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# PFAS compounds PFOA and Gen X are teratogenic to sea urchin embryos

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BOSTON UNIVERSITY  
GRADUATE SCHOOL OF ARTS AND SCIENCES

Thesis

**PFAS COMPOUNDS PFOA AND GEN X ARE  
TERATOGENIC TO SEA URCHIN EMBRYOS**

by

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B.A., Boston University, 2022

Submitted for partial fulfillment of the  
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**KELLEY MCCUTCHEON**

**ABSTRACT**

Per- and polyfluorinated substances (PFAS) are synthetic chemicals that are used to make fluoropolymer coatings found in many products, such as non-stick pans, clothing, cosmetics, and food packaging. These highly persistent molecules are known as “forever chemicals,” since they neither degrade environmentally nor break down enzymatically within biological systems. PFAS compounds easily contaminate water sources, and as a result, PFAS molecules have bioaccumulated in all species including humans. The purpose of this study was to define the effect of two PFAS molecules, the more toxic perfluorooctanoic acid (PFOA) and the more recent, reportedly safer chemical hexafluoropropylene oxide dimer acid (Gen X) in *Lytechinus variegatus* embryos. We examined the effects of PFOA and Gen X on development and patterning using morphological analysis, immunostaining, and HCR-FISH. We find that both PFOA and Gen X are sufficient to perturb skeletal patterning and primary mesenchyme cell (PMC) migration in *L. variegatus* embryos, although at relatively high doses, likely reflecting the hydrophilic nature of PFAS compounds that limits their diffusive entry into cells. We find that zygotes are sensitive to PFOA at a lower dose than Gen X. We defined the temporal window of action for both agents by performing timed additions and removals of the chemicals. These results indicate that Gen X is functionally effective between 7 and 13 hpf (hours post-fertilization), which correspond with hatching and mesenchyme

blastula stages. In contrast, PFOA is most effective between 21 and 36 hpf, which correspond to prism and early pluteus stages. Both chemicals mildly perturbed ciliary band restriction, as well as expression of Chordin and IxA, which are dorsal-ventral (DV) marker genes. In addition, both chemicals perturbed some PMC subset genes and ectodermal patterning cues, to varying degrees of severity. Together, these data show that PFAS compounds are teratogenic to sea urchin embryos and that PFOA and Gen X provoke distinct outcomes and function at different intervals during development. Though Gen X is thought to be a “safer” alternative to other PFAS like PFOA, our findings indicate that Gen X has an earlier and more severe effect on DV specification, albeit at a higher dose. However, because these chemicals are so stable and persistent in the environment and within biological systems, they will continue to accumulate and their concentrations will continue to increase if strategies to remediate them are not activated, even if their production were to cease. Thus, determining their effects on biological systems at relatively high doses is ecologically relevant; seeking methods for their removal is crucial for the prevention of widespread teratogenesis both environmentally and among humans.

## TABLE OF CONTENTS

<i>LIST OF FIGURES</i> .....	viii
<i>LIST OF ABBREVIATIONS</i> .....	ix
<i>INTRODUCTION</i> .....	1
<i>MATERIALS AND METHODS</i> .....	4
<i>Animals, Drug Treatments, and Imaging</i> .....	4
<i>Chemicals</i> .....	4
<i>Time Course Experiments</i> .....	5
<i>Immunostaining</i> .....	6
<i>Hybridization Chain Reaction Florescent In Situ Hybridization</i> .....	6
<i>RESULTS</i> .....	8
<i>PFOA and Gen X perturb sea urchin skeletal patterning, PMC migration, and ciliary band restriction</i> .....	8
<i>PFOA and Gen X do not perturb sea urchin serotonergic neural specification</i> .....	11
<i>PFOA and Gen X exert their effects during defined temporal windows</i> .....	12
<i>PFOA and Gen X perturb DV spatial territories</i> .....	14
<i>PFOA and Gen X perturb some PMC subset genes and ectodermal patterning cues</i> ..	16
<i>DISCUSSION</i> .....	22
<i>REFERENCES</i> .....	26
<i>VITA</i> .....	32

## LIST OF FIGURES

Figure 1: Chemical structures of PFOA and Gen X. ....	8
Figure 2. Treatment with PFOA and Gen X is sufficient to perturb skeletal patterning, PMC migration, and ciliary band restriction during larval development in <i>Lytechinus variegatus</i> . ....	9
Figure 3. Treatment with PFOA or Gen X is not sufficient to perturb neural specification in <i>L. variegatus</i> . ....	11
Figure 4: PFOA and Gen X treatment exert their effects during defined and distinct temporal windows. ....	13
Figure 5: PFOA and Gen X are sufficient to perturb DV spatial territories. ....	15
Figure 6: PFOA and Gen X are sufficient to perturb Jun, Univin, and Pks2 expression. ....	19
Figure 7: PFOA and Gen X are sufficient to perturb VEGF but not VEGFR expression. ....	20
Figure 8: Most skeletal matrix genes are unaffected by PFOA or Gen X. ....	21

## LIST OF ABBREVIATIONS

<i>Abbreviation</i>	<i>Meaning</i>
anon	Anonymous rod
AR	Aboral rod
ASW	Artificial sea water
BR	Body rod
CB	Ciliary Band
Chd	Chordin
DIC	Differential image contrast
DMSO	Dimethyl sulfoxide
DV	Dorsal-ventral
DVC	Dorsal-ventral connecting rod
Fig	Figure
Gen X	Hexafluoropropylene oxide dimer acid fluoride
HCR-FISH	Hybridization Chain Reaction Fluorescent In Situ Hybridization
HFPO-DA	Hexafluoropropylene oxide dimer acid
hpf	Hours post fertilization
MSP130	Matrix-spicule protein 130
OR	Oral rod
PFAS	Per-and polyfluoroalkyl substances
PKS2	Phytochrome kinase substrate 2
PMC	Primary mesenchyme cell
RR	Recurrent rod
SCH	Scheitel
SM	Spicule matrix
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VT	Ventral-transverse rod

## INTRODUCTION

Per- and polyfluoroalkyl substances (PFAS) are synthetic chemicals that are used to make fluoropolymer coatings found in many products, such as non-stick pans, clothing, cosmetics, fire retardants and food packaging (Brandsma et al., 2019; Buck et al., 2011; Ding et al., 2020; Gaballah et al., 2020; Gebreab et al., 2020; Landrigan et al., 2020; Wang et al., 2017). Known colloquially as “forever chemicals,” PFAS are highly stable and persistent in the environment, allowing them to bioaccumulate in exposed species. Humans are exposed to PFAS through a variety of pathways, including drinking water, the air, food packaging materials, and diet (Blake & Fenton, 2020; Cousins et al., 2020; Davidsen et al., 2021). Nearly all humans have some percentage of PFAS in their bloodstreams (Davidsen et al., 2021; Ding et al., 2020; Gebreab et al., 2020). PFAS accumulate in human placenta, allowing them to be passed to the fetus *in utero*; this necessitates extensive study of their effects during embryonic development (Blake et al., 2020; Davidsen et al., 2021; Foguth et al., 2020; Gebreab et al., 2020; Mamsen et al., 2019). In humans, PFAS have detrimental effects on immune function, thyroid function, and development; PFAS have also been linked to liver and kidney diseases and various cancers (Blake & Fenton, 2020; Fenton et al., 2021; Kotlarz et al., 2020).

