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Investigation of biomolecular condensates as novel targets mediating germ cell toxicity from per- and polyfluoroalkyl substance exposure

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Investigation of biomolecular condensates as novel targets mediating germ cell toxicity from per- and polyfluoroalkyl substance exposure

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Molecular Toxicology

by

Abigail Pendleton Bline

2022

ABSTRACT OF THE DISSERTATION

Investigation of biomolecular condensates as novel targets mediating germ cell toxicity from per- and polyfluoroalkyl substance exposure

by

Abigail Pendleton Bline

Doctor of Philosophy in Molecular Toxicology

University of California, Los Angeles, 2022

Professor Patrick Allard, Chair

Per- and polyfluoroalkyl substances (PFAS) are among the most prevalent and persistent environmental toxicants worldwide. The scale of human exposure to PFAS is vast and available data indicate that PFAS can target most organ systems in the body and produce a wide range of health effects. The molecular mechanisms eliciting these effects are incompletely understood and further characterization is needed to better protect public health. Considering that many PFAS are surface active substances, they have the potential to alter phase separation dynamics within a cell and may particularly target biomolecular condensates (BCs), subcellular compartments that are dependent upon their physical organization to execute their regulatory functions. In this work, the reproductive system of *Caenorhabditis elegan*s was used as a model to examine the ability of two surfactant PFAS, PFOS and F-53B, to disrupt BC structure and function. Both

PFOS and F-53B were found to alter the structure of P granules and the synaptonemal complex (SC), two BCs essential for proper germ cell development in *C. elegans*. In sensitized P granule and SC mutants, PFOS and F-53B exposures exacerbated infertility and embryonic lethality defects. These results indicate that the impacts of PFAS exposure on BC structure have functional consequences and that BCs may play a role in mediating PFAS-induced reproductive toxicity. Furthermore, this work suggests it is plausible that BC structural disruption could serve as a molecular initiating event for PFAS-induced toxicity in other cell types and organisms.

The dissertation of Abigail Pendleton Bline is approved.

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2022

Dedicated to my husband Andy and my orange cat Orange Cat

TABLE OF CONTENTS

Chapter 1: Introduction to the Dissertation 1

Impetus for the Dissertation Project 2

Per- and Polyfluoroalkyl Substances 3

Biomolecular Condensates 7

Hypothesis and Aims of the Dissertation 14

Chapter 2: The Importance of Germ Cells in Toxicology 17

Introduction 18

What is Lost in the Weismann Barrier 18

Chapter 3: Characterization of Effects of PFAS on Germ Cell Development and Reproduction in Caenorhabditis elegans 31

Introduction 32

Methods 34

Results 37

Discussion 40

Conclusion 42

Chapter 4: Biomolecular Condensates as Potential Mediators of Reproductive Toxicity from PFAS Exposure 55

Introduction 56

Methods 58

Results 62

Discussion 65

Conclusion 70

Chapter 5: Summary and Conclusions 82

References 85

LIST OF FIGURES

Chapter 3

Figure 1 44
Figure 2 46
Figure 3 48
Figure 4 49
Figure 5 50
Figure 6 52
Supplemental Figure 1 53
Supplemental Figure 2 54

Chapter 4

Figure 7 71
Figure 8 73
Figure 9 75
Figure 10 77
Figure 11 78
Figure 12 80

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[1] Bline AP, Le Goff A, Allard P. 2020. What is lost in the Weismann barrier? Journal of Developmental Biology. 8(4):35. doi:10.3390/jdb8040035.

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CHAPTER 1:

Introduction to the Dissertation

IMPETUS FOR THE DISSERTATION PROJECT

According to the United States Environmental Protection Agency (USEPA), there are over 12,000 per- and polyfluoroalkyl substances (PFAS) known to exist (USEPA 2022a). Because of the design of the current regulatory framework in the US, the vast majority of PFAS have little toxicity data available or lack toxicity data entirely. Under the Toxic Substances Control Act (TSCA), chemicals initially produced prior its passage in 1976 were presumed safe and exempt from requiring toxicological review (Richter et al. 2021). For new chemicals produced after this time, the pre-manufacture notices that must be submitted to the USEPA prior to production are not required to include toxicity data (Richter et al. 2021). As a result, despite the fact that PFAS are widely distributed in the environment, present in an array of industrial and consumer products, and regularly detected in human blood and tissues, remarkably little is known about the how the vast majority of these substances impact human bodies.

The enormous scale of PFAS production and human exposure presents daunting toxicological and public health challenges. Retroactively conducting in-depth toxicological assessments of each of the thousands of PFASs that have already been put into production is not economically or practically feasible. An alternative approach would be to evaluate PFAS toxicity as a group based upon a broadly shared physicochemical property and determine if this property may produce a common biological effect. Specifically, many PFAS have been used because they are particularly effective surfactants (Glüge et al. 2020), compounds that preferentially partition to phase boundaries and reduce surface tension. Within a cell, biomolecular condensates (BCs) have the potential to be especially sensitive to the effects of surfactants since their assemblage and stability are dependent upon relatively weak intermolecular forces, such as electrostatic and hydrophobic interactions, that can be altered by surfactants (Aguirre-Ramírez et al. 2021). In this

work, BCs are presented as potential molecular targets in germ cells that could be impacted in similar ways by many PFAS due to their shared surfactant properties.

