated with decreased testis weights in rats (58, 59) and is also observed in the androgen receptor knockout mouse (60). It is possible that our one dose of flutamide did not show antagonist effects for this end point, although it did act to fully retain nipples. Increased testis weights and decreased sperm counts and/or impacted spermatogenesis are also observed in cases of prenatal hypothyroidism (61–64), often via in utero exposure to propylthiouracil or other thyroid antagonists (65, 66). However, adult mice did not exhibit altered thyroid-regulated gene expression in the liver that we might expect if the mixture were acting through a thyroid mechanism in vivo. Importantly, this does not preclude other potential thyroid programming effects. These outcomes have also been observed in the estrogen receptor knockout mouse (67). In this case, estrogen inhibition is suspected to lead to the accumulation of testicular secretions in the lumen of the seminiferous tubule, transiently increasing testicular weight prior to the long-term atrophy of testes due to the backpressure of the luminal fluids (67). Because antiestrogenic activity was the most prevalent activity observed in vitro, further targeted investigation

should assess whether the supplementation of low-dose agonists can reverse this effect. Reproductive or developmental effects have been previously reported for several of the 23 oil and gas chemicals used in our in vivo experiment at high doses typical of occupational exposure, as reviewed previously (2).

Furthermore, more than 300% and 500% increased concentrations in serum T were noted in the Mix30 and Mix300 groups, respectively (Figure 5). Yoshida et al (68) reported increased testes weights, decreased sperm counts, and increased serum T (on the scale reported herein) in male mice after in utero exposure to diesel exhaust. Diesel exhaust is a complex chemical mixture that contains, among others, many of the chemicals we have tested herein: benzene, toluene, ethylbenzene, and xylenes, trimethylbenzene, naphthalene, phenol, styrene, and others (reviewed in reference 69). Significant enlargement was also observed in accessory male reproductive organs, which were not assessed in this study. We suggest that future studies assess the complete male reproductive system and potential mechanisms for increased T (ie, LH, aromatase, etc) to identify the mechanism(s) of action for the observed effects.

Increased birth weights in Colorado children have been associated with maternal residence proximity to natural gas development during pregnancy (33), although a study in the Marcellus Shale region reported the opposite trend (70). We observed increased body weights at PND21 for



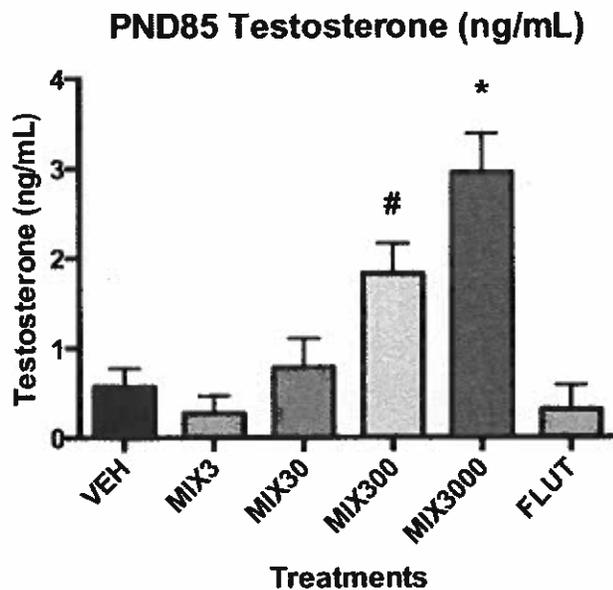
**Figure 4.** Testes weights and sperm assessments in developmentally exposed mice. Estimated marginal means  $\pm$  SEM of paired testes weights for developmentally exposed C57BL/6J mice collected at both PND21 (A) and PND85 (B). Sperm quantity assessment was performed at PND85 (C), provided in millions of cauda sperm per milliliter and sperm motility performed at PND85 (D) defined as percentage motile sperm using a motile/nonmotile assessment method described in the Supplemental Information. \*, Different from untreated controls (vehicle) alone at  $P \leq .05$ ; #, different from untreated controls (vehicle) alone at  $0.05 < P \leq .10$  ( $n = 11, 10, 10, 11, 10, 10$  litters for vehicle, Mix3, 3Mix0, Mix300, Mix3000, and flutamide, respectively). FLUT, flutamide; VEH, vehicle.

Mix300 (Figure 6A) and a tendency for increased body weights at PND7 and PND13 for Mix3 and Mix300 (Supplemental Information and Supplemental Table 2). There is some evidence that several of the chemicals tested herein are potential obesogens and can promote adipogenesis, result in increased body weights in animal studies, or have been associated with obesity in epidemiological studies (71). Specifically, nonylphenol and octylphenol have been shown to promote adipogenesis both in vitro and in vivo (72, 73), and naphthalene has been associated with increased rates of obesity in children (74).

We observed increased heart weights for Mix3 and a trend for increase in Mix300 ( $P < .10$ ; Figure 6B). We also report increased cardiac myocyte size at PND85 (Figure 6D), suggesting that despite the apparent transient effect on heart weight, architecture of the heart was still impacted in adulthood. Both increased heart weights and

increased cardiac myocyte sizes can be indicative of cardiac hypertrophy (75–77), which has been previously associated with exposure to glucocorticoids (78), androgens (79), estrogens, and progestogens (80). Hypertrophy has not specifically been assessed in humans near unconventional oil and gas development, although structural heart defects have been associated with maternal proximity to natural gas development during pregnancy (33). These structural defects are also associated with gestational exposure to EDCs and bioactive polycyclic aromatic hydrocarbons, reinforcing a potential endocrine-mediated mechanism of action (34, 35, 81). Taken together, adverse cardiac outcomes are a likely outcome after exposure to these chemicals, although identifying the mechanism requires further investigation.

A trend for increased splenic weights in Mix3 and Mix300 was noted at PND21, an end point that has been



**Figure 5.** Body and organ outcomes in developmentally exposed mice. Estimated marginal means  $\pm$  SEM of body weights (A), thymus weights (B), and heart weights (C) for developmentally exposed C57BL/6J mice collected at PND21. Estimated marginal means  $\pm$  SEM of cardiac myocyte diameters (micromoles) for developmentally exposed C57BL/6J mice collected at PND85 (D). \*, Different from untreated controls (vehicle) alone at  $P \leq .05$ ; #, different from untreated controls (vehicle) alone at  $0.05 < P \leq .10$  ( $n = 11, 10, 10, 11, 10, 10$  litters for vehicle, Mix3, 3Mix0, Mix300, Mix3000, and flutamide, respectively) FLUT, flutamide; VEH, vehicle.

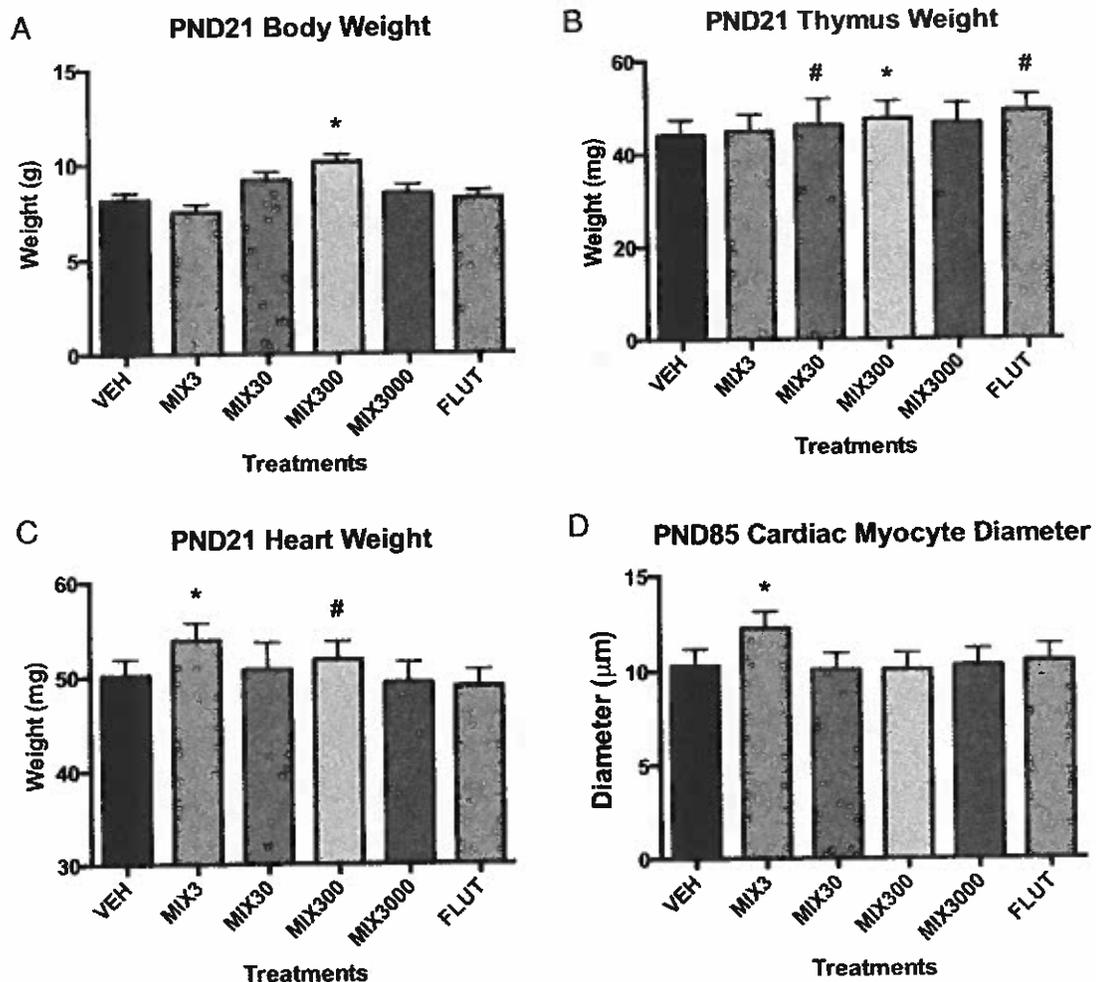
shown to result from androgen deprivation in C57BL/6 mice (82, 83). Given the antiandrogenic mechanism of action for *in vitro* results in both uterine and liver cell lines as well as many of the *in vivo* outcomes presented herein, this is a potential causative mechanism for the observed trend.

Consequences of exposure to multiple EDCs simultaneously are not well understood. Many of these chemicals have the potential to act additively through a common biological pathway, as has been described *in vitro* and *in vivo* for the estrogen and androgen receptors (84–86). Due to these combinatory receptor interactions, effects can be observed, even when each chemical in the mixture is present at levels below an observed effect threshold (85, 87). The indirect interactions between chemicals and receptors further complicate the issue, such as the modulation of cytochrome P450 enzymes. It is well understood that P450 expression can increase or decrease exposure to pharmaceuticals by modulating metabolism or by producing active metabolites of parent compounds (88–90). Our analysis of these 24 hydraulic fracturing chemicals in HepG-2 cells, a metabolically active liver cell line, showed similar results but some individual chemicals showed altered receptor activities relative to our Ishikawa cell results (Supplemental Figure 2), demonstrating this effect. Future

work should assess expression of key metabolic genes to assess the likely contribution of this to the observed effects.

Our results support the idea that mixture interactions are complex and difficult to predict. When removing an estrogen agonist from the 24-mix (BPA), the response was synergistic rather than the anticipated additive (23-mix, Table 2). This apparent synergistic behavior may be due to induction of metabolic enzymes and subsequent alteration in the ratios of agonists to antagonists in the cells and at the level of the receptors. However, removing a nonactive thyroid receptor compound from the 24-mix (BPA) resulted in an apparent synergistic response for the resulting mixture as well (23-mix, Table 3). The increased potency and efficacy for this mixture was not expected and may be due to unknown indirect interactions of the chemicals with the receptor. Alternatively, BPA may be altering serum binding and/or cellular transport of individual hormones, resulting in these disparate effects. Most our chemical mixtures displayed less than additive activity, despite greater potencies than individual mixture components. These mixture potencies correlated well with the presence of agonists; less potent responses were observed for mixtures when partial or weak agonists were present. This suggests the utility of isobolographic mixture assessments: the degree of interaction is not readily determined through relative potencies alone. Many of the chemicals that we evaluated, despite exhibiting more potent activities than the component chemicals, were actually displaying less than additive behavior. Without a defined method to calculate expected behavior and thus deviations from it, one may assume additive interactions when other than additive interactions are present.

Despite knowledge gaps addressed in this study, more research is needed to assess the many additional chemicals used in and produced by this process, better characterize environmental concentrations, and assess reproductive and other health outcomes via other exposure routes and during varying windows of development. Humans and animals may be exposed to the chemicals examined herein from oil and natural gas operations and/or from other personal and/or industrial sources (Table 1). Also, inhalation and dermal absorption are potential additional routes of exposure to these chemicals and were not examined in this study. A comprehensive understanding of routes of exposure is critical to assessing bioactivity, and the current understanding of adverse health effects due to oil and gas operations is limited. Analytical limitations prevented complete characterization of all 23 *in vivo*-tested chemicals in the Colorado WW samples, limiting knowledge on realistic environmental concentrations for some constituents. Whereas this study is an overview of potential adverse reproductive and developmental effects



**Figure 6.** Body and organ outcomes in developmentally exposed mice. Estimated marginal means  $\pm$  SEM of body weights (A), thymus weights (B), and heart weights (C) for developmentally exposed C57BL/6J mice collected at PND21. Estimated marginal means  $\pm$  SEM of cardiac myocyte diameters (micrometers) for developmentally exposed C57BL/6J mice collected at PND85 (D). \*, Different from untreated controls (vehicle) alone at  $P \leq .05$ ; #, different from untreated controls (vehicle) alone at  $0.05 < P \leq .10$  ( $n = 11, 10, 10, 11, 10, 10$  litters for vehicle, Mix3, Mix30, Mix300, Mix3000, and flutamide, respectively). FLUT, flutamide; VEH, vehicle.

from disruption of several receptor systems, more targeted assessments are necessary in many cases to determine which receptor pathway disruption resulted in which outcomes. As noted, adverse outcomes were noted at varying concentrations both between end points and within individual dose responses. Further work is needed to characterize whether disruption of multiple receptor systems at varying concentrations has resulted in the dose-response curves observed in this study.

In conclusion, we report for the first time that chemicals used in and produced by oil and gas operations can act as EDCs both in vitro and in vivo. We report adverse health outcomes in C57BL/6J male mice exposed during gestation to a mixture of these chemicals at concentrations that may be found in impacted drinking water. We further report mechanistic information on the interactions of these chemicals with the estrogen, androgen, glucocorti-

coid, P4, and thyroid receptors in a human endometrial cancer reporter gene assay system. Our results suggest adverse health outcomes that may be observed in humans and animals in areas impacted by extraction operations and provide mechanistic evidence for these effects. Further work should examine these end points in both laboratory experiments and epidemiological studies to better characterize the complex mixtures of EDCs used in and produced by oil and natural gas operations.

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