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Developmental exposure to a mixture of unconventional oil and gas chemicals: A review of experimental effects on adult health, behavior, and disease

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ABSTRACT

Unconventional oil and natural gas extraction (UOG) combines directional drilling and hydraulic fracturing and produces billions of liters of wastewater per year. Herein, we review experimental studies that evaluated the potential endocrine-mediated health impacts of exposure to a mixture of 23 UOG chemicals commonly found in wastewater. The purpose of this manuscript is to synthesize and summarize a body of work using the same UOG-mix but with different model systems and physiological endpoints in multiple experiments. The studies reviewed were conducted in laboratory animals (mice or tadpoles) and human tissue culture cells. A key feature of the *in vivo* studies was the use of four environmentally relevant doses spanning three orders of magnitude ranging from concentrations found in surface and ground water in UOG dense areas to concentrations found in UOG wastewater. This UOG-mix exhibited potent antagonist activity for the estrogen, androgen, glucocorticoid, progesterone, and thyroid receptors in human tissue culture cells. Subsequently, pregnant mice were administered the UOG-mix in drinking water and offspring were examined in adulthood or to tadpoles. Developmental exposure profoundly impacted pituitary hormone concentrations, reduced sperm counts, altered folliculogenesis, and increased mammary gland ductal density and preneoplastic lesions in mice. It also altered energy expenditure, exploratory and risk-taking behavior, the immune system in three immune models in mice, and affected basal and antiviral immunity in frogs. These findings highlight the diverse systems affected by developmental EDC exposure and the need to examine human and animal health in UOG regions.

1. Introduction

Unconventional oil and gas extraction (UOG) combines directional drilling and hydraulic fracturing or "fracking." Directional drilling differs from conventional vertical drilling using diagonal or horizontal drilling relative to the earth's surface. This is followed by fracking, where millions of gallons of water and thousands of gallons of chemicals are injected under high pressure to release previously inaccessible oil and gas. The use of these methods has resulted in significantly more domestic natural gas production since 2000 (Energy Information Administration, 2016). However, oil and gas extraction results in over two billion gallons of wastewater per day in the USA alone, and the wastewater contains numerous chemicals, some of which have endocrine disrupting activity (Colborn et al., 2011; Elliott et al., 2017,USEPA). Spills and leaks have been reported to occur at 31% of UOG wells over a ten-year period, and spills are a common route of surface and ground water contamination (Maloney et al., 2017). Contamination of water and air has been shown to occur at every step of the process (Burton et al., 2014,Colborn T and Herrick, 2014,Czolowski et al., 2017; Ingraffea et al., 2014; Kassotis et al., 2016c; Mauter et al., 2014; Rozell and Reaven, 2012; Vengosh et al., 2014). Across the industry > 1000 chemicals are used, and many can disrupt the endocrine system, reproduction and/or development (Colborn et al., 2011; Elliott et al., 2017). Elliott et al. (2017) performed a systematic evaluation of

Abbreviations: UOG, Unconventional oil and natural gas; UOG-mix, Mixture of 23 common UOG chemicals

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UOG chemicals in the ReproTox database. Out of 1,021 chemicals identified in hydraulic fracturing fluids and/or wastewater, only 240 chemicals had toxicity information available. Of these 240, > 40% were reproductive and/or developmental toxicants (Elliott et al., 2017). Thus, while there are economic benefits of UOG, it also releases chemicals into the environment that have the potential to alter reproduction, development and adult health and disease.

Over 17 million Americans live within one mile of a UOG well, and residential and occupational exposure to UOG chemicals has been associated with negative health impacts (Rabinowitz et al., 2015; Steinzor et al., 2013; Werner et al., 2015). Hormones in the hypothalamic, pituitary and gonadal axis control human reproduction and development, thus disruption by endocrine disrupting chemicals (EDCs) can alter development, reduce fertility, and contribute to changes in health (Chiang et al., 2017; Gore et al., 2015; Rattan et al., 2017). We previously performed a systematic review of the human literature and found evidence that exposure to oil and gas industry activities is associated with increased risk of preterm birth, miscarriage, prostate cancer, birth defects, and decreased semen quality (Balise et al., 2016). Three studies have recently found maternal residential exposure to UOG was associated with an increased risk of preterm birth and/or low birth weight (Casey et al., 2016; Hill, 2018; Stacy et al., 2015). McKenzie et al. (2014) found maternal residential proximity to natural gas wells within a 10-mile radius was associated with congenital heart defects [high vs low exposure tertile: OR = 1.3] and neural tube defects [OR = 2.0] (McKenzie et al., 2014). In addition, two studies have found an increased risk of miscarriage in women either residentially or occupational exposed to oil and gas industry activities (OR = 2.4 and OR = 2.7, respectively) (Sebastian et al., 2002; Xu et al., 1998).

In this review, we have assembled and discuss our published work to begin to dissect potential underlying endocrine-mediated mechanisms for UOG-related health effects. The research performed in our laboratories to evaluate the endocrine activity associated with UOG operations has comprised three main areas. The first phase of our research evaluated the potential endocrine disrupting activity of commonly used UOG chemicals and mixtures with *in vitro* bioassays. We found that 23 of 24 individual UOG chemicals could activate or inhibit one or more of the estrogen (ER), androgen (AR), progesterone (PR), glucocorticoid (GR) and/or thyroid hormone (TR) receptors in a human cell-based reporter gene assay (Tables 1 and 2) (Kassotis et al., 2014; Webb et al., 2014).

The second phase consisted of evaluating surface, ground, and municipal water near UOG operations for the presence of endocrine bioactivities to evaluate potential contamination and exposure pathways for humans and animals living in these regions. We found fracturing fluid spills and disposal were associated with elevated endocrine disrupting activity in natural waters, particularly antagonism for ER, AR, PR, TR and GR (Kassotis et al., 2014, 2016b).

The third phase evaluated the health of mice exposed during gestation to an environmentally relevant mixture of commonly used UOG chemicals. We found that developmental exposure to a mixture of 23 UOG chemicals via maternal drinking water altered adult health. In females, prenatal exposure altered folliculogenesis, suppressed pituitary hormones [prolactin (PRL), luteinizing hormone (LH), and follicle stimulating hormone (FSH)], altered body and organ weights, altered mammary gland ductal epithelium and increased preneoplastic lesions, and disrupted the immune system's response to infection in adulthood (Boule et al., 2018; Kassotis et al., 2015, 2016a; Sapouckey et al., 2018). In males, prenatal exposure altered body weights, serum testosterone, and reduced epidydimal sperm production (Kassotis et al., 2015). Further, combined prenatal and postnatal exposure altered activity and energy expenditure in adult females (Balise et al. submitted). These studies are described below in more detail.

Table 1
EDC activity of 23 UOG chemicals [a].

Receptor	Agonist	Antagonist
Estrogen	**0**	**21**
Androgen	**0**	**20**
Progesterone	**1**	**11**
Glucocorticoid	**0**	**9**
Thyroid	**2**	**6**

[a] Number of chemicals active for each type

2. Endocrine disrupting potential of UOG chemicals *in vitro* and selection of chemicals for mixture

Our initial research focused on measuring the endocrine activities associated with common-use UOG chemicals. We selected 24 chemicals based on 1) hydraulic fracturing chemicals reported to the United States Congress by the UOG industry (Waxman et al., 2011), 2) published data compiled by the Endocrine Disruption Exchange (Colborn et al., 2014; Exchange, 2018) on the chemical's potential to interfere with some aspect of hormone action, and 3) UOG chemicals self-reported by industry to be used in multiple chemical products in Colorado, as described previously (Kassotis et al., 2014, 2015, 2016a,b,c).

To determine the UOG chemicals ability to interact with hormone receptors, we utilized transient transfection assays with human receptors and luciferase reporter linked hormone response elements inserted into human endometrial cells to examine the potential agonist and/or antagonist activities for ER, AR, PR, GR, and TR, respectively. For antagonist assays, the fixed concentration of positive control hormone was chosen as the EC50 of the positive control hormone in each assay. This approach allows for a sensitive measurement of antagonist activity and more closely resembles physiologically active concentrations in the dynamic range of the dose response curve as opposed to saturating concentrations that are rarely seen in living organisms. This analysis revealed that 21, 21, 12, 10, and 7 chemicals, respectively, antagonized these receptors, and 1, 0, 1, 0, and 2 chemicals, respectively, acted as agonists [Tables 1 and 2 (Kassotis et al., 2014, 2015),]. Additional research has followed our investigation that assessed agonism and antagonism of ER, AR, PR, GR, and the peroxisome proliferator activated receptor gamma, PPARγ and reported receptor antagonism for some UOG chemicals, with some chemical overlap (Bain and Kumar, 2018).

UOG chemicals are used in complex mixtures and found as mixtures in impacted natural water, so we assessed mixtures of UOG chemicals for EDC activity. We chose equal concentrations of each chemical for the mixture as there is no one ratio of the selected 23 chemicals found in wastewater. Each company uses different chemicals and concentrations depending on their protocols and the geography, and each geography contributes its own unique set of natural contaminants to the overall mixture. Since it is impossible to select one mix that represents contaminated water, we chose to create an unbiased approach with each chemical present at the same concentration. The UOG-mix contained potent antagonism for ER > PR > TR > AR > GR (Fig. 1). A combination of 23 UOG chemicals (UOG-mix) at equi-molar concentrations, was compared to anticipated additive outcomes using methods described previously [59]. It is generally anticipated that for chemicals operating through the same mechanism of action, combinations at concentrations that individually produce no observable effects may produce a measurable effect when combined (Silva et al., 2002). In this case, the 23-mix generally acted additively for the AR, PR, and GR, but appeared to act synergistically (greater than expected additive response) for the ER and TR (Kassotis et al., 2015), illustrating the complexity of assessing environmental mixtures. Taken together, these studies demonstrate that UOG chemicals are capable of disrupting hormone receptor activity (either directly or indirectly) and that combinations can result in greater than anticipated responses. However, given the heterogeneous chemical complexity of UOG chemical mixtures, further research needs to be performed on additional chemicals

Table 2
Efficacy and potency of individual UOG chemicals for hormone receptors.

Chemical	% AE max	AE EC_{10} (μM)	% AA max	AA EC_{10} (μM)	% AP max	AP EC_{10} (μM)	% AG max	AG EC_{10} (μM)	% AT max	AT EC_{10} (μM)
Ethylene glycol butyl ether	84%	0.1	52%	0.6	0%	N/A	0%	N/A	20%	8
2-Ethyl-1-hexanol	62%	0.2	38%	1	50%	10	41%	2.8	0%	N/A
Naphthalene	39%	2.7	37%	2.5	35%	20	48%	0.4	40%	1.7
Ethylene glycol	59%	0.2	67%	0.4	23%	30	37%	5	37%	1.5
Ethoxylated nonylphenol	73%	0.2	29%	3	100%	0.2	54%	0.4	37%	1.1
Diethanolamine	77%	0.3	54%	2.3	19%	35	0%	N/A	0%	N/A
Sodium tetraborade decahydrate	56%	0.3	21%	5	0%	N/A	0%	N/A	0%	N/A
2-(2-methoxyethoxy)ethanol	47%	0.1	49%	2.5	0%	N/A	39%	4	0%	N/A
N,N-Dimethylformamide	44%	0.3	28%	4	18%	40	0%	N/A	0%	N/A
Cumene	43%	0.9	38%	0.6	25%	30	0%	N/A	0%	N/A
Bronopol	34%	2.3	5%	N/A	0%	**30**	31%	0.3	0%	N/A
Styrene	66%	0.3	0%	N/A	0%	N/A	24%	4.5	24%	4.5
Propylene glycol	27%	27.5	0%	N/A	0%	N/A	0%	N/A	0%	N/A
Acrylamide	0%	N/A	11%	77.5	0%	N/A	0%	N/A	0%	N/A
Triethylene glyol	20%	36.6	19%	32.5	0%	N/A	0%	N/A	0%	N/A
Methyl-4-isothiazolin	13%	7.5	0%	N/A	0%	N/A	0%	**3**	0%	N/A
Ethoxylated octylphenol	74%	0.1	39%	1.3	90%	1.5	25%	3.5	29%	1.8
Phenol	26%	15	18%	31	16%	50	0%	N/A	0%	N/A
Toluene	8%	N/A	10%	N/A	0%	N/A	0%	N/A	0%	N/A
Xylenes	33%	4.5	13%	63.3	38%	21	0%	N/A	0%	N/A
Benzene	20%	9	33%	6	0%	N/A	0%	N/A	0%	N/A
Ethylbenzene	14%	55	30%	15	0%	N/A	0%	N/A	0%	N/A
1,2,4-Trimethylbenzene	0%	N/A	0%	N/A	0%	N/A	0%	N/A	0%	N/A
23-mix	**55%**	**0.13**	**25%**	**0.3**	**45%**	**0.15**	**23%**	**0.28**	**25%**	**0.23**

% inhibition of positive control hormone.
AE = percent inhibition of estradiol.
AA = percent inhibition of DHT.
AP = percent inhibition of progesterone.
AG = percent inhibition of dexamethasone.
AT = percent inhibition of triiodothyronine.

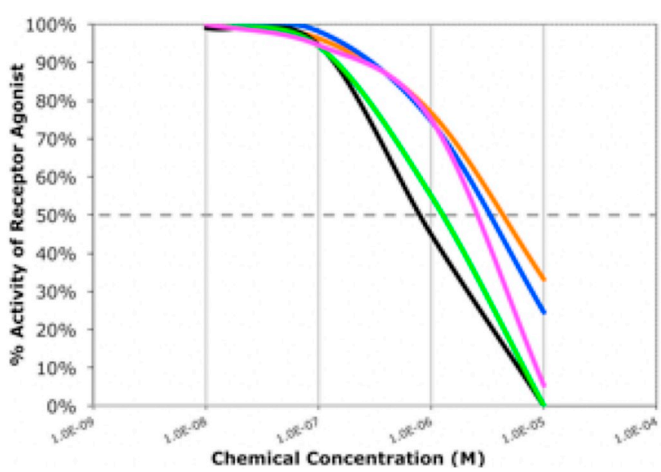

Fig. 1. Receptor antagonist activities of laboratory-created mixture of 23 UOG chemicals for anti-ER (black), anti-AR (blue), anti-PR (green), anti-TR (purple), and anti-GR (orange) activities in Ishikawa cells. Antagonist activities presented as the percent suppression of 20 pM E2, 300 pM DHT, 100 pM P4, 2 nM T3, 50 pM DEX for anti-ER, anti-AR, anti-PR, anti-TR, and anti-GR.

and mixtures to better elucidate additive, synergistic, and antagonistic (lower than expected additivity) responses to better understand these interactions.

3. Endocrine disrupting activities of surface and ground water near UOG operations

The second phase of this research evaluated the potential endocrine activities in surface and groundwater near UOG operations. Our initial investigation focused on a drilling-dense region of Garfield County, Colorado—a county with over 11,000 oil and gas wells. In this initial study, we assessed surface and groundwater near UOG spills (Kassotis et al., 2014), to evaluate the potential for UOG-related impacts in areas of greatest concern. In this study, the water around drilling-dense spill sites exhibited greater estrogenic, anti-estrogenic, and anti-androgenic activities relative to nearby reference sites, suggesting a potential role of UOG operations on water-associated EDC activities. We next evaluated the impact of an injection wastewater disposal facility on EDC activity in surface water in West Virginia. We found greater ER, AR, GR, PR, and TR antagonism at the site of and downstream from the wastewater injection disposal well [Fig. 2 (Kassotis et al., 2016b)] than water samples collected upstream and in a reference stream nearby, which contained minimal antagonism (and some agonism). Collaborative work with the US Geological Survey confirmed contamination by UOG wastewater being stored and disposed of at the site (Akob et al., 2016; Orem et al., 2017), more specifically linking altered endocrine activities with UOG operations. In another collaboration with the USGS in the Williston Basin in North Dakota, we reported geochemical alterations in a creek following a large UOG fluid spill (Cozzarelli et al., 2017). These tracers co-occurred with increased concentrations of UOG-associated organic contaminants and an increased anti-estrogenic activity in water downstream from the spill site (Cozzarelli et al., 2017). This series of research studies demonstrates a role for UOG operations impacting surface and ground water sources following spills/unintentional releases related to these operations.

Next, we assessed the potential for more widespread effects, less dependent on defined/reported spills. To address this, a small-scale project was undertaken in Wyoming to examine potential UOG-specific endocrine bioactivities in groundwater, relative to reference sites and sites with a history of conventional oil and gas (COG) operations (Kassotis et al., 2018c). We found greater ER antagonist activities in groundwater near UOG and conventional oil operations relative to reference sites and areas with conventional natural gas production. It also appeared that PR antagonist activities were greater in the UOG region relative to all others, suggesting a UOG-specific association, though this assessment was limited by the small scale of the study (Kassotis et al., 2018a,b,c) and should be substantiated further in future research.

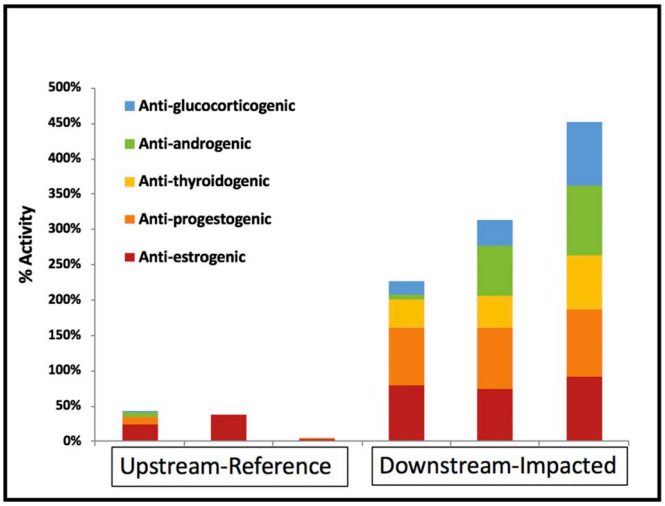

Fig. 2. Combined receptor antagonist activities of 4X surface water samples associated with UOG wastewater injection well. Combined total antagonist activities as percent suppression of half maximal positive control response for each receptor. Water samples labeled "upstream" were surface water samples collected upstream or in a reference stream. Water samples labeled "downstream" were either surface water samples collected on the site or downstream of the injection well.

A recent follow-up from our group evaluated the activation of PPARγ and promotion of adipogenesis (in 3T3-L1 cells, a mouse pre-adipocyte model) by surface water known to be impacted by UOG fluids in WV and CO (Kassotis et al., 2018b). The most-impacted samples and wastewater exhibited the greatest degree of adipogenic activities (via both increased triglyceride accumulation, a marker for adipocyte differentiation, and increased pre-adipocyte proliferation), suggesting chemicals present in UOG wastewaters could disrupt the development of fat cells (Kassotis et al., 2018a). While PPARγ activation occurred at equivalent concentrations to those that promoted adipogenic responses in some environmental samples, others that exhibited adipogenic activities lacked PPARγ activity, suggesting a differing molecular mechanism promoting the effects in these samples. Interestingly, a concurrent investigation of nonionic ethoxylated surfactants, reported at high levels in UOG wastewater (Ferrer and Thurman, 2015; Getzinger, O'Connor, Hoelzer et al., 2015; Thurman et al., 2014), promoted potent and efficacious adipogenic effects that were independent of PPARγ activation (Kassotis et al., 2018a,b,c), suggesting a potential causative factor for these effects.

4. Body and organ weights and reproductive parameters in prepubertal and adult mice following prenatal exposure to a UOG chemical mixture

The third phase of our initial research evaluated the potential developmental and reproductive health effects following prenatal exposure to a mixture of 23 common UOG chemicals (UOG-mix). C57Bl/6 mice were mated and pregnant dams were exposed via their drinking water from gestational day 11 through birth to the UOG-mix. Chemicals were tested at concentrations deemed environmentally relevant. The two highest concentrations were defined by levels reported in wastewater samples (1 and 10 mg/L) (Agency, 2015,Akob et al., 2015; Cozzarelli et al., 2017; He et al., 2017; Kassotis et al., 2015; Orem et al., 2014; Rosenblum et al., 2017; Thurman et al., 2014). The two lowest concentrations (10 and 100 μg/L) have been reported in surface, ground, and drinking water in UOG production regions (Agency, 2015,Cozzarelli et al., 2017; DiGiulio et al., 2011; DiGiulio and Jackson, 2016a,b; DiGiulio and Jackson, 2016a,b; Gross et al., 2013; Orem et al., 2017) (Drollette et al., 2015; Hildenbrand et al., 2015).

Table 3
Effects in mice after developmental exposure to UOG chemicals.

Concentration in water (μg/ml)	0.01	0.1	1	10
Impact of prenatal exposure to UOG mixture in prepubertal and adult mice				
Dose (μg/kg bw)	**3**	**30**	**300**	**3000**
Males (3 weeks)				
Body weight	/	/	increased	/
Testes weight	increased	/	increased	/
Thymus weight	/	increased^	increased	/
Heart weight	increased	/	increased^	/
Females (3 weeks)				
Altered folliculogenesis	/	Decreased[a]	Increased[b]	/
Body weight	increased	/	increased	/
Uterine weight	decreased	/	/	increased^
Ovary weight	increased^	/	decreased^	/
Heart weight	increased^	/	increased^	/
Mammary gland size	/	/	/	/
Males (3 months)				
Body weight	increased	increased	increased^	/
Testes weight	increased^	/	/	increased
Sperm count	decreased^	decreased	decreased	/
Serum testosterone	/	/	increased^	increased
Cardiomyocyte diameter	increased	/	/	/
Females (3 months)				
Serum prolactin	decreased	decreased	decreased	decreased
Serum FSH, LH	decreased	decreased	increased	decreased
Serum GH, TSH	/	/	increased	/
Altered folliculogenesis	Increased[c]	Increased[d]	/	Increased[e]
Heart collagen deposition	/	increased	/	/
Mammary gland:				
Epithelial volume	/	/	increased	increased
Ductal volume	/	increased^	increased	increased^
Epithelial cell proliferation	increased			/
Intraductal hyperplasias[f]	observed	observed	observed	observed
Energy expenditure, activity and behavior following prenatal and postnatal exposure to a mixture of UOG chemicals in female mice				
Dose (μg/kg body weight)**	**1.5**	**15**	**150**	**1500**
Females (7 months)				
Total energy expenditure	decreased	/	decreased	/
Resting energy expenditure	decreased	/	decreased	/
Females (12 months)				
Non-resting energy expenditure	increased	increased	increased	increased
Spontaneous activity	increased	increased	increased	increased
Time in open arms-Elevated Plus Maze	increased	increased	increased	/

Dose = μg/kg maternal body weight per day.
Endpoints noted as increased, decreased or observed were different ($p < 0.05$) from control.
/- not different from vehicle (0.2% ethanol) control.
^ $p < 0.1$.
Did not assess this endpoint at this concentration.
**The same concentration in water was used to expose pregnant mice; the dams were older and heavier so the dose was lower per kg body weight.
[a] Decreased primordial and primary follicles
[b] Increased antral, decreased secondary follicles
[c] Increased primordial, primary, and atretic follicles
[d] Increased secondary follicles
[e] Increased primary follicles
[f] Intraductal hyperplasia not observed in vehicle control mice

Gestationally exposed offspring were then evaluated from birth and into adulthood to assess potential impacts of exposure to the UOG chemicals across multiple endpoints and developmental stages.

Male offspring were evaluated throughout development for body weight; anogenital distance, sex, and nipple retention early in life; pubertal development; and organ weights, serum hormone concentrations, reproductive health, and other endpoints in early adulthood (Table 3). Mice exhibited increased testis weights at PND21 and 85, decreased epididymal sperm counts at PND85, and dramatically increased serum testosterone levels at PND85 (Kassotis et al., 2015). Certain exposure groups exhibited increased body weights at PND21, though they were not different by PND85, suggesting a possible transient effect. Heart weights were also increased in the lowest exposure group at PND85, and further analysis revealed increased cardiomyocyte diameter in these mice, suggesting persistent effects on cardiac architecture. All exposure groups tended to exhibit shorter anogenital distance ($p < 0.10$), and many of these outcomes were mirrored in the anti-androgenic flutamide control. No apparent differences were observed in litter size, sex ratio, nipple retention, sperm morphology or motility, age of preputial separation, or thyroid-regulated hepatic gene expression in any of the UOG exposure groups.

Female offspring were also evaluated throughout development for body and organ weights, anogenital distance, pituitary and reproductive hormone concentrations (always during estrus), heart architecture, and ovarian follicle development (at PND21 and 85). Mice exposed to the lowest and highest doses had a 50–70% reduction of serum prolactin, follicle stimulating hormone (FSH), and luteinizing hormone (LH) across almost all UOG exposure groups [Fig. 3 (Kassotis et al., 2016a,b,c),]. Notably, the female offspring of dams exposed to the middle dose group (1 mg/L UOG-mix) did not exhibit suppressed FSH and LH, but instead exhibited increased growth hormone and thyroid stimulating hormone (TSH) concentrations. Ovarian follicle development appeared to be accelerated at PND21 and 85, with higher proportions of later developmental stage follicles and a higher rate of follicle atresia at PND85 (Kassotis et al., 2016a,b,c). Exposure to the UOG-mix also resulted in non-monotonic dose response curves for both uterine and ovary weights (greater ovary weights at lower levels and lower at higher levels, and vice versa for uterine weights). Increased heart weights were also noted at both PND21 and 85. Further analysis of hearts revealed that cardiomyocyte size was not different between groups, though significantly increased collagen deposition was noted in several treatment groups by PND85 (Kassotis et al., 2016a,b,c). No differences were observed in serum estradiol concentrations, age of vaginal opening, or age of first vaginal estrus between treatment groups. These preliminary effects suggested potential reproductive and metabolic health effects, which our laboratory investigated further through more targeted studies described below.

5. Mammary gland development and morphology following prenatal exposure to a mixture of UOG chemicals

The mammary gland has proven to be an excellent model to evaluate the effects of single chemicals with one main mode of action (e.g., the ER agonist diethylstilbestrol) or single chemicals with several modes of action (e.g., bisphenol A, which is an ER agonist, AR antagonist, thyroid hormone antagonist, and activator of aryl hydrocarbon receptor [AhR], among other activities) (Macon and Fenton, 2013). Because mammary development, function (e.g., lactation), and disease (e.g., breast cancer) can be influenced by exposures to EDCs at different life stages, the outcomes that can be evaluated in this tissue are complex. *In utero* production of endogenous hormones is responsible for the striking sexual dimorphism observed in the rodent mammary gland. Production of androgens in the fetal testes induces a detachment of the mammary epithelial stalk from the overlying skin, producing male rodents that lack nipples (Kratochwil and Schwartz, 1976). This AR-dependent sexual dimorphism of the mammary gland produces a clear outcome by which androgenic and anti-androgenic chemicals can be evaluated: exposure to androgenic chemicals in female rodents during embryonic development will result in loss of nipples and less-developed underlying mammary epithelium (Kratochwil and Schwartz, 1976). In contrast, exposure to anti-androgenic chemicals in male rodents during embryonic development will result in nipple retention and more-developed underlying mammary epithelium (Hass et al., 2007).

Although ER is expressed in the embryonic mammary mesenchyme starting at approximately embryonic day 12, estrogen is not required for the early development of the female mammary gland (Bocchinfuso and Korach, 1997). Yet, numerous studies have demonstrated that in utero exposure to ER agonists can alter the development of the mammary gland; although these alterations appear subtle during early development, the long-term consequence of developmental estrogen exposures can be severe (Soto et al., 2008). The effects of developmental exposures to ER antagonists are less clear; gestational exposures to tamoxifen increased female rats' responses to mammary carcinogens (Halakivi-Clarke et al., 2000). Although tamoxifen is used as an anti-estrogenic treatment for breast cancer in women, it is a selective estrogen receptor modulator, and can also act as an ER agonist, making it difficult to differentiate the mechanism for observed effects after fetal exposures.

A small number of studies have examined the effects of chemical mixtures on the developing mammary gland (Schwarzman et al., 2015). Most of these studies examine mixtures of only 2–5 chemicals. In our study of UOG mixtures, we examined the effects of gestational exposure on the developing female mouse mammary gland prior to puberty and in early adulthood (Sapouckey et al., 2018). Using morphometric tools to evaluate changes to shape and complexity of the mammary epithelial tree, we found no effects of the UOG-mix on any morphological outcome prior to puberty at PND21. In contrast, there were both subtle and striking effects of the UOG-mix on the female mouse mammary gland in

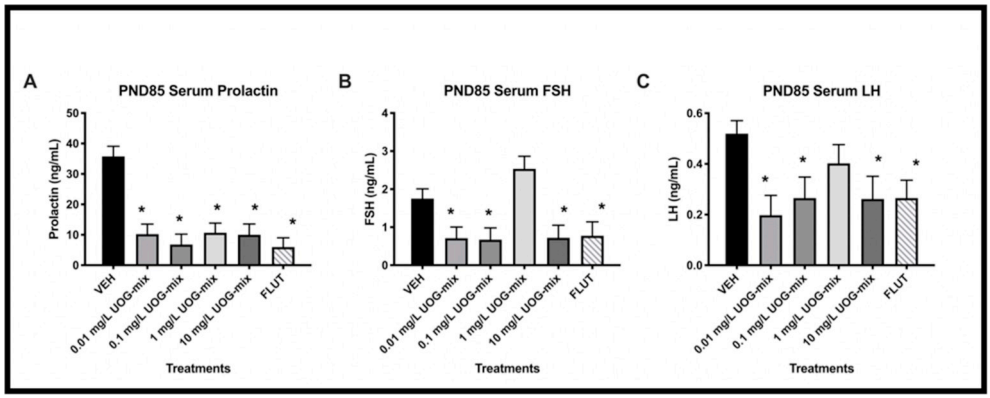

Fig. 3. Developmental exposure to UOG chemicals alters pituitary hormones in adulthood. Estimated marginal mean ± SEM of prolactin (A), follicle stimulating hormone (FSH) (B), luteinizing hormone (LH) (C) for developmentally exposed mice collected at PND85. *, different than untreated controls (vehicle) at $P < 0.05$; n = 9, 9, 7, 8, 6, and 10 litters for vehicle (VEH), 0.01, 0.1, 1.0, 10 mg/L, and flutamide (FLUT).

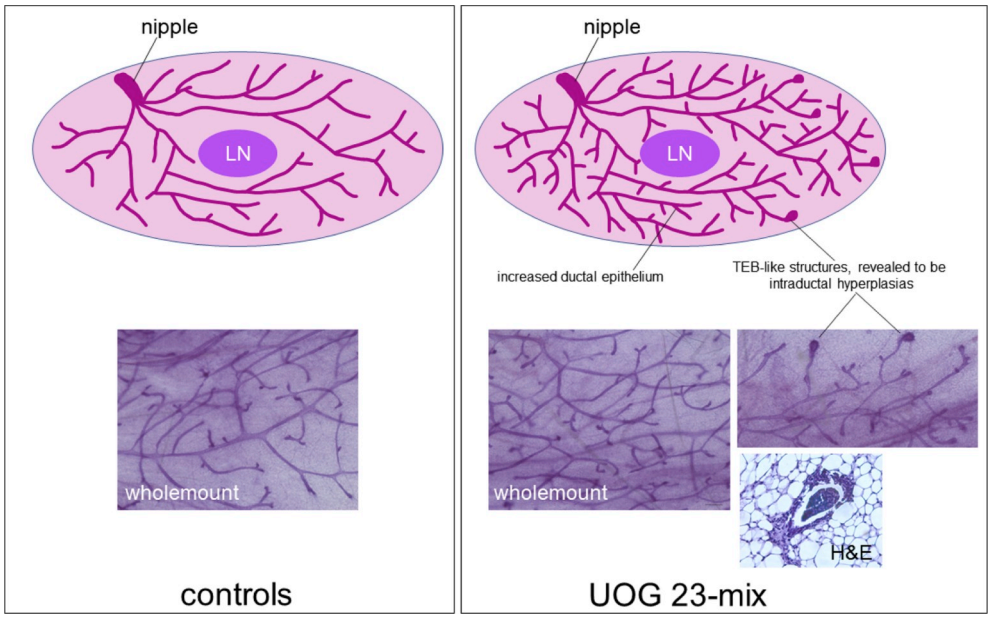

Fig. 4. Prenatal exposures to the UOG-mix alter morphology of the female mouse mammary gland. On the left is a schematic diagram and representative whole mount mammary gland from a control animal in early adulthood. On the right is a schematic that illustrates the morphological changes in the UOG-mix exposed glands, representative whole mount mammary glands from 1 mg/L UOG-mix illustrating the increased density of ducts and the presence of bulb-like structures, and a histological image stained with hematoxylin and eosin (H&E) of one of these precancerous intraductal hyperplasia from 10 mg/L UOG-mix.

early adulthood (Table 3). We observed modest but significant increases in the total volume of mammary epithelium, as well as rounded ductal ends that we confirmed were pre-cancerous intraductal hyperplasia (Fig. 4). These lesions had an appearance similar to terminal end buds (TEBs), *i.e.*, mammary epithelial structures that are typically present only during puberty; TEBs are highly proliferative structures that are responsible for the rapid growth of the ductal tree during puberty, but they are also known to be highly sensitive to carcinogenic insults. Further molecular evaluation revealed effects on cell proliferation and apoptosis that were consistent with non-monotonicity (e.g., increased proliferation in females exposed to the lowest concentration of the UOG-mix). Collectively, these results indicate that developmental exposure to a 23-chemical UOG-mix alters the growth and morphology of the adult mammary gland, including the induction of highly proliferative epithelial lesions.

6. Energy expenditure, activity and behavior following prenatal and postnatal exposure to a mixture of UOG chemicals

Hormones including estrogens, androgens, glucocorticoids, thyroid hormones, and insulin, among others regulate energy expenditure, energy intake, and adipogenesis (Heindel et al., 2017). Chemicals that disrupt this regulation have been termed metabolic disrupting chemicals (MDCs), and MDCs have been shown to disrupt metabolism by altering energy balance or promoting adipogenesis (Heindel et al., 2017). MDC exposures during development have been reported to alter energy expenditure, energy intake, activity, body composition, and glucose and insulin sensitivity (Heindel et al., 2017).

Our prior work described above suggested that the UOG-mix could disrupt hormone receptors that regulate metabolism. We also found that the UOG-mix and UOG wastewater increased adipogenesis in tissue culture and prenatal exposure to the UOG-mix increased body weight in prepubertal female mice (Kassotis et al., 2016,b,c, 2018b). Building upon this data, we sought to study how a preconceptional, gestational, and lactational exposure to the UOG-mix would program the fetus and alter metabolism in adulthood. Pregnant and lactating mice were exposed to the same 23 UOG chemical mixture at equimass concentrations via drinking water at a range of doses from pregnancy day 1 to lactational day 21. Body composition, energy expenditure, activity, and glucose tolerance were examined in the offspring at seven months of age (Balise et al., 2019a,b) and again at 12 months of age after an acute high fat and high sugar diet challenge (Balise et al., 2019a,b).

Developmental exposure to the UOG-mix altered body weight in prepubertal mice and altered energy expenditure and activity in adulthood. Female mice displayed lower body weight at PND7 in almost all exposure groups (Balise et al., 2019a,b). At 7 months of age, female mice displayed both lower total energy expenditure in a 12 h cycle and resting energy expenditure (lowest energy expenditure in 12 h cycle) in the dark cycle in the 0.01 and 1 mg/L treatment groups compared to vehicle. However, these changes in energy expenditure did not result in alterations in body composition at 7 months.

We next gave the females a metabolic challenge in an attempt to stress the tightly regulated homeostatic mechanisms beyond compensation to reveal other possible metabolic effects. The metabolic challenge included aging the females and exposing them to an acute six-day high fat high sugar diet. After this challenge at 12 months of age, females in all treatment groups displayed an increase in total and non-resting energy expenditure during the light phase (Balise et al., 2019a,b). The increase observed in energy expenditure corresponded with an increase in spontaneous activity (activity in x, y, and z directions) and ambulatory activity (activity in x and y directions), also seen in the light phase in all treatment groups; and females in the 0.01, 0.1, and 10.0 mg/L treatment groups displayed lower periuterine adipose tissue weight.

Developmental exposure to the UOG-mix was associated with increased spontaneous activity in the light phase (Table 3). Since mice are more active at night to avoid predation during the day, being more active during the day can be related to risk-taking behavior. To test this hypothesis, we used the elevated plus maze that measures exploratory behavior. An elevated plus maze consists of two open arms and two closed arms. Mice routinely spend more time in the closed arms as it is evolutionarily safer to be in protected spaces. Female mice developmentally exposed to the UOG-mix spent > 200% more time in open arms than vehicle control mice in all but the highest treatment group (Fig. 5A), indicative of an increase in exploratory and risk-taking behavior (Balise et al., 2019a,b; Walf and Frye, 2007). The 0.1 mg/L females also spent more time in the center, and less time in the closed arms when compared to the controls (Fig. 5B and C). Interestingly, 100% of animals in the 0.1 and 1.0 mg/L treatment groups chose to enter open arms, while about 45% of the control animals never entered an open arm throughout the experiment, which also suggests an increase in risk-taking behavior after UOG-mix exposure (Fig. 5D). These data together suggest that females developmentally exposed to the UOG-mix have increased exploratory and risk-taking behaviors.

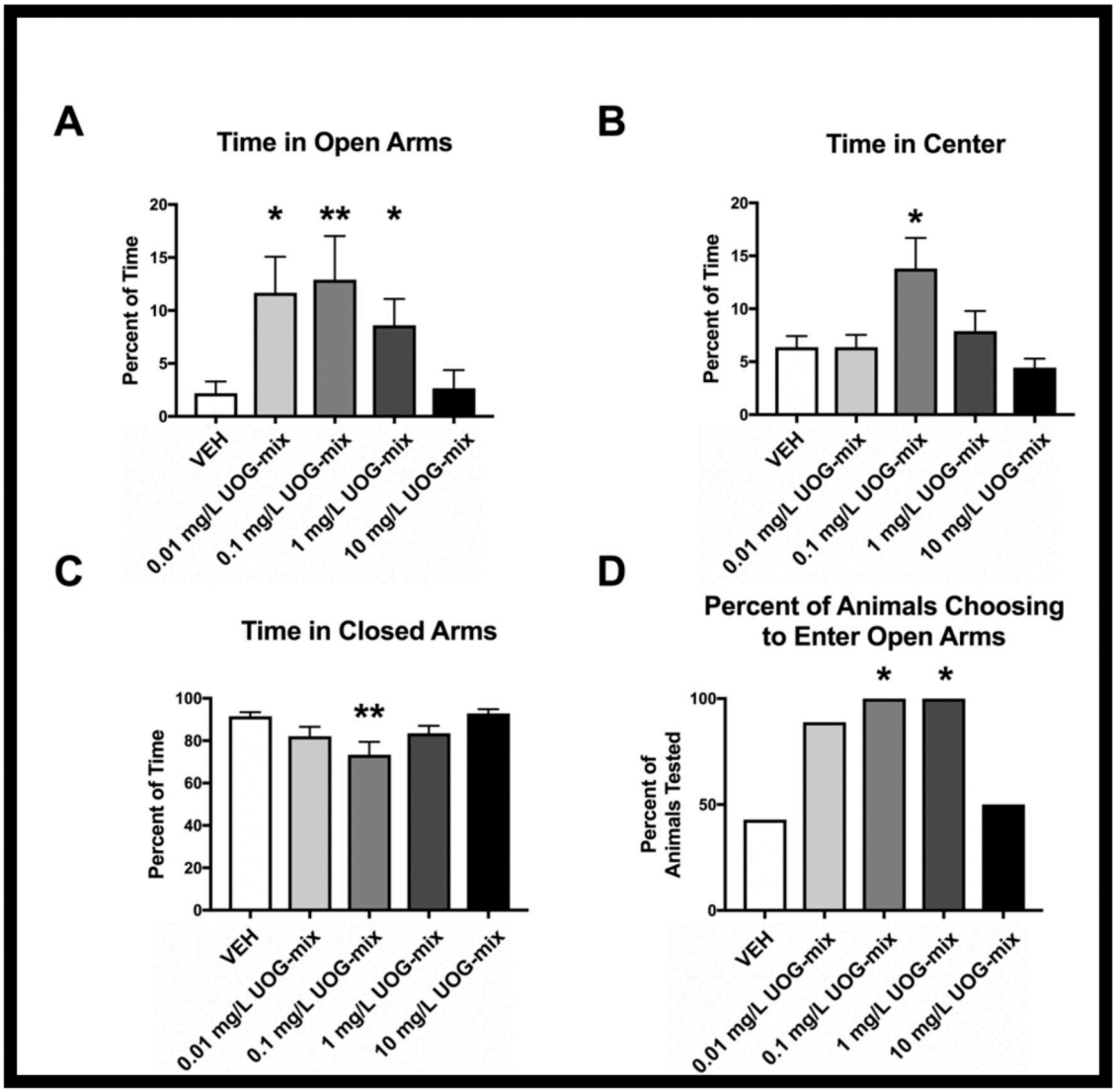

Fig. 5. Developmental exposure from gestation day 1 to postnatal day 21 to the UOG-mix increased time spent in open arms in elevated plus maze test. Female mice at 11 months of age were placed in elevated plus maze and behavior was filmed and time spent in each area tabulated.

Exploratory behavior is sexually dimorphic: Females tend to be less anxious and exhibit more exploratory behavior by spending more time in the open arms of the EPM test than males (Leret et al., 1994; Palanza et al., 2016). Perinatal exposure to either exogenous estrogens or androgens has been shown to decrease time spent in open arms thus masculinizing females (Leret et al., 1994; Palanza et al., 2016). In our study, we found the opposite: Females spent more time in open arms after perinatal exposure to the UOG-mix (Balise et al., 2019a,b). This suggests the UOG-mix may have antagonized the estrogen and/or the androgen receptors to increase this behavior. Overall, these studies demonstrated that the UOG-mix altered developmental programming that resulted in sex-dependent alteration in energy expenditure, activity, and behavior in adulthood.

7. Immune system function following developmental exposure to a mixture of UOG chemicals

The immune system is a highly integrated network designed to find and eliminate invading pathogens and cancer cells (Fig. 6). Even subtle weakening in immune detection or responsiveness can reduce resistance to infection, diminish vaccine efficacy, and lessen tumor surveillance. Overzealous and mis-directed immune responses also have detrimental consequences, including autoimmune and hypersensitivity diseases and chronic inflammation. Moreover, minute perturbations during development, which may be difficult to measure at the time of exposure, can profoundly impact immune function later on. Thus, health effects of exposures that modify the immune system are far reaching, and it is important to determine the impact of developmental exposure to chemicals associated with UOG on the immune system.

While the possible immunotoxicity of each of the many chemicals used in UOG has not been explored, emerging evidence suggests that some UOG chemicals are EDCs, and that EDC mixtures affect the immune system (Boule and Lawrence, 2016; Kuo et al., 2012). Although the mechanisms are not fully understood, the endocrine and immune systems influence each other. As such, there is growing attention on understanding the effects of EDCs on the developing immune system. Given that immune system function includes cellular migration among anatomical sites, and because of the complexity of the endocrine-immune connections, and of the effects of exposure to mixtures of chemicals, reliable animal model systems are paramount to creating rigorous and reproducible knowledge to inform risk assessment of exposure to EDC mixtures during early life. Comparative insights into genetically and evolutionarily distant species such as mice and frogs (*Xenopus*) used in parallel have considerable potential to increase the

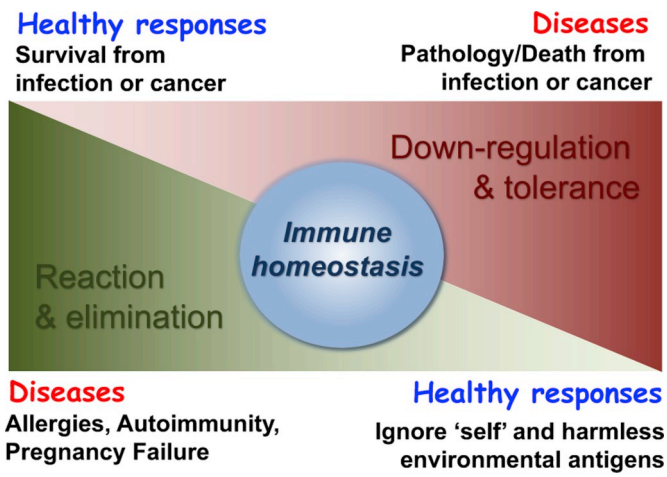

Fig. 6. Immune system balance is key to maintaining health and preventing disease. Illustration of the complex regulation required to control the immune system function. On the one hand, efficient detection and rapid responses are required to fight and eliminate pathogens or tumors, but misguided detection, disproportionate or excessive responses may lead to diseases such allergy, autoimmunity or pregnancy failure. Likewise, although the immune system needs to be controlled to prevent its overreaction and avoid its response against self or innocuous agents, too much control can increase susceptibility to infection and cancers. Thus, even minute alterations of immune homeostasis or to the signals that control the up and down regulation of immune function resulting from exposure to UOG chemicals may tilt the balance toward diseases.

pace of discovery and advance the field more rapidly. Notably, the endocrine and immune systems are remarkably conserved between *Xenopus*, mouse, and human (reviewed in (Buchholz, 2015; Robert and Ohta, 2009)). Moreover, the external post-embryonic development in *Xenopus*, which occurs free of maternal influences, allows direct exposure to water contaminants (De Jesus Andino et al., 2017). This provides rich opportunity for comparing and contrasting the effects of maternal influences and vertical exposure of the fetus and offspring, which occur in mammalian models. Indeed, despite these differences in development, recent findings from separate studies using *Xenopus* and mice demonstrate that developmental exposure to a mixture of EDCs in water has lasting effects on the immune system (Boule et al., 2018b; Robert et al., 2018).

In mice, developmental exposure to the UOG-mix also affected the immune system (Boule et al., 2018b). In these studies, after prenatal exposures to the UOG-mix immune functions were examined in adult male and female mice using three established models of distinct human diseases: allergic airways disease, provoked using house dust mite (HDM) sensitization and challenge; respiratory infection with influenza A virus (IAV); and experimental autoimmune encephalomyelitis (EAE), a mouse model of the autoimmune disease multiple sclerosis. Given that all of these diseases require T cells, cellular studies focused on this particular type of immune cell. In all 3 disease models, developmental exposure to the UOG-mix altered frequencies of T cell sub-populations in female, but not male, offspring (Table 4). Also, in the EAE model, disease onset occurred earlier and was significantly more severe in female offspring. Thus, developmental exposure to this mixture caused durable deleterious immunological effects. This suggests that developmental exposure to a complex mixture of water contaminants, such as those derived from UOG operations, could contribute to immune dysregulation and disease later in life.

In *X. laevis*, immunomodulatory effects of waterborne environmental pollutants, such as UOG chemicals, on the development of effective immune defenses have also been examined (Robert et al., 2018; Robert et al., 2019). When *Xenopus* tadpoles were exposed to the UOG-mix there were adverse effects on the immune system at this life stage, and also later in life, after metamorphosis (Table 4). For instance,

Table 4
Immune system function following developmental exposure to a mixture of UOG chemicals in mice.

Concentration in water (μg/ml)	0.1	1
Dose (μg/kg body weight)	**30**	**300**
Experimental Autoimmune Encephalomyelitis		
Female Mice		
Disease Severity	increased	increased
Disease Onset	expedited	expedited
T cell subsets	skewed	/
Male Mice		
Disease Severity	/	/
Disease Onset	/	/
T cell subsets	/	/
Allergic Airway Disease		
Female Mice		
Airway inflammatory cells	increased	increased
T cell subsets	skewed	skewed
Male Mice		
Airway inflammatory cells	/	/
T cell subsets	/	/
Mild acute respiratory virus infection		
Female Mice		
Host resistance	/	/
T cell subsets	/	skewed
Male Mice		
Host resistance	/	/
T cell subsets	/	/
Immune system function following developmental exposure to a mixture of UOG chemicals in Xenopus		
Concentration in water (μg/ml)	**0.1**	**1**
Tadpole		
Survival to exposure	/	/
Antiviral IFN-I response	decreased	decreased
Antiviral TNF-a and IL-1b response	altered	altered
FV3 viral loads	Increased	Increased
Frog		
Body weight post-metamorphosis	decreased	decreased
Numbers splenic innate leukocytes	decreased	decreased^
Number B and T lymphocytes	decreased	decreased
Leukocyte surface MHC class II	decreased	decreased
FV3 viral loads	Increased^	Increased
Antiviral immun response	Weaker	Weaker

/- not different from vehicle (0.2% ethanol) control.
Endpoints noted as increased, decreased, expedited, altered, skewed, or weaker were different ($p < 0.05$) from control.
^ $p < 0.1$.

tadpoles that were exposed to the UOG-mix via their swimming water for three weeks demonstrated both immediate and delayed impairment in defense against infection with an emerging ranavirus, Frog Virus 3 (FV3 (Grayfer et al., 2015);). Alterations to the immune response to FV3 infection included diminished survival of tadpoles, increased viral load, and decreased *tnfα, Il1β and ifn* gene expression response (Robert et al., 2018). Deregulated expression of these innate immune genes persisted after metamorphosis, as did increased viral load when exposed tadpoles reached maturity in clean water (Robert et al., 2019). Interestingly, tadpole's exposure to the UOG-mix also affected the myeloid lineages at steady state, both during tadpole life and later after metamorphosis, as evidenced by significant changes in gene expression of critical myeloid receptors such as the Granulocyte-colony stimulating factor receptor (GCSF-R). Exposure to the UOG-mix also changed weight gain and time to complete metamorphosis, which correlated with a decreased number of innate leukocytes expressing MHC class II and B and T lymphocytes in the spleen. This suggests that UOG-associated water pollutants at low

but environmentally–relevant levels have the potential to induce acute as well as long-term alterations of immune function and antiviral immunity.

8. Limitations and future directions

Taken together the studies described above provide compelling evidence that UOG chemicals at low, but ecologically–relevant concentrations, have the potential to induce long term alterations in endocrine-mediated health and disease. The summarized set of experiments including *in vitro* receptor and *in vivo* endpoints provides an excellent set of data (Tables 3 and 4) for future adverse outcome pathway (AOP) analyses. Future studies should build on these data to probe the underlying mechanisms of action and consequences on environmental and human health.

One strength of the current set of studies is also a limitation. We used only one synthetic mix of 23 commonly used UOG extraction chemicals out of an infinite number of potential mixtures of real-world chemicals used by the industry and found in waste-water, so while it is representative of chemicals used and at concentrations found in the environment, it is only one scenario. The precise relevance to human exposure is unclear, and people may not be concurrently exposed to these 23 chemicals in the real world at these concentrations. Future studies should aim to examine exposure to real-world UOG wastewater to examine these mixtures of chemicals and at different phases of production.

Previous studies, including our own, have not examined effects of UOG wastewater on fertility. The next steps in this line of research will be to directly measure the impacts of developmental UOG exposure on fertility in offspring. In developmentally-exposed females, we observed suppressed PRL, FSH, and LH, which all may impact ovulation and other aspects of fertility. Suppressed PRL concentrations in UOG-mix groups may result in profound reproductive effects in these animals; PRL is critical for lactation, female receptivity and parenting behavior, as well as immune function, angiogenesis, metabolism, and more (Freeman et al., 2000). Suppressed FSH may result in impaired fertility; FSH receptors are localized to ovarian granulosa cells and activation is considered essential for folliculogenesis (Kumar et al., 1997). Suppressed LH may also impact fertility; LH is critical for folliculogenesis, ovulation, and for maintenance of luteal function (Westergaard et al., 2000). In developmentally-exposed males, we observed enhanced testosterone and reduced sperm counts, which may also contribute to fertility concerns. Exposure to the UOG-mix resulted in 17–35% reductions in epidydimal sperm counts, 6–10% increased testis weights, and 300–500% increased serum testosterone in the two highest dose groups. Taken together, the obvious question is how the observed effects on serum hormones, folliculogenesis, and sperm production impact fertility and reproductive senescence.

Results on the mammary gland also leave a number of important open questions (Table 5). Our first mammary gland study focused on morphology and development of the female mammary gland (Sapouckey et al., 2018), yet as discussed above, the male mammary gland is likely also affected by EDC mixtures that include anti-androgenic chemicals. Further studies should also evaluate older adult females to determine if intraductal hyperplasia manifesting as 'beaded ducts' arise, if there are changes to the appearance of alveolar buds, or other relevant morphological disruptions. Moreover, additional studies evaluating whether UOG mixtures alter the function of the mammary gland, or long-term disease in this organ, would also be important to pursue.

Developmental UOG exposure resulted in long term alterations in host-pathogen interactions and antiviral immunity that also warrant further investigation. By extension, these results provide evidence that even at low concentrations, waterborne anthropogenic chemicals can serve as contributing factors to the recent increase in susceptibility of aquatic vertebrates to infectious diseases and consequently in the emergence of these diseases. Likewise, these studies provide evidence of the ability of EDCs in water to durably influence the mammalian immune system, and may contribute to clinically significant health effects due to imbalances in immune function. It will be important in future experiments to define, in more detail, whether UOG chemicals impact host defense against other pathogens, such as bacteria, and whether they affect particular immune pathways or function that could serve as potential biomarkers for evaluating effects of UOGs on human immune function and health. Also, helpful will be the use of less complex mixtures based on a common EDC mechanism of action, such as thyroid hormone disruptor activity. Taken together, pinpointing the cellular mechanisms affected by UOG mixtures will help inform testing and use of biomarkers to determine the impact of chemical exposures on human and wildlife immune function.

Metabolic perturbations observed *in vitro* and *in vivo* should also be assessed in greater detail in future studies. Our studies have found that UOG chemicals and mixtures promote fat cell differentiation and precursor cell proliferation, and that gestational exposure resulted in greater body weights through early adulthood in mice. We further demonstrated that the UOG-mix altered developmental programming and resulted in altered energy expenditure, activity, and behavior in adulthood. While these phenotypes might typically be deemed beneficial, these changes were seen during the light phase when mice are typically less active. From an evolutionary perspective, this is maladaptive and has been interpreted as risk taking behavior and may be related to attention deficit and hyperactivity disorders, or other major depressive disorders (Martel et al., 2009). Future studies should be specifically designed to assess these endpoints in animal models and in people. Additional research on human and animal metabolic health and behavioral effects in drilling dense areas is needed.

9. Conclusions

To identify potential health impacts and underlying mechanisms of exposure to complex mixtures of UOG chemicals found in wastewater, we designed a mixture of 23 chemicals. The UOG-mix exhibited potent antagonist activity for the estrogen, androgen, glucocorticoid, progesterone, and thyroid hormone receptors. We administered the UOG-mix in drinking water to pregnant mice and examined the health and behavior of their offspring in adulthood. Perinatal exposure had a profound impact on adult pituitary hormone concentrations, and reduced sperm counts and altered folliculogenesis and increased mammary

Table 5
Additional research directions to pursue in the mammary gland.

Type of Study	Important questions to pursue
Developmental, morphological	• Do UOG exposures alter the male mammary gland including nipple retention and size of the ductal tree? • Do developmental UOG exposures alter morphology of the female mammary gland at puberty? • Do UOG exposures alter the onset of alveolar bud formation in adult females?
Function	• Do UOG exposures alter differentiation of the lactating mammary gland? • Do UOG exposures alter milk production, milk quantity, or milk composition?
Disease	• Do UOG exposures induce carcinomas in sensitive rodent strains? • Do UOG exposures sensitize animals to chemical carcinogens?

gland ductal density and preneoplastic lesions. Developmental exposure altered the immune system in a female specific manner in three different immune system models, and exposure altered energy expenditure and exploratory and risk-taking behavior.

Taken together, these studies suggest that UOG extraction may be an important source of human EDC exposure and altered health parameters. Given that > 17 million people in the US live within one mile of an UOG well (Czolowski et al., 2017), and human health impacts have been associated with maternal residential proximity up to 10 miles (McKenzie et al., 2014), the potential number of people impacted is substantial. By assembling an interdisciplinary team of collaborators, we were able to share tissues and reagents to increase efficiency and answer novel questions. This approach yielded fruitful, synergistic collaborations that led to research showing that health impacts from UOG operations are likely and identified endpoints should be directly investigated in future studies. Taken together, these data suggest a strong need to examine the impacts of residential and occupational UOG exposure in humans and other wildlife in drilling areas. Additionally, these experiments may shed light on the effects of development exposure to other EDCs that have similar mechanisms of action to antagonize nuclear receptors.

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CRediT authorship contribution statement

S.C. Nagel: Conceptualization, Writing - original draft, Writing - review & editing. **C.D. Kassotis:** Writing - original draft, Writing - review & editing. **L.N. Vandenberg:** Writing - original draft, Writing - review & editing. **B.P. Lawrence:** Writing - original draft, Writing - review & editing. **J. Robert:** Writing - original draft, Writing - review & editing. **V.D. Balise:** Writing - original draft, Writing - review & editing.

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