Our lab is focusing on two PFAS, perfluorooctanoic acid (PFOA), and hexafluoropropylene oxide dimer acid (HFPO-DA) fluoride, known as Gen X. PFOA has been linked to toxic effects in humans and model organisms and is a developmental

toxicant (Fenton et al., 2021; Gaballah et al., 2020; Kotlarz et al., 2020; Liu & Irudayaraj, 2020; Yu et al., 2022). As such, it has been generally phased out of production and replaced with Gen X. Gen X was chosen as a replacement due to initial studies that indicated it may bioaccumulate less than PFOA, however; its effects have been understudied across species (Brandsma et al., 2019; Davidsen et al., 2021; Gebbink et al., 2017; Gebbink & van Leeuwen, 2020). Despite its reportedly shorter half-life, studies have indicated Gen X's toxic effects are similar to those of PFOA (Blake et al., 2020; Brandsma et al., 2019; Fenton et al., 2021; Gebbink & van Leeuwen, 2020; Gomis et al., 2018). A 2021 study by Davidsen et al. showed that both PFOA and Gen X impaired human cardiomyocyte differentiation, suggesting Gen X may also be a developmental toxicant. Additionally, Gomis et al. found that when differences in toxokinetics were considered, Gen X appeared to have higher toxic potency in modeled serum and liver than PFOA (Gomis et al., 2018). Further studies of the effects of Gen X on development are warranted, as it may not be the "safer" replacement it was thought to be.

This study utilizes *Lytechinus variegatus* embryos as a model organism to study the impacts of PFOA and Gen X. *L. variegatus*, commonly known as the green sea urchin, is an ideal model organism for studying effects on development. *L. variegatus* embryos are transparent and morphologically simple, making them easy to study. Development and embryogenesis are rapid, with larval development well-underway 48 hours post-fertilization (hpf) (Adonin et al., 2021). Additionally, female sea urchins release millions

of eggs at a time which are fertilized *ex vivo*, allowing large sample sizes for research (Pennisi, 2006). Aside from their practical advantages, the surprisingly high genetic similarity of sea urchins to more complex organisms, including humans, makes them a suitable model for genetic analysis (Adonin et al., 2021; Davidson, 2006; McClay, 2011; Sodergren et al., 2007). By virtue of being a simple model with high genetic similarity to complex mammalian animals, *L. variegatus* may help elucidate the molecular pathways through which PFAS act. In addition to the effects of PFAS on human health, it is also important to understand their adverse environmental and ecosystem effects. Run-off from factories, production plants, airports, and military bases have caused wide-spread PFAS contamination of fresh waters; PFAS have also entered the oceans in large quantities. Despite this, little is known about the consequences of PFAS accumulation in marine species (Hayman et al., 2021; Landrigan et al., 2020).

The goal of this study is to define the effects of PFOA and Gen X on development and skeletal patterning in *L. variegatus* embryos, and to explicate more information on how PFAS chemicals are perturbing these processes.

## MATERIALS AND METHODS

### *Animals, Drug Treatments, and Imaging*

Adult *Lytechinus variegatus* sea urchins were obtained from Pelagic Corp (Sugarloaf Key, FL, USA) or the Duke University Marine Lab (Beaufort, NC, USA). Gametes were collected by injecting adult sea urchins with 0.5 M potassium chloride solution. Embryos were cultured in artificial sea water (ASW) or ASW and PFAS. ASW was buffered with 1  $\mu$ L saturated NaHCO<sub>3</sub> solution per 1 mL ASW. PFAS working doses were determined as 300  $\mu$ M (PFOA) and 875  $\mu$ M (Gen X) by dose-response experiments geared towards finding the minimum dose that produced reproducible, penetrant, non-lethal perturbations. Those doses were employed in each experiment except when stated otherwise. Initial Gen X experiments were performed using pre-diluted chemical whose true concentration is ambiguous, and based on follow up studies, likely higher than initially thought. Ambiguous concentrations from experiments using this original chemical solution are marked with an asterisk. Chemicals were added directly to the sea urchin cultures at fertilization, except where otherwise noted. Sea urchin skeletal images were obtained via illumination with plane-polarized light to capture the birefringence of the skeletal crystal. Images of each embryo's skeleton were taken in multiple focal planes, then assembled manually in Photoshop (Adobe; v 22.0.1) to visualize the complete skeleton in focus.

### *Chemicals*

Perfluorooctanoic acid (PFOA, CAS No. 335-67-1) of  $\geq 95\%$  purity and hexafluoropropylene oxide dimer acid (Gen X, CAS No. 13252-13-6) of  $\geq 96\%$  purity were obtained from our collaborator Jennifer Schlezinger (Boston University School of Public Health, Boston, MA, USA). Solid PFOA was originally reconstituted in dimethyl sulfoxide (DMSO; CAS No. D8418) from Fisher Bioreagents to obtain a stock solution of 0.3 M, while Gen X (liquid) was diluted in deionized water to obtain a stock solution of 0.5 M. Later, PFOA was reconstituted in deionized water for consistency, and this change did not appear to have any effect on the treated embryos' phenotypes. In experiments where DMSO-diluted PFOA was used, the largest equivalent volume of DMSO was added to the vehicle control embryo cultures. Chemical stock solutions were frozen in single-use aliquots to avoid repeated freeze-thaw cycles, and the appropriate volume of the stock solution was added to the embryo cultures to achieve the desired final concentrations.

### ***Time Course Experiments***

For PFAS removal experiments, PFOA or Gen X was added at fertilization and removed at various developmentally relevant time points. For PFAS addition experiments, sea urchin cultures were made using solely ASW, then PFOA or Gen X was added at various time points. For both experimental series, the embryos were developed to the pluteus stage, photographed, then analyzed from the images. All images were

collected at 48 hpf. Embryos were scored and counted in Canvas X Draw (Canvas GFX; v 7.0.2).

### ***Immunostaining***

Primary antibodies include PMC-specific monoclonal 6a9 (used at 1:50; from Prof. Charles Ettensohn, Carnegie Mellon University, Pittsburgh, PA, USA), ciliary band-specific monoclonal 295 (1:1; from David McClay, Duke University, Durham, NC, USA), and serotonergic neuron-specific polyclonal  $\alpha$ -ser (1:1000, Sigma). Secondary antibodies include goat anti-mouse antibodies labeled with Alexa Fluor 488 (1:500; Thermo Fisher), Alexa Fluor 546 (1:500; Thermo Fisher), and DyLight 405 (1:500; Jackson Laboratories), and Cy2-conjugated goat anti-rabbit (1:900; Jackson Laboratories). Hoechst nuclear stain was added along with secondary antibody (1:500; Sigma Aldrich) for experiments in which its excitation wavelength was available. For PMC immunostaining, embryos were fixed in 4% paraformaldehyde in ASW for one hour, otherwise fixation and staining were performed as described (Bradham et al., 2009; Gross et al., 2003). Confocal microscopy was performed on Nikon C2 or Olympus FV10i-DOC microscopes. All images are full projections of z-stacks; z-stack projections were performed in FIJI (v 2.1.0).

### ***Hybridization Chain Reaction Florescent In Situ Hybridization***

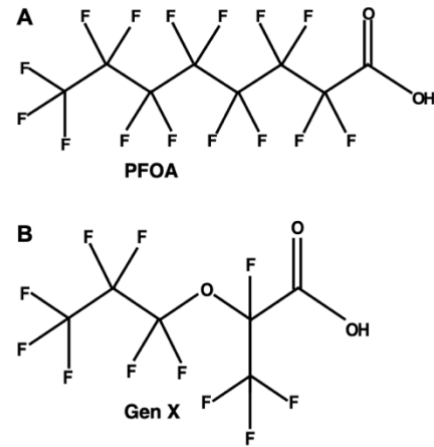
Embryos were fixed in 4% paraformaldehyde in ASW for one hour, otherwise fixation was performed as described (Gross et al., 2003). HCR-FISH was performed as

described (Choi et al., 2016). Confocal microscopy was performed on Nikon C2 microscope. All images are full projections of z-stacks; z-stack projections were performed in FIJI (v 2.1.0). For dorsal-ventral HCR-FISH analyses, the extent of Chordin and IrxA spatial expression was measured radially from the center of the gut using an angle measurement tool in ACD Canvas, and the ciliary band size was calculated by subtracting the sum of chordin and IrxA angle measurements from 360° on a per-embryo basis.

## RESULTS

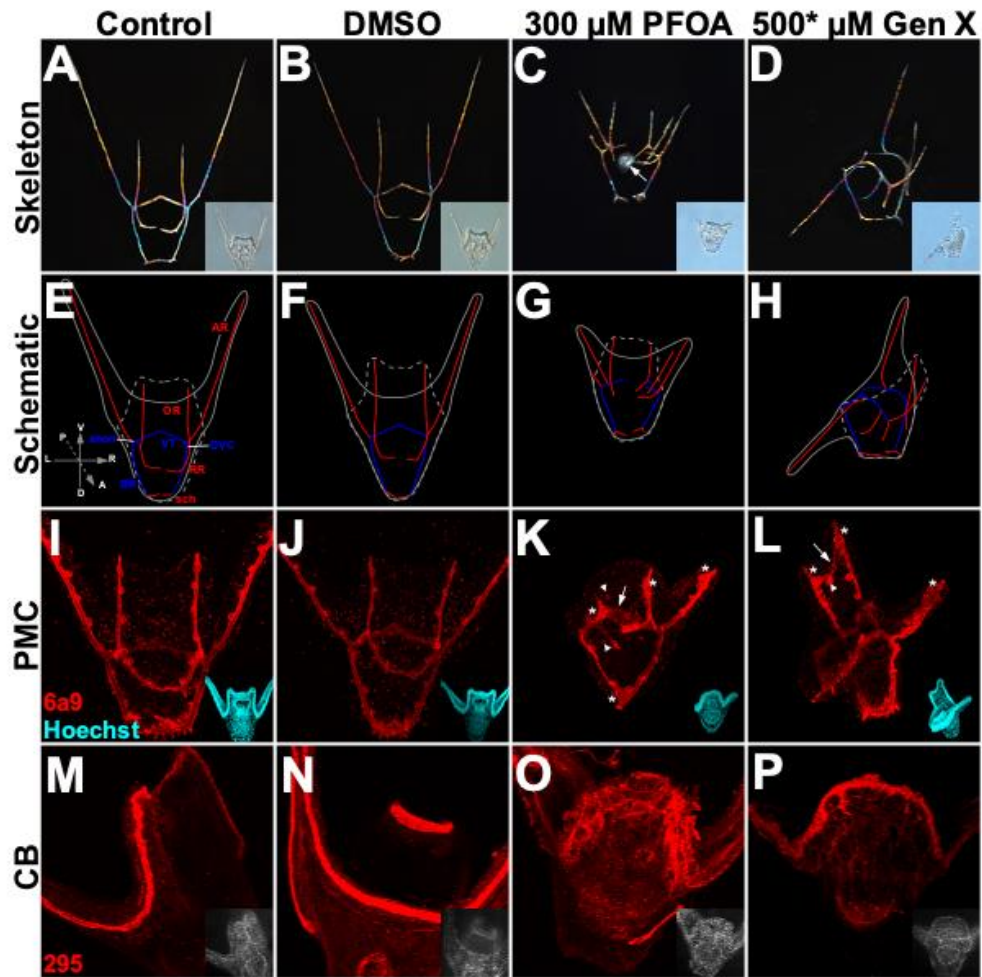
### *PFOA and Gen X perturb sea urchin skeletal patterning, PMC migration, and ciliary band restriction*

To determine whether exposure to PFOA or Gen X (structures shown in Fig. 1) perturbs development in sea urchins, *Lytechinus variegatus* embryos were treated at fertilization with either PFOA or Gen X. We observed skeletal patterning defects in the treated embryos, and we therefore imaged the embryos using plane-polarized light to illuminate the skeletons (Fig. 2A-D). Both PFOA and Gen X-treated embryos exhibit duplications, transformations, and deletions of secondary skeletal elements, as well as rotations around the dorsal-ventral axis. These patterning defects are schematized in Fig. 2E-H. Thus, PFAS compounds are sufficient to elicit skeletal patterning defects in sea urchin embryos. Some embryos also exhibited a birefringence signal within their guts (Fig. 2C, arrow). We determined that this signal does not contain calcium via calcein staining (not shown); we therefore conclude that this reflects ingested PFOA, which is naturally birefringent (Butnor et al., 2020).



**Figure 1: Chemical structures of PFOA and Gen X.**

A. Perfluorooctanoic acid (PFOA),  $C_8HF_{15}O_2$ , molecular weight (MW) = 414.07 g/mol. B. Perfluoro(2-methyl-3-oxahexanoic) acid (Gen X),  $C_6HF_{11}O_3$ , MW= 414.07 g/mol.



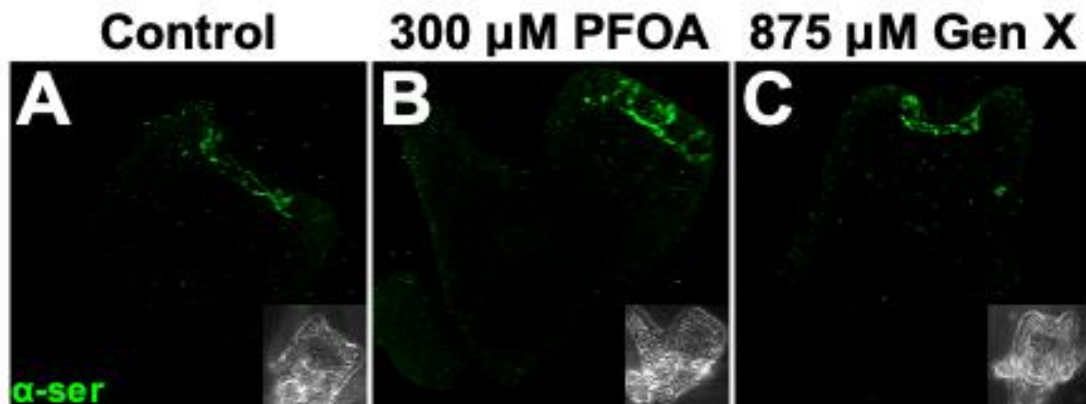
**Figure 2. Treatment with PFOA and Gen X is sufficient to perturb skeletal patterning, PMC migration, and ciliary band restriction during larval development in *Lytechinus variegatus*.** Embryos were treated post-fertilization with artificial sea water (ASW), Dimethylsulfoxide (DMSO, loading control), PFOA, or Gen X as indicated, allowed to grow to 48 hours post fertilization (hpf), then imaged or fixed and immunostained. A-D. Skeletal morphology was imaged with plane-polarized light; corresponding differential image contrast (DIC) images are inset. Arrow in panel C indicates PFOA accumulation in the gut. E-H: Schematized skeletons from panels A-D, with primary skeletal elements in blue and secondary skeletal elements in red. I-P: Embryos were immunostained for primary mesenchyme cells (PMCs, I-L) or ciliary band (CB, M-P); full confocal z-stacks are shown. Inset images show Hoechst staining for nuclei (I-L) or phase-contrast images (M-P). Arrows, arrowheads, and asterics in K and L indicate filopodia, ectopically positioned PMCs, and large PMC clusters, respectively.

The sea urchin skeleton is secreted by a lineage called the primary mesenchyme cells (PMCs) (Kahil et al., 2020; Lyons et al., 2014). The PMCs extend thin filopodia that appear to sense patterning cues expressed by the ectoderm, which directs their migration and differentiation (Armstrong et al., 1993; Miller et al., 1995; Piacentino et al., 2015; Piacentino et al., 2016). The PMCs also extend thick cables that join to form a tubular syncytium that connects the cells, and into which the skeleton is secreted (Lyons et al., 2014). To determine whether the PMCs were similarly perturbed by PFAS treatments, immunostains were performed to visualize the PMCs (Fig. 2I-L). PFOA- and Gen X-treated embryos display PMCs with abnormal filopodia, ectopically positions that do not align with the syncytium, and abnormally large clusters at rod tips that are consistent with delayed migration (Fig. 2K-L). Together, these findings suggest that both PFOA and Gen X treatment results in abnormal migration and spatial organization of the PMCs, consistent with the skeletal patterning defects elicited by these chemicals. To test whether the aberrant PMC migration in PFAS-treated embryos is due to broad ectodermal perturbations, immunostains were performed to visualize the ciliary band (Fig. 2M-P). The ciliary band is a morphologically distinct structure comprised of apically constricted, ciliated cells and neurons that divides the ventral and dorsal (DV) ectodermal regions (Fig. 2M-N). Perturbations to DV specification result in spatially aberrant ciliary bands (Bradham et al., 2009; Yaguchi et al., 2010). The ciliary bands in PFOA-treated embryos are dramatically expanded in the oral hood structure (Fig. 2O), while the Gen X-treated

embryos have ciliary bands that are ragged, with uneven and sometimes indistinct edges. (Fig. 2P). These ciliary band perturbations suggest mild DV specification defects contribute to PFOA- and Gen X-induced patterning defects.

***PFOA and Gen X do not perturb sea urchin serotonergic neural specification***

Neurons are derived from the ectoderm, and most neurons in sea urchin larvae are found in the ciliary band (Slota & McClay, 2018), while the easily detected serotonergic neurons are apically localized (Yaguchi et al., 2006). To investigate whether the ectodermal perturbations that are disturbing the ciliary band also perturb neuronal



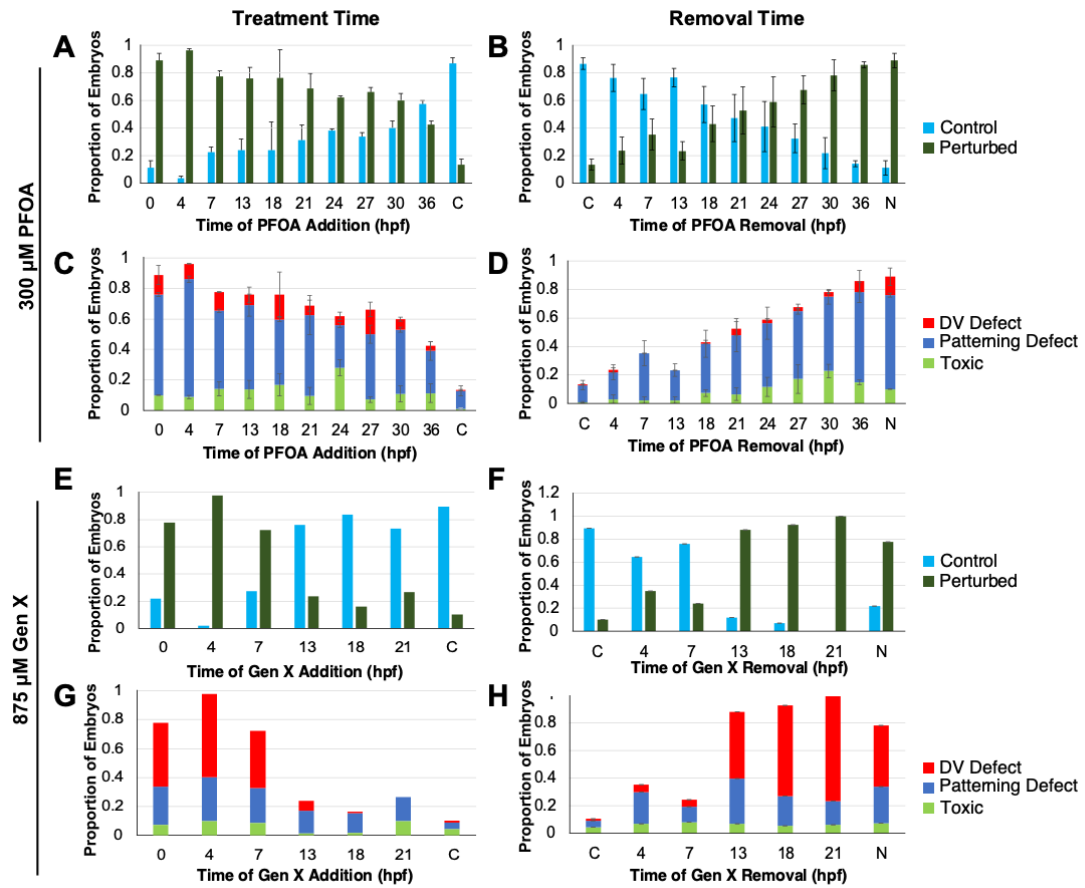
**Figure 3. Treatment with PFOA or Gen X is not sufficient to perturb neural specification in *L. variegatus*.** Embryos were treated post-fertilization with sea water (A), PFOA (B), or Gen X (C). The resulting plutei were stained for serotonergic neurons (green) and imaged via confocal microscopy. Corresponding phase-contrast images are inset.

specification, immunostaining for the serotonergic neurons was performed in both PFOA and Gen X-treated embryos (Fig. 3). The serotonergic neurons appear control-like in both

PFOA- and Gen X-treated embryos, implying that the ectodermal defects caused by these chemicals are not sufficient to perturb serotonergic neuronal specification and patterning.

***PFOA and Gen X exert their effects during defined temporal windows***

To determine when PFOA and Gen X perturb *L. variegatus* development, PFOA (Fig. 4A-D) or Gen X (Fig. 4E-H) were either added to or washed from sea urchin cultures at various developmentally relevant time points. Embryos were then scored as either control or perturbed after developing to 48 hpf. PFOA perturbs development when added before 36 hpf (Fig. 4A), while removing PFOA ceases to rescue normal development after 18 hpf (Fig. 4B). This broad window of effect suggests that PFOA is acting on multiple developmental processes and pathways, since 18-36 hpf encompasses the transition between the late gastrula and early pluteus stages (Hogan et al., 2020) and also encompasses the known interval for skeletal patterning (Piacentino et al., 2015, 2016). However, this temporal window is not consistent with a direct impact on DV specification, since that process is completed by 13 hpf, at the onset of gastrulation (Bradham & McClay, 2006; Hardin et al., 1992; Piacentino et al., 2015). Defects were specifically scored to see if they were DV perturbations or general skeletal patterning



**Figure 4: PFOA and Gen X treatment exert their effects during defined and distinct temporal windows.** Embryos were treated with PFOA (A-D) or with Gen X (E-H) at the indicated time points (left side) or treated at fertilization, then washed at the indicated time points (right side). The resulting embryos were scored at 48 hpf as either normal or perturbed to determine effective windows (A, B, E, F), or scored for DV versus patterning defects (C, D, G, H).

defects. Most defects observed in PFOA-treated embryos are broad skeletal patterning defects, with relatively few DV defects (Fig. 4C-D), consistent with the timing results.

Gen X perturbs development when added before 13 hpf (Fig. 4E) and fails to rescue development when removed after 7 hpf (Fig. 4F). This early window of effect is

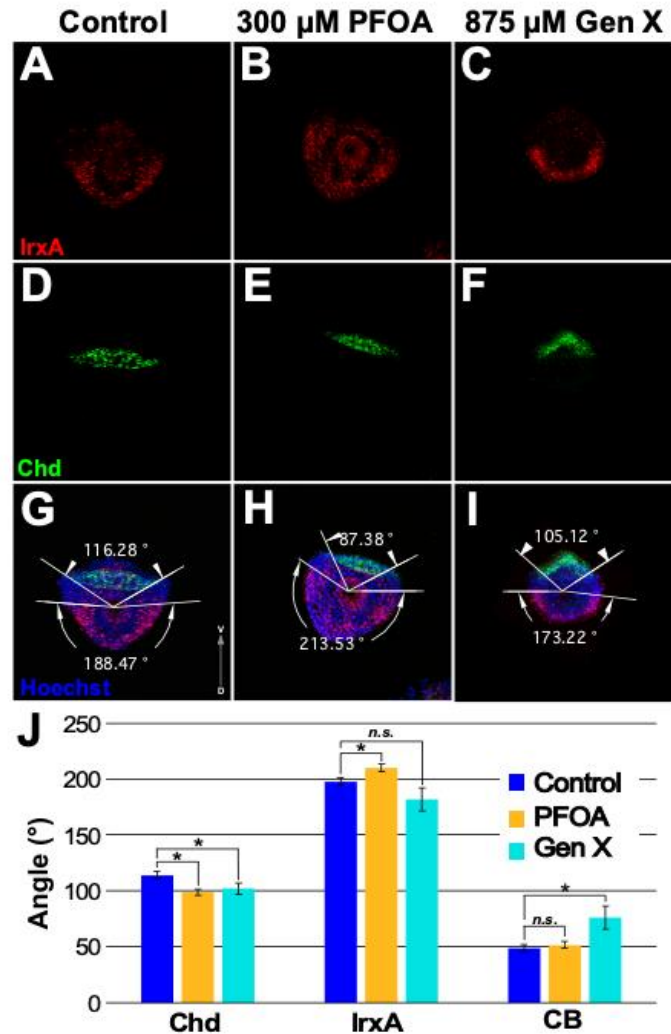
consistent with DV perturbations, as DV specification starts at 7 hpf, with the onset of genes downstream from Nodal, and is completed by 13 hpf with the onset of gastrulation (Bradham & McClay, 2006; Hardin et al., 1992; Piacentino et al., 2015). This is consistent with the scoring results shown in Fig. 4G-H, in which most of the perturbations observed in Gen X-treated embryos are DV defects, including partial defects such as additional aboral rods (AR) extending from the ventral-transverse rods (VTs) and rotations around the DV axis that represent ventral or dorsal expansion, and full defects that are reflected by skeletal radialization. These results suggest that Gen X has an early, potentially direct effect on ectodermal DV specification that indirectly perturbs skeletal patterning, while PFOA has a later, direct effect skeletal patterning.

#### ***PFOA and Gen X perturb DV spatial territories***

To further investigate the impacts of PFOA and Gen X on DV specification, the expression of DV marker genes was tested *in situ* using HCR-FISH (Fig. 5). We measured the expression of Chordin, a target of nodal and a ventral marker, and IrxA, a target of BMP2/4 signaling and a dorsal marker (Bradham et al., 2009; Su et al., 2009) (Fig. 5). The ciliary band is restricted to the space between the expression domains of these two genes. In both PFOA- and Gen X-treated embryos, Chordin's spatial domain is significantly contracted, while IrxA shows statistically significant expansion only in PFOA-treated embryos (Fig. 5J). This unexpected and surprising outcome indicates that

PFOA treatment impacts both Chordin and IrxA expression, potentially explaining the perturbed ciliary band in these embryos, despite the lack of effect of PFOA at the time that these DV genes are expressed.

The ciliary band itself (calculated as the space between the IrxA and chordin territories, Fig. 5G-I) does not exhibit significant spatial change in PFOA-treated embryos but is significantly expanded in Gen X-treated embryos (Fig. 5J). In a second surprise, the effects of Gen X treatment on



**Figure 5: PFOA and Gen X are sufficient to perturb DV spatial territories.** Control and treated embryos were subject to HCR FISH for IrxA (red, A-C) and chordin (Chd, green, D-F) at 18 hpf. Merged images including nuclear Hoechst staining (G-I, blue) were used to measure the angles of Chd and IrxA expression domains as shown. (J) The spatial extent of ventral Chd, dorsal IrxA, and the ciliary band (CB) are plotted as the average angle; \*  $p < 0.05$  in t-tests,  $n \geq 30$  across 4 biological replicates.

the expression of these genes are mild, since Chordin but not IrxA is affected, although the CB territory is also significantly enlarged by Gen X, which does act while these genes function to specify DV.

***PFOA and Gen X perturb some PMC subset genes and ectodermal patterning cues***

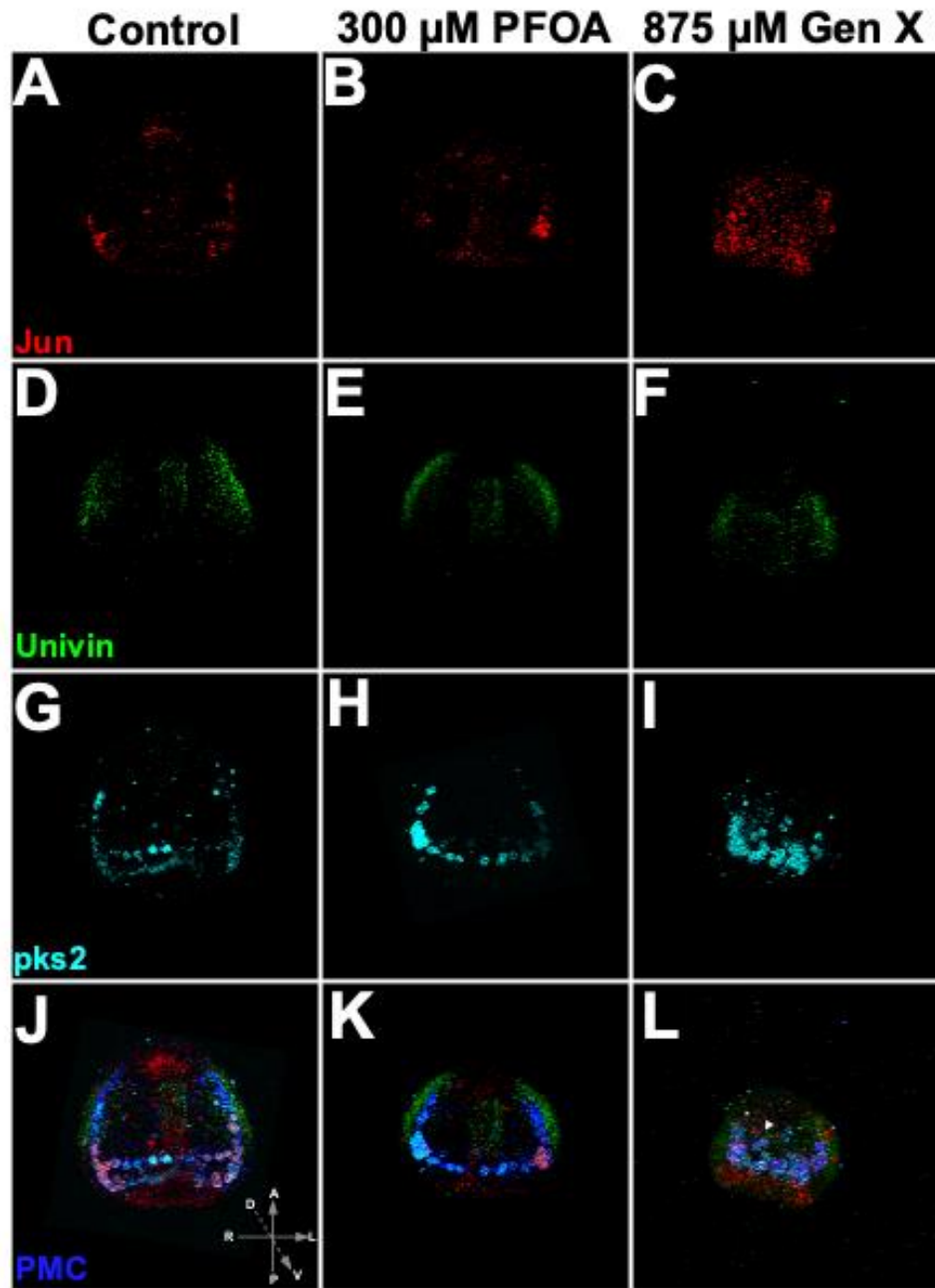
*L. variegatus* skeletal patterning is controlled by ectodermal expression of patterning cues, which include VEGF and Univin, with the corresponding receptors expressed by the PMCs (Duloquin et al., 2007; Piacentino et al., 2015, 2016). Our unpublished results show that VEGF activates Jun expression in the PMC clusters, while LOX activity restricts Pks2 expression primarily to the ventral midline by inhibiting it elsewhere. The other spicule matrix (SM) genes (including Msp130, "matrix-spicule protein 130") are well-known proteins that are incorporated into the biomineral skeletal spicules (Lyons et al., 2014). To investigate the effects of PFOA and Gen X on these ectodermal and PMC-specific patterning cues, the expression of Univin, Jun, Pks2, VEGF, VEGFR, Msp130, SM50, and SM30b were measured *in situ* using HCR-FISH (Figs. 6-8). In control embryos at 18 hpf, Jun is expressed primarily in the PMC clusters, as well as the apical pole (Fig. 6A). PFOA-treated embryos tend to exhibit unilateral reduction or loss of Jun (Fig. 6B), while Gen X-treated embryos show complete loss of Jun in the PMCs (Fig. 6C). Univin, which is expressed in the ectoderm along the cords and in the gut at 18 hpf (Fig. 6D), appears relatively unperturbed in PFOA-treated embryos (Fig. 6E), though some embryos exhibit a mild, unilateral reduction. In Gen X-

treated embryos, Univin expression overall is diminished (Fig. 6F). In control embryos, Pks2 is expressed primarily in the center of the ventral side of the ring, though it is weakly expressed in almost all PMCs (Fig. 6G). PFOA and Gen X-treated embryos exhibit diminished Pks2 signal in the central ventral ring, and many PFOA-treated embryos show increased Pks2 expression in one or both PMC clusters (Fig. 6H-I). The lack of restriction of Pks2 during PFOA and Gen X treatment may be indicative of a reduction of LOX signaling.

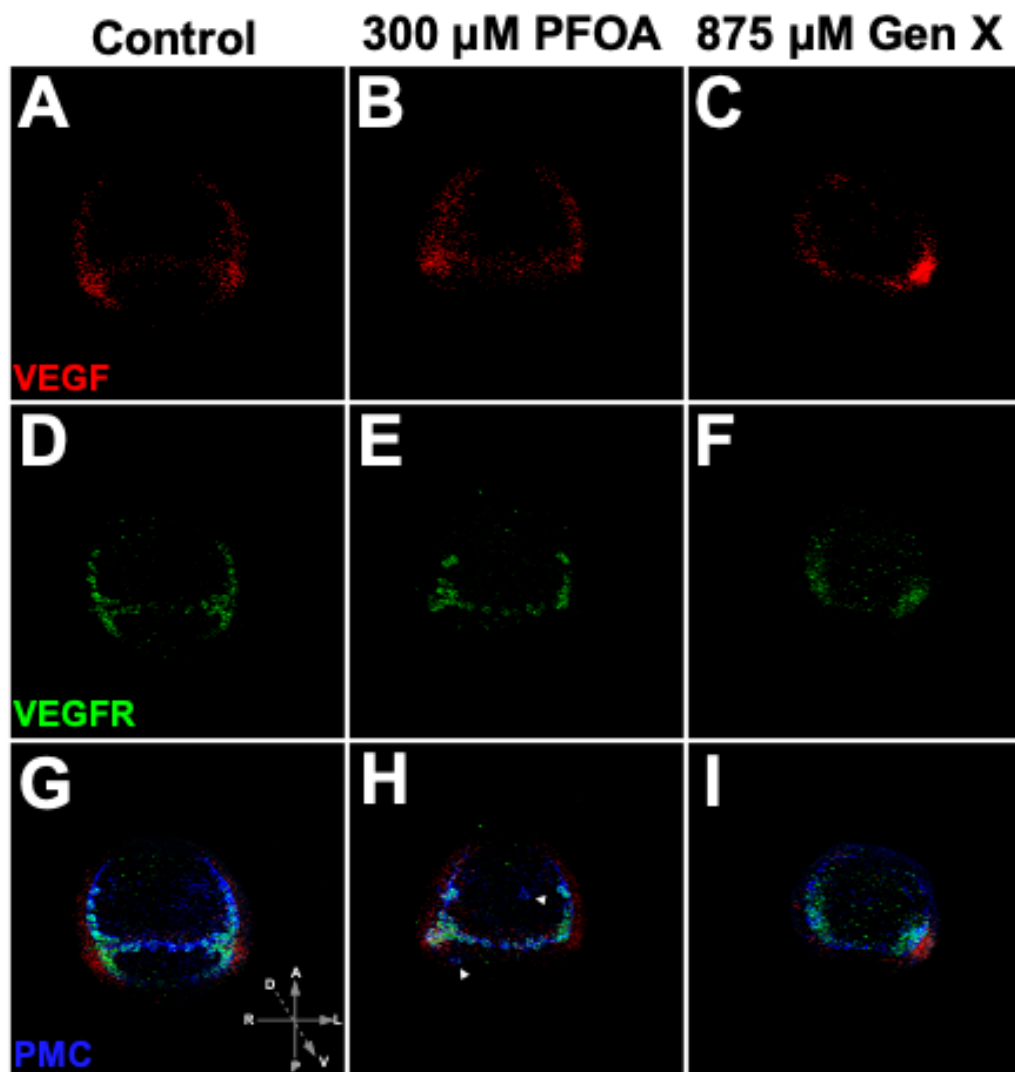
Given the perturbations to Jun expression in both PFOA- and Gen X-treated embryos, we expected similar perturbations to VEGF. VEGF is expressed in the ectoderm adjacent to the PMC clusters at 18 hpf (Duloquin et al., 2007; Piacentino et al., 2015, 2016) (Fig. 7A). Many PFOA- and Gen X-treated embryos show unilateral reduction of VEGF expression (Fig. 7B-C), consistent with the unilateral Jun expression losses observed with PFOA (Fig. 6B), and suggesting that, despite some VEGF expression, VEGF signaling does not successfully activate Jun transcription in Gen X-treated embryos. Strong DV perturbations cause radialization of VEGF expression (Duloquin et al., 2007); this is not the phenotype seen with Gen X treatment, suggesting that the effects of Gen X exposure on DV specification are mild. VEGFR, which is typically expressed in all PMCs and is brightest in the clusters and chords (Fig. 7D), is not perturbed with PFOA nor Gen X treatment (Fig. 7E-F).

In control embryos, spicule matrix protein-encoding genes are generally expressed by all PMCs. *Msp130* is most highly expressed in the PMC clusters and weakly marks the cords (Fig. 8A), *SM50* is expressed especially by the ventral PMCs and also marks the cord tips (Fig. 8D), while *SM30b* expression is strongest in the PMC clusters (Fig. 8G). These genes all appear control-like in both PFOA and Gen X-treated embryos (Fig. 8), except for *Msp130*, which is mildly expanded into the PMC ring with Gen X treatment (Fig. 8C).

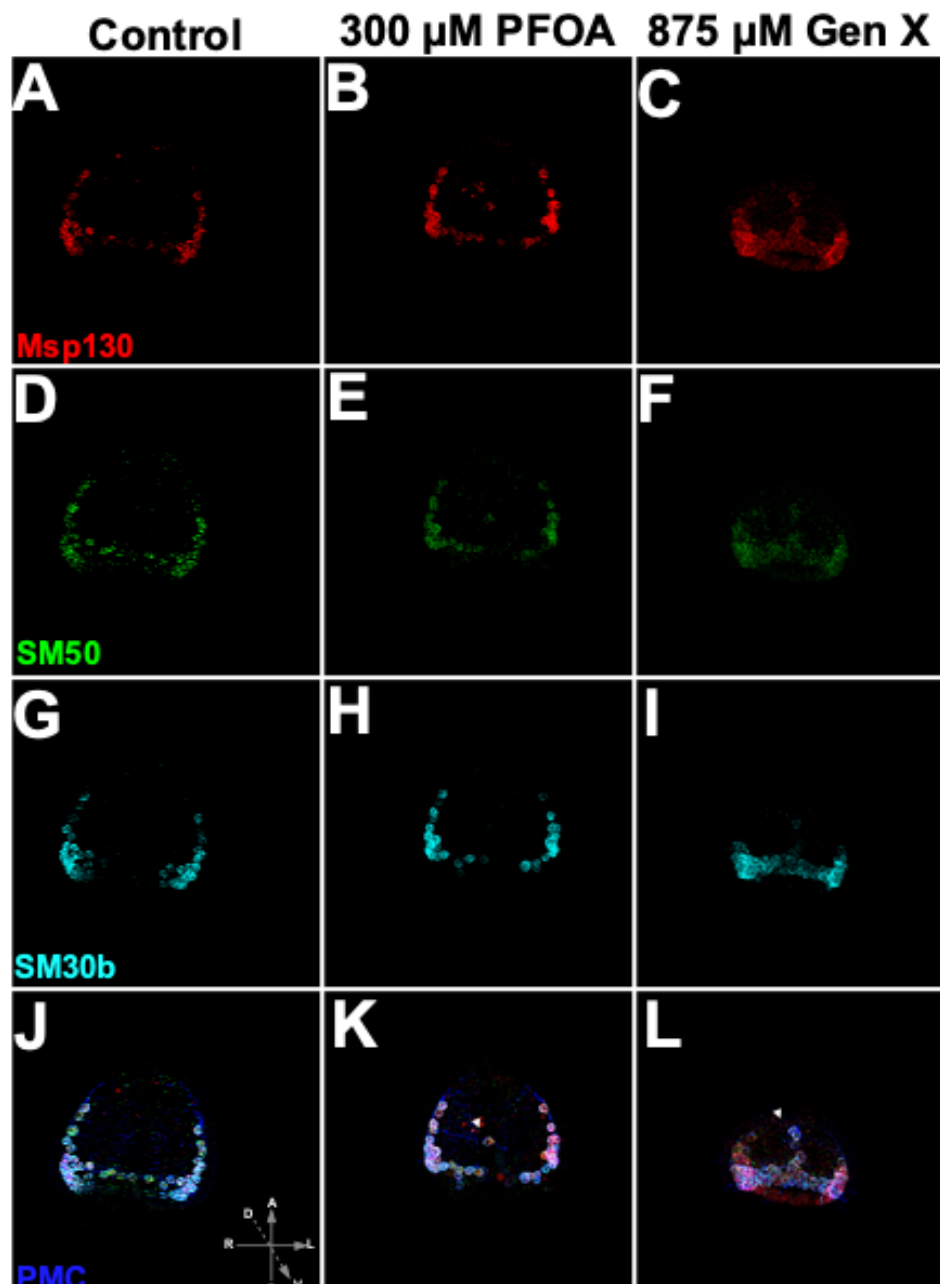
Both PFOA and Gen X-treated embryos frequently exhibit ectopically positioned PMCs (arrowheads in Fig. 6L, Fig. 7H, and Fig. 8K-L). Of the PMC subset genes analyzed in this experiment, *pks2*, *msp130*, and *SM50* are expressed in the ectopic PMCs, while *jun*, *VEGFR*, and *sm30b* are not.



**Figure 6: PFOA and Gen X are sufficient to perturb Jun, Univin, and Pks2 expression.** Control and treated embryos were subject to HCR FISH for Jun (red, A-C), Univin (green, D-F), and Pks2 (cyan, G-I) at 18 hpf. Merged images also show PMC staining (blue) in panels J-L. Arrowhead (L) shows ectopically positioned PMC.



**Figure 7: PFOA and Gen X are sufficient to perturb VEGF but not VEGFR expression.** Control and treated embryos were subject to HCR FISH for VEGF (red, A-C) and VEGFR (green, D-F) at 18 hpf. Merged images also show PMC immunostaining (blue, G-I). Arrowheads (H) show ectopically positioned PMCs.



**Figure 8: Most skeletal matrix genes are unaffected by PFOA or Gen X.** Control and treated embryos were subject to HCR FISH for Msp130 (red, A-C), SM50 (green, D-F), and SM30b (cyan, G-I) at 18 hpf. Merged images also show PMC staining (blue) in panels J-L. Arrowheads (K-L) show ectopically positioned PMCs.

## DISCUSSION

In this study, we characterize and compare the effects of the PFAS chemicals PFOA and Gen X on *L. variegatus* development. We demonstrate that both are sufficient to perturb *L. variegatus* skeletal patterning and they each impact the migration and patterning of the PMCs as well as the restriction of the ectodermal ciliary band. We establish that PFOA and Gen X act in distinct temporal windows, suggesting they differ mechanistically in the way they perturb skeletal patterning. The timing results and specific skeletal abnormalities combined with the impacts of PFAS exposure on ectodermal and PMC gene expression patterns suggest that PFOA more directly perturbs skeletal patterning, while Gen X acts earlier, albeit mildly, on the DV specification pathway, each of which results in skeletal patterning defects.

These results indicate that Gen X is not safer than PFOA, and in fact may be more teratogenic since it acts earlier in development and impacts axial specification. Gen X has a mild effect on DV genes and the ciliary band, without strongly affecting the serotonergic neurons. Chordin, whose expression was significantly reduced in Gen X-treated embryos, is necessary for non-serotonergic neuronal specification (Bradham et al., 2009). This suggests non-serotonergic neuronal specification could be perturbed by Gen X; this hypothesis will be tested via visualizing the expression of a pan-neural marker. Prior work achieved this with a monoclonal antibody which has since been lost; we will therefore obtain a substitute to label this important population. Gen X also perturbs

multiple ectodermal and PMC genes involved in skeletal patterning, such as *Univin*, *VEGF*, *Jun*, *Pks2*, and *Msp130*. The complete loss of *Jun* in the PMCs at 18 hpf in Gen X-treated embryos is interesting. First, *Jun* is a marker of PMC clusters. Since Gen X-treated embryos tend to exhibit larger PMC clusters than control embryos, this indicates that *Jun* is not required for PMC clustering. However, *Jun* may be required to inhibit other PMCs from adopting a cluster identity to maintain a small cluster size.

Alternatively, this may reflect either the absence of *VEGF* signaling or an inability of *VEGF* to specifically activate *Jun* expression. Equally surprising is Gen X's effect on *Msp130* transcription, which encodes a spicule matrix protein found in the PMCs.

*Msp130* expression is expanded in Gen X-treated embryos, suggesting Gen X may perturb a different ectodermal patterning gene outside of *Univin* and *VEGF*.

*PFOA* acts much later in development than Gen X, suggesting that it directly perturbs ectodermal and PMC patterning cues rather than effecting DV specification. It perturbs the expression of *Univin*, *Pks2*, *Jun*, and *VEGF*; these perturbations likely cause the PMCs to migrate improperly in *PFOA*-treated embryos. The effects of *PFOA* on the expression of DV genes and the spatial restriction of the ciliary band were surprising, and in combination with the timing results, remain difficult to explain. *PFOA* begins perturbing *L. vareigatus* development around 18 hpf, after the DV axis has already been specified (Bradham & McClay, 2006; Hardin et al., 1992; Piacentino et al., 2015).

Further repetitions of the time course experiments and an expansion of the DV FISH experiments will be necessary to reconcile these results.

Overall, these results identify both PFOA and Gen X as teratogens to echinoderms. This is likely to become an ecological issue as environmental concentrations of these chemicals rise, especially given the lack of regulation surrounding Gen X. These results have also provided some insights into how PFOA and Gen X act mechanistically on developing organisms. Recent studies have also suggested differences in their toxicological mechanisms (Bangma et al., 2020; Blake et al., 2020; Wen et al., 2020). In a study based in a placental trophoblast model, Bangma et al. found that while the parent chemical for Gen X accumulates intercellularly at lower levels than PFOA, it has comparatively stronger effects on gene expression (Bangma et al., 2020; Bischel et al., 2011). Additionally, Blake et al. found that the placenta is more sensitive to the adverse effects caused by Gen X than by PFOA, further suggesting that the two chemicals have distinct reproductive toxicity mechanisms.

Further confirmation and clarification of these mechanisms could be achieved by performing transcriptomics or single-cell transcriptomics on embryos treated with these chemicals. This would be the most efficient way to understand how each chemical impacts gene expression to produce their effects, as well as defining their tissue(s) of action. Because sea urchins are evolutionarily conserved with a gene complement that is similar to vertebrates and mammals (Sodergren et al., 2007), these mechanistic findings

are highly likely to be relevant both ecologically and to more complex species, including humans.

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**VITA**