PER- AND POLYFLUOROALKYL SUBSTANCES

Overview

PFAS are anthropogenic chemicals that have been commercially manufactured since 1949 (Richter et al. 2018). While it is generally agreed that there are thousands of different PFAS compounds, precisely what a PFAS is does not have a uniform definition. PFAS was originally defined as "aliphatic substances containing one or more C atoms on which all the H substituents present in the nonfluorinated analogues from which they are notionally derived have been replaced by F atoms" (Buck et al. 2011). In recent years, alternative definitions have been developed by the Organization for Economic Cooperation and Development (OECD), the USEPA, and US state legislatures, with the most expansive definition being any organic chemical with at least one fully fluorinated carbon atom (Hammel et al. 2022). For the purposes of this dissertation, the definition proposed by the USEPA Office of Pollution Prevention and Toxics (OPPT) is used:

> PFAS...means any chemical substance or mixture that structurally contains the unit R-(CF2)-C(F)(R′)R″. Both the CF2 and CF moieties are saturated carbons. None of the R groups (R, R′ or R″) can be hydrogen. (40 CFR Part 705 §705.3)

PFAS by this definition have been and continue to be used in countless consumer products and industrial processes. In consumer products, PFAS are mainly used in surface coatings (notably Scotchgard and Teflon) or in product formulations to bestow them with stain,

grease, and/or water resistance (Wang et al. 2017; Whitehead 2021). PFAS-treated consumer products include food packaging, cosmetics, paints, cookware, and clothing (Kwiatkowski et al. 2020; Whitehead et al. 2021). Industrially, PFAS are used in aerospace, automotive, chemical, electronics, and semiconductor manufacturing, as well as in metal plating and oil and gas extraction (USEPA 2022b). They're also used in firefighting foams and in pesticide formulations (Wang et al. 2017). Part of what makes PFAS desirable in so many different applications is their high stability, which is attributable to the strength of the C-F bond (Glüge et al. 2020). However, this stability also makes them extremely resistant to both abiotic and biotic transformation, and what transformations do occur mainly generate other PFAS (ITRC 2021). While manufacturing of the "legacy" PFAS perfluorooctane sulfonic acid (PFOS) and perfluorooctanoic acid (PFOA) was globally restricted after their listing as persistent organic pollutants (POP) under the Stockholm Convention in 2009 and 2019, respectively, other PFAS have been used as replacements for these compounds (Ng et al. 2021) and PFAS production and use remains largely unrestricted (Richter et al. 2021).

Human exposures and health effects

Consistent with the widespread use and environmental persistence of PFAS, human exposure is common and nearly all people in the US have detectable levels of PFAS in their blood (Jian et al. 2018; CDC 2017). In addition to direct contact with PFAS-treated consumer products, human exposures can occur from PFAS-contaminated house dust, food, and drinking water (Jian et al. 2017; Boone et al. 2019). After entering the body via ingestion, inhalation, or dermal transfer, PFAS can distribute widely throughout the body (ATSDR 2018) and have been detected in all tissues and fluids tested, including lung, liver, brain, bone, seminal fluid, follicular fluid, and amniotic fluid (Pérez et al. 2013; Stein et al. 2012; Cui et al. 2020; Kang et al. 2020).

However, PFAS tend to be detected at highest concentrations in the blood, liver, and kidneys, in part to their preferential binding to serum albumin and other proteins (ATSDR 2018). Concentrations of individual PFAS compounds detected in blood vary but generally range from 0.1-10ng/mL (Jian et al. 2018), though concentrations exceeding 100ng/mL in blood have be detected in occupationally exposed or other highly exposed populations (Gomis et al. 2016; Li et al. 2018).

PFOS and PFOA continue to be among the most ubiquitously detected PFAS in human samples, despite the restrictions in place on their production (Jian et al. 2018; CDC 2017). However, most biomonitoring studies typically analyze samples for twenty or fewer targeted PFAS and do not quantify any of the other thousands of PFAS. Non-targeted analyses have indicated that unidentified PFAS may constitute 50% or more of the total organic fluorine (TOF) in present blood samples (Xiao et al. 2017), suggesting that many additional PFAS may be present in human bodies but not routinely quantified. However, it should be noted that over 300 pharmaceutical compounds are fluorinated and could contribute to the TOF load detected (Hammel et al. 2022). Individual PFAS that have been analyzed and studied have been linked to a wide array of human health effects, including altered immune and thyroid function, liver disease, lipid and insulin dysregulation, kidney disease, adverse reproductive and developmental outcomes, and cancer (Fenton et al. 2020). However, the mechanisms by which PFAS exposure leads to these effects remain incompletely understood (Ng et al. 2021).

Mechanisms of PFAS toxicity

The most extensively studied potential mechanism underlying PFAS-induced toxicity is modulation of nuclear receptor activity, with a particular focus on peroxisome proliferator activated receptor (PPAR) isoforms. Examination of nuclear receptor activity modulation has

been facilitated by high-throughput *in vitro* assays, including those developed under the USEPA's Toxicity Forecaster (ToxCast) project. Of 142 individual PFAS tested with ToxCast, 69 exhibited activity modulation for one or more nuclear receptors, including PPARα, estrogen receptor-α (ER-α), and Pregnane X receptor (PXR) (Houck et al. 2021). The half-maximal activity concentrations (AC_{50}) for most of the PFAS-receptor combinations exceeded 10μM, which is approximately 1,000-10,000 higher than the concentrations typically detected in human blood and tissues (Houck et al. 2021). Another study similarly found AC_{50} values for several PFAS-receptor combinations exceeded 25μM, leading the authors to question whether nuclear receptor activation is a human-relevant toxicity mechanism (Behr et al. 2020).

Other proposed mechanisms of PFAS-induced toxicity include increased oxidative stress, dysregulation of mitochondrial function, and inhibition of gap junction intercellular communication (GJIC) (ATSDR 2018), all of which may be a consequence of effects on phospholipid bilayers. PFAS have been shown to be capable of partitioning into phospholipid bilayers, increasing bilayer permeability, and/or altering bilayer fluidity (Fitzgerald 2018; Hu et al. 2003; Kleszczyński & Składanowski 2009; Liu et al. 2016; Starkov & Wallace 2002; Droge et al. 2019). The ability of PFAS to incorporate into lipid bilayers appears to vary by PFAS chain length and functional group (Nouhi et al. 2018). PFAS may also sorb to the surface of phospholipid bilayers and exert effects through their surface activity (Droge et al. 2019).

Physicochemical properties

Surface activity is a key PFAS feature and many PFAS are particularly effective surfactants (Glüge et al. 2020), preferentially partitioning to phase boundaries (Aguirre-Ramírez et al. 2021). The surface activity of PFAS is due to their amphiphilic structures, with heads

consisting of a hydrophilic functional group and tails consisting of hydrophobic carbon-fluorine tails (ITRC 2021). The unique properties of carbon-fluorine bonds also make PFAS chemically stable and subject to interacting weakly with other molecules via Van der Waals forces and hydrogen bonding (ITRC 2021). The functional head groups of PFAS can bestow them with ionic properties and most polyfluoroalkyl acids (PFAAs) are fully ionized at physiological pH ranges, leading to their tendency to bind to proteins (ITRC 2021). PFAS are generally soluble in water, though PFAS with longer carbon-fluorine chains are less soluble than those with shorter chains (ITRC 2021). The physicochemical properties of PFAS, especially their surface activity, have potential implications for physiological phase separation in cells and the behavior of biomolecular condensates.

BIOMOLECULAR CONDENSATES

Overview

BCs are dynamic assemblages of proteins and nucleic acids that can have liquid- or gel-like properties (Holehouse & Pappu 2018). BCs are found in all eukaryotes and are involved in a diverse range of cellular functions, including transcriptional and post-transcriptional gene regulation (Brangwynne 2011; Buchan et al. 2014). Also termed membraneless organelles, rather than using a membrane, BCs compartmentalize their constituents into a dense phase that demixes from the surrounding bulk phase (Holehouse & Pappu 2018). The concentration of biomolecules into the dense phase likely facilitates interactions between BC constituents and the functional roles that BCs perform (Brangwynne 2011). In cells, phase separation is largely driven by hydrophobic and electrostatic interactions between RNA and multivalent proteins with intrinsically disordered regions (IDRs) (Vernon et al. 2018; Rana et al. 2021; Garcia-Jove

Navarro et al. 2019). Protein and RNA concentrations, as well as their post-translational and post-transcriptional modifications, respectively, are important in modulating phase separation behavior and droplet size (Van Treeck & Parker 2018; Hofweber & Dormann 2019; Ries et al. 2019). Temperature, salt concentration, pH, and other aspects of the cellular environment also influence phase separation (Rana et al. 2021). However, active regulation of BCs can also be achieved via molecular chaperones and RNA helicases (Snead & Gladfelter 2010). Changes in the biophysical properties of BCs can affect their regulatory functions and activity (Elbaum-Garfinkle et al. 2015). The ability of BC behavior and biophysical properties to be modulated by a variety of factors has led some to propose that BCs function as tunable signaling hubs that respond quickly and with high sensitivity to changing cellular conditions (Nott et al. 2015; Holehouse & Pappu 2018).

Germ granules

All metazoans appear to have germ cell-specific BCs, termed germ granules, that share certain components, including DEAD-box RNA helicases, (particularly VASA orthologs), P-element wimpy testis (PIWI) orthologs, PIWI-interacting RNA (piRNA), Tudor domain-containing proteins, and translation initiation complex proteins (Arkov & Ramos 2010; Schisa 2012; Whittle & Extavour 2017). Different germ granules also appear to share similar functions, including translational regulation, mRNA storage and metabolism, and small RNA pathway modulation (Schisa 2012).

Germ granules have been extensively studied in the nematode *Caenorhabditis elegans*, with P granules being among the best characterized. In the one-cell zygote, P granule components are distributed throughout the cytoplasm (Wang et al. 2014). Upon symmetry

breaking, P granule components are enriched in the posterior region of the zygote due to a countering gradient created by accumulation of MEX-5 in the anterior region (Marnik & Updike 2019). As the P granule component MEG-3 binds to RNA, it promotes condensation of granules that recruit PGL-1 (Lee et al. 2020). During the first cellular divisions, the posterior cytoplasm containing P granules partitions asymmetrically with the P cell lineage to the P4 cell, the founding primordial germ cell (PGC) that divides symmetrically into Z2 and Z3 cells (Strome 2005). Sometime between the 2- and 8-cell stage, P granules become localized to the nuclear periphery and specifically associate with nuclear pores (Marnik & Updike 2019). The localization of P granules to nuclear pores is dependent upon active transcription resulting from zygotic genome activation as well as association with the VASA ortholog GLH-1 (Sheth et al. 2010). Once perinuclear, P granules appear to lose their outer MEG-3 shell while maintaining a PGL-1/3 core (Wang et al 2014). P granules remain perinuclear during germ cell proliferation in *C. elegans* larvae as well as in the mitotic and early meiotic regions of the adult gonad, ensuring that they are distributed to each immature germ cell despite the shared syncytium of the gonad (Marnik & Updike 2019). As individual oocytes cellularize and become transcriptionally quiescent in the proximal adult gonad, P granules detach from the nucleus and are freed into the cytoplasm but remain intact (Sheth et al. 2010). P granules are lost entirely in spermatogonia (Sheth et al. 2010). Immediately following fertilization, P granules from the mature oocyte dissociate into the cytoplasm before being reassembled in the zygote, marking the oocyte-to-zygote transition (Wang et al. 2014).

Although P granules segregate with P lineage cells during embryonic development, they are not required to specify germ cells (Seydoux 2018). Rather, the primary functions of P granules appear to be the suppression of the somatic program, maintenance of totipotency, and

promotion of genomic integrity in germ cells (Updike et al. 2014; Knutson et al. 2017). These functions of P granules are supported by studies that completely abrogate P granules by using RNA interference (RNAi) to knock down in combination *pgl-1*, *pgl-3*, *glh-1*, and *glh-4,* four core P granule proteins; knock down or knockout of any of these genes in isolation leads to smaller, but not completely absent, P granules with altered function (Updike et al. 2014). Worms subject to this P granule RNAi treatment still produce proliferating germ cells, but these germ cells fail to initiate oogenesis and aberrantly express sperm and somatic cell-specific transcripts (Updike et al. 2014). Therefore, loss of P granules can lead to loss of germ cell identity and other germ cell defects.

The role of P granules in maintaining germ cell identity may in part be linked to their perinuclear localization, which allows them to efficiently engage in post-transcriptional regulation of mRNAs exported through the nuclear pore (Sheth et al. 2010). Numerous proteins involved in post-transcriptional regulation are constituent components of P granules, including different Argonaute and Pumilio proteins (Phillips & Updike 2022). P granules harbor several Argonaute proteins, including ALG-5, CSR-1, and PRG-1, as well as their associated small RNA (sRNA) guides, micro RNAs (miRNA), endogenous small interfering RNAs (endo-siRNA), and piRNAs, respectively (Dallaire et al. 2018; Brown et al. 2017; Gerson-Gurwitz 2016; Batista et al. 2008; Spichal et al. 2021). Different Argonaute-sRNA complexes can vary in their effects on the mRNA they target, including suppressing somatic gene expression, licensing germline gene expression, or recognizing non-self genes (Updike et al. 204; Gerson-Gurwitz 2016; McEnany et al. 2022). Additionally, licensing by binding to CSR-1 specifically protects mRNA from transcriptional silencing that is initiated by binding to PRG-1 (Cornes et al. 2022). Binding of mRNA to PRG-1 (*C. elegans* PIWI ortholog) in P granules initiates the generation of secondary

siRNA that can be transported into the nucleus, target nascent transcripts, and recruit the NRDE-1/2/4 complex, which in turn recruits histone modifying enzymes (Spichal et al. 2021; Mao et al. 2015). The deposited post-translational modifications (PTMs) on the histone tails can alter chromatin conformation (as discussed further below) and influence transcription. Aside from directing histone tail PTMs, Argonaute-sRNA complexes in P granules can also mediate transgenerational inheritance by regulating the heritable pool of sRNAs (Lev et al. 2019; Dodson & Kennedy 2019).

The Pumilio protein FBF-2 binds to the mRNA of several other constituent P granule proteins as well as the mRNA of genes regulating the cell cycle and meiosis (Prasad et al. 2016). Another Pumilio protein, PUF-8, also binds to mRNA of genes involved in regulating the cell cycle in addition to genes important for maintaining germ cell identity (Mainpal et al. 2011). Both FBF-2 and PUF-8 are capable of either promoting or suppressing translation of their mRNA targets, and their specific activity appears to be dependent on their recruitment of different protein cofactors (Wang & Voronina et al. 2020). In the case of FBF-2, binding to core P granule protein PGL-1 appears to be important for its repressive activity (Voronina et al. 2012). Repressive activity of the Argonaute protein WAGO-1 is also also dependent on binding to PGL-1, specifically in the context of a perinuclear P granule (Aoki et al. 2021). The physiological functioning of both Argonaute-sRNA complexes and Pumilio proteins appears to be dependent on their localization to P granules (Ouyang & Seydoux 2022; Voronina et al. 2012), highlighting the importance of P granule structure in regulating the germ cell program and producing functional gametes.

Aside from the P granules of *C. elegans*, other germ granules have been well studied in *Drosophila melanogaster*, *Danio rerio*, and *Xenopus laevis*. However, less is known about germ

granules in mammals. In XX human embryos, the onset of meiosis in germ cells at 14–17 weeks of gestation corresponds with the formation of cytosolic punctae that contain the RNA-binding protein fragile X mental retardation protein (FMRP) (Rosario et al. 2016). FMRP punctae at least partially overlap with GW182, which interacts with Argonaut proteins in miRNA-mediated gene silencing (Rosario et al. 2016). Further granule dynamics later in oocyte development are unknown. In prospermatogonia of embryonic mouse testes, components of the piRNA, miRNA, and siRNA pathways localize to perinuclear granules that resemble P granules (Watanabe et al. 2011). Adult mouse and human XY germ cells contain chromatoid bodies (CBs), perinuclear granules that are conserved in other mammals (Ginter-Matuszewska et al. 2011). CBs contain miRNA, miRNA-processing proteins, the VASA ortholog DDX4, and other RNA-binding proteins (Messina et al. 2012). While germ granules across different organisms vary in their exact composition and dynamics during germ cell development, they appear to share a common function in post-transcriptional regulation of RNA that is essential for proper germ cell development and function (Voronina et al. 2011).

Other BCs

In both *C. elegans* and mice, the constituent proteins of germ granules exhibit a high degree of overlap with P bodies and stress granules (Gallo et al. 2008; Anbazhagan et al. 2022), additional cytoplasmic BCs present in both germ and somatic cells. P bodies harbor mRNA associated with proteins involved in translational repression and 5'-to-3' mRNA decay, though they appear to act primarily as sites of mRNA storage (Luo et al. 2018; Standart & Weil 2018). While P bodies exist under ambient conditions, stress granules are induced in response to acute abiotic or biotic stress, including oxidative stress, thermal stress, and viral infection (Advani & Ivanov 2019). These acute stress conditions initiate the integrated stress response, leading to

translational arrest, disassembly of the polysome, ribosomal runoff from mRNA, and free mRNA in the cytosol (Reineke & Neilson 2019). Free mRNA initiates the formation of stress granules (SGs) through binding to and oligomerizing the key SG nucleating proteins TIA-1/TIAR and G3BP1/2 (Yang et al. 2019). Mature stress granules contain a mixture of proteins, mRNA, miRNA, and long non-coding RNA (lncRNA) (Mahboubi & Stochaj 2017). It appears that SGs serve to both facilitate a stress-specific translational program (Matsuki et al. 2013) as well as sequester and protect translationally stalled mRNA (Yang et al. 2019). SGs promote survival in the short term but, under chronic stress conditions, form irreversible, persistent aggregates that can lead to pathology and cell death (Reineke & Neilson 2019). These effects highlight the importance of BC dynamics in physiology and pathophysiology.

Aside from cytoplasmic BCs, several nuclear BCs additionally exist. The genome itself has been described as an "emulsion of condensates" consisting of multiple BCs across hierarchical levels (Feric & Misteli 2021). These BCs include histone tail-driven condensates, transcriptional condensates, topologically associating domains (TADs), and heterochromatin at the largest scale (Feric & Misteli 2021). Histone tail-DNA condensates specifically appear capable of undergoing liquid-liquid phase separation, the dynamics of which can be modulated by a range of histone tail PTMs (Gibson et al. 2019; Ghoneim et al. 2021). The changes in chromatin conformation caused by different histone tail PTMs can make stretches of DNA more or less accessible to chromatin-associated proteins and complexes, ultimately affecting transcriptional activity (Ghoneim et al. 2021).

A germ cell-specific nuclear BC is the synaptonemal complex (SC). Early in meiosis, the SC forms between paired homologous chromosomes, where it both stabilizes the homolog pairs and recruits additional signaling molecules (Libuda et al. 2013; Gordon et al. 2021). While the

SC was originally conceptualized as a fixed structure due to its rigid, ladder-like appearance, more recent research indicates that the SC is a dynamic, liquid-like structure that likely undergoes liquid-liquid phase separation (Rog et al. 2017). As a BC, assembly of the SC is dependent on hydrophobic and electrostatic interactions (Rog et al. 2017). The SC recruits DNA repair as well as crossover-promoting factors and ensures proper chromosome segregation in mature gametes (Rog et al. 2017; Gordon et al. 2021). Loss of any SC core component and/or alteration to the SC structure leads to severe chromosome segregation errors (Libuda et al. 2013; Gordon et al. 2021).

In summary, BCs serve multiple regulatory roles essential for maintaining germ cell identity, promoting germ cell development, and generating functional gametes competent for reproduction. Alterations to BC structure and/or composition can impair their functions, compromising germ cell integrity. Therefore, substances that are capable of altering BCs have the potential to cause germ cell toxicity.

HYPOTHESIS AND AIMS OF THE DISSERTATION

Hypothesis and aims

The central hypothesis of this dissertation is that the surfactant activity common to many PFAS alters BC structure and function, leading to cell- and tissue-level dysfunction. To test this hypothesis, two PFAS employed specifically for their surfactant properties were used to examine the role of BCs in mediating PFAS toxicity using the reproductive system of *C. elegans* as a model. Since limited data are available on PFAS toxicity to *C. elegans* germ cells, the first aim of the dissertation was to characterize the toxic effects of PFAS exposure on germ cell development

and reproduction. The second aim was to examine the effects of PFAS exposure on two germ cell BCs and their potential to act as mediators of germ cell and reproductive toxicity.

PFAS selection

Of the targeted PFASs commonly analyzed in biomonitoring samples in the United States over the past ten years, PFOS continues to be detected most consistently and at highest concentrations (Jian et al. 2018; CDC 2017). Despite the fact that production of this PFAS has been phased out in the United States, human exposure continues due to its persistence in the environment and long elimination half-life in the human body. Even though PFOS is one of the most studied PFAS, there are still gaps in understanding of its mechanisms of toxicity. Due to its prevalence in the environment and biota, as well as the limited knowledge on its mechanisms of toxicity, PFOS was selected as one of the PFAS to test in the subject research.

Non-targeted PFAS analysis of biomonitoring samples collected in China first indicated that chlorinated polyfluorinated ether sulfonates (Cl-PFAES) may constitute a substantial fraction of the organic fluorine in the general Chinese population (Shi et al. 2016). Some of these detections may be linked to the widespread use of the PFAS F-53B in China since the late 1970s (Liu et al. 2018). F-53B is a Cl-PFAES-based formulation composed of about 90% 6:2 Cl-PFAES and 10% 8:2 Cl-PFAES by weight (Ti et al. 2018). Note that F-53B will be used interchangeably with its primary component 6:2 Cl-PFAES in the rest of the dissertation. F-53B is mainly used as a mist suppressant in the chrome plating industry with approximately 60% of Chinese chrome plating plants using F-53B and an estimated 13 metric tons of F-53B produced in China in 2015 (Ti et al. 2018). Similar to PFOS, F-53B is resistant to degradation in the environment and has been detected in environmental media and wildlife globally (Wang et al. 2013; Shi et al. 2018). F-53B is also estimated to have an extremely long elimination half-life at

15.3 years, which is more than twice that of PFOS (Shi et al. 2016). Since there is extensive human exposure to F-53B, F-53B shares many similarities with PFOS, and relatively few studies of F-53B toxicity are available, F-53B was selected as the second PFAS to test in the subject research.

BC selection

It has previously been shown that both P granules and the SC can be disrupted by treatment with 1,6-hexanediol, an aliphatic alcohol surfactant (Updike et al. 2011; Rog et al. 2017). Additionally, the effects of genetic loss of P granule and SC constituents on germ cell development and reproduction have been well characterized. Furthermore, transgenic fluorescent reporter and loss-of-function mutant strains of *C. elegans* are available for core P granule and SC proteins from the Caenorhabditis Genetics Center (CGC) at the University of Minnesota. Since their basic biology is fairly well understood, and since they are essential for the production of functional gametes, P granules and the SC were selected as model BCs to test in a toxicological context.

Dissertation organization

The remainder of the dissertation is organized into four additional chapters. Chapter 2 discusses how a long-held conceptual model for germ cell identity and heritability has limitations and inaccuracies, particularly when evaluating the importance of toxicant exposures to germ cells. Chapters 3 and 4 present the findings of experiments designed to address aims 1 and 2, respectively. Chapter 5 provides a summary and conclusions of the dissertation.

CHAPTER 2:

The Importance of Germ Cells in Toxicology

INTRODUCTION

Germ cells are the sole progenitors of each successive generation of sexually reproducing animals. Each generation, germ cells must be specified, proliferate, and undergo a germ cell-specific developmental program to produce functional gametes capable of reproducing. All of these processes must be tightly regulated to ensure germ cell competency. While germ cells have many mechanisms for maintaining cellular homeostasis, chemical toxicants and other stressors can lead to dysregulation with negative effects on reproductive outcomes. Furthermore, germ cell toxicants are able to not only impact the fertility of the initially exposed generations but can also have negative effects on all aspects of organismal health in later generations originating from those cells. Gaining a better understanding how germ cells respond to stress induced by environmental toxicants should be a pressing goal in the field of toxicology. However, standard testing paradigms for reproductive toxicity are generally poorly equipped to adequately evaluate germ cell effects and the temporal scales at which effects may persist.

WHAT IS LOST IN THE WEISMANN BARRIER

The Weismann barrier is a conceptual model of germs cells as fundamentally separable from the somatic cells by which they are surrounded. The Weismann barrier also positions germ cells as isolated from broader environmental influences, which would imply that they are not susceptible to toxicant effects. The following article, published the *Journal of Developmental Biology*, presents a case for why the Weismann barrier is an outdated and counterproductive model for understanding germ cell biology, particularly in the context of toxicant exposures and intergenerational inheritance.

Journal of Developmental Biology

MDPI

Review

What Is Lost in the Weismann Barrier?

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Abstract: The Weismann barrier has long been regarded as a basic tenet of biology. However, upon close examination of its historical origins and August Weismann's own writings, questions arise as to whether such a status is warranted. As scientific research has advanced, the persistence of the concept of the barrier has left us with the same dichotomies Weismann contended with over 100 years ago: germ or soma, gene or environment, hard or soft inheritance. These dichotomies distract from the more important questions we need to address going forward. In this review, we will examine the theories that have shaped Weismann's thinking, how the concept of the Weismann barrier emerged, and the limitations that it carries. We will contrast the principles underlying the barrier with recent and less recent findings in developmental biology and transgenerational epigenetic inheritance that have profoundly eroded the oppositional view of germline vs. soma. Discarding the barrier allows us to examine the interactive processes and their response to environmental context that generate germ cells in the first place, determine the entirety of what is inherited through them, and set the trajectory for the health status of the progeny they bear.

Keywords: Weismann barrier; germline; germ cells; soma; transgenerational epigenetic inheritance

1. Introduction

The idea of the Weismann barrier provides a conceptual framework for the relationship between germ cells and somatic cells and the larger extraorganismal environment. The considerable influence of this framework is, perhaps unsurprisingly, matched by manifold ways of understanding it depending on the time, the writer and the scientific area. The basic premise of the Weismann barrier, that is shared across its varied uses, is that germ cells are fundamentally separate from somatic cells. The larger implications of the barrier concept bear on the nature of germ cells, the mechanisms of inheritance, and the course of evolution.

The Weismann barrier is typically applied as a self-evident label and rarely defined, suggesting that it rests on an uncontroversial piece of evidence [1,2]. Taking a historical view on the origins of the Weismann barrier shows that it is hardly the case. August Weismann was trained as a medical practitioner and chemist in the late 1800s prior to conducting research in zoology and embryology [3]. In a pre-genetics era, he worked to understand how species can be relatively stable in their characteristics across generations, yet exhibit enough variation for substantial change to occur over evolutionary time in accordance with Charles Darwin's theory of natural selection. Weismann's solution to this problem was described in his seminal 1893 work *The Germ Plasm: A Theory of Heredity*, in which he proposed a nuclear substance called the "germ plasm" as the essential unit of inheritance. The germ plasm was the theoretical foundation upon which Weismann built his inferences.

Although Weismann's model of the germ plasm was never widely accepted, the concept of a barrier named for him has been widely used as a default framework in which new scientific evidence and theories must be constructed. Modern-day scientific articles still refer to "crossing the Weismann barrier" [1,4] and evidence undermining the existence of such a barrier is described in this framework as an exception to the rule or a "leak" in the barrier. In this article, we seek to reevaluate the framework of the barrier itself and its limitations in light of contemporary scientific knowledge and to explore how scientific pursuits might advance differently without the framework of the Weismann barrier.

2. Weismann's Model of Inheritance

With his germ plasm theory, Weismann sought to explain how species transform over evolutionary time depending on their environment yet preserve relative constancy between each generation. To understand this theory, it is necessary to understand the hypothetical physical organization and evolutionary origins of Weismann's unit of inheritance: the germ plasm. According to Weismann, the germ plasm itself was an assembly of three "vital units", that is, a unit exhibiting the primary "vital forces" of assimilation and metabolism, growth, and multiplication by fission. The smallest unit was the "biophor" [5]. Biophors were made up of varying types, numbers, and arrangements of molecules that could occur in unlimited combinations. The particular composition of a given biophor determined the structure that it formed in the cell and Weismann considered the biophors the "bearers of the cell qualities" [5]. Biophors could only be produced from pre-existing biophors and bore "historical" qualities formed over time through heredity and selection.

Weismann thought that in the earliest unicellular organisms, the biophors were freely dispersed throughout the cell [5]. In this scenario, changes induced to a biophor in any part of the cell could be inherited with the next cell division by binary fission. However, with the evolution of the nucleus, the heritable biophors capable of reproducing the entirety of the organism were sequestered to this structure alone. The separation of the heritable material into the nucleus also meant that external influences that acted upon and changed the cell body could no longer be transmitted to offspring; only changes to the nuclear substance were heritable. It appears that Weismann believed the nucleus provided a degree of protection that made the nuclear contents less susceptible to external influences and thus more stable over time. Although different biophors could grow and reproduce unevenly in response to factors such as nutrient availability and temperature due to their different physicochemical properties, Weismann thought that substantial transformations of the biophors did not occur easily or rapidly. Thus, the relative constancy of the nuclear substance between generations provided a defining feature of a particular phylogenetic lineage.

As organisms became more complex and evolved different cell types, Weismann reasoned that the organization of the nuclear substance also became more complex. While different biophors corresponded to different structures in a cell, they did not correspond directly to a particular cell type [5]. Instead, different combinations of biophors were organized into a higher-order vital unit called a "determinant" (Figure 1). As the name implies, Weismann thought that determinants conditioned the character of a cell; one type of determinant might correspond to a muscle cell while another determinant might correspond to a skin cell. In addition to organizing the biophors, determinants also served to control their activity. Within the structure of a determinant, biophors were maintained in an "inactive" state where they could only grow and reproduce. However, once released from a determinant, biophors were small enough to move through the nuclear pores and into the cytoplasm, where they were physically used up to produce the structures of the cell. This regulatory power over the biophors' activity provided the nuclear contents with the ability to control "the body of the cell" [5].

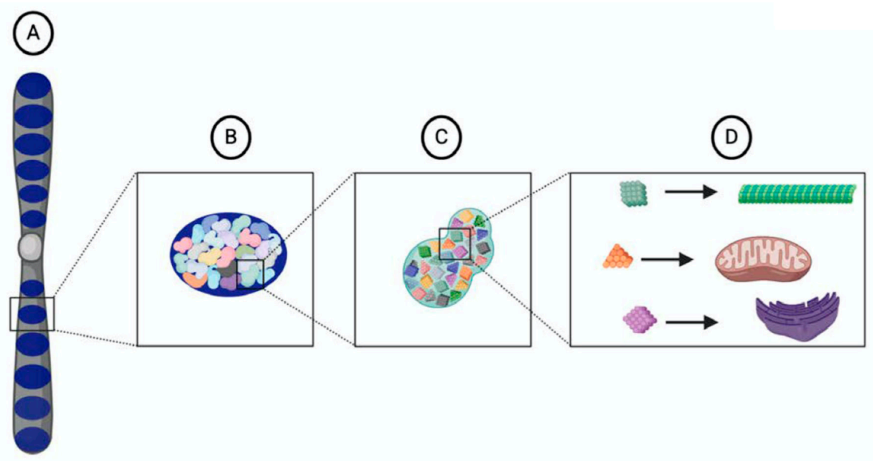

Figure 1. Illustration of Weismann's vital units. Each chromosome, or idant (**A**), is composed of several ids (dark blue) (**B**) that are inherited intact from the parental germ cells within the germ plasm. Each id is composed of determinants (**C**) that are arranged in a particular architecture. Each determinant corresponds to a specific cell type in an organism and contains a particular assemblage of biophors (**D**), a group of molecules that, when released from the nucleus and into the cytoplasm, provide the starting material from which cellular components develop.

The determinants were organized into the highest-order vital unit called the "id." Several ids were aligned along the length of each chromosome (Figure 1), which Weismann in turn called an "idant" [5]. In sexually reproducing organisms, half of a zygote's ids originated from the mother while the other half originated from the father. Weismann thought that the different combinations of ids inherited from the parents was one of the main sources of variation in a species. Although all the ids of a mature gamete were very similar, they were not identical and had very slight differences in their precise organization and composition. These differences occurred in part due to the different ancestral lineage of each id. Weismann thought that the "reducing divisions" of meiosis randomly determined which ids were retained in a gamete. Therefore, even though a zygote received half of its ids from each parent, the share of ids inherited from each grandparent could not be predicted. Because each id had its own heritage and contained all the determinants necessary to reproduce an organism, Weismann alternatively called an id the "ancestral germ plasm" [5].

All of the ids together constituted the germ plasm proper, which Weismann argued was the sole heritable substance transmitted to each new generation via the germ cells [5]. Similar to the three vital units composing it, Weismann thought that the germ plasm could only be propagated from a pre-existing germ plasm. Most importantly, what was transmitted in the germ plasm was its "fixed architecture", or the particular arrangements and proportions of the different vital units [5]. The architecture of the germ plasm was essential because it determined the entire course of ontogeny. While evolutionary time built up the structure of the germ plasm, it was deconstructed in an individual over developmental time. Weismann referred to this deconstruction process as "blastogenesis", or origin from a germ plasm. From the zygote, Weismann thought that cells could replicate through "ordinary" cell divisions, where exact copies of the nuclear contents are replicated in the daughter cells, or "embryogenic" cell divisions, where daughter cells receive unequal shares of the nuclear contents (Figure 2). Ordinary divisions producing exact copies of the complete germ plasm created a "reserve" that was maintained intact in cells of the germ lineage. Embryogenic divisions generated somatic "idioplasm" that retained only part of the germ plasm. The original architecture of the germ plasm

dictated the order in which the idioplasmic ids were deconstructed and their determinants distributed into daughter cells.

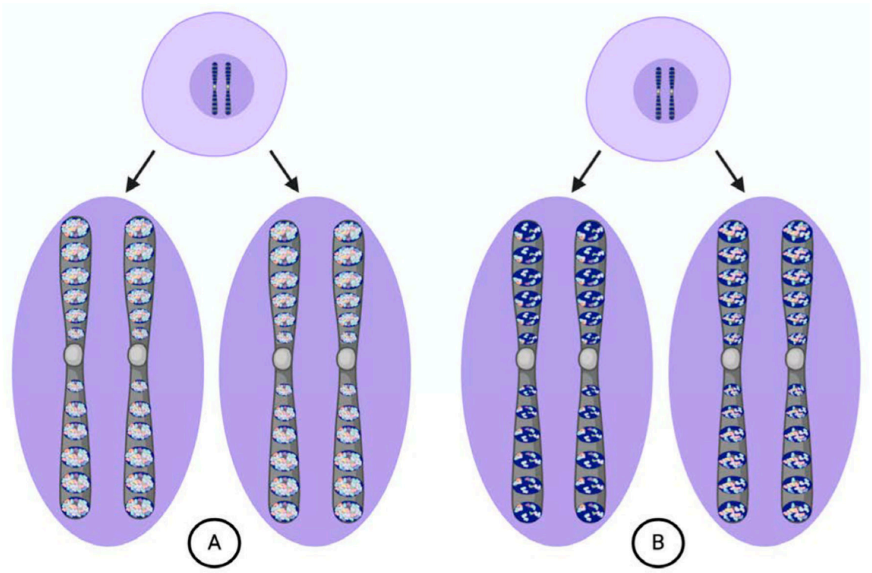

Figure 2. According to Weismann, cells can undergo either ordinary (**A**) or embryogenic divisions (**B**). (**A**) Ordinary divisions produce daughter cells with chromosomes bearing vital unit composition identical to the parental cell and maintain the germ plasm intact from the zygote through mature germ cells. (**B**) Embryogenic divisions produce daughter cells with chromosomes that bear different determinants in their ids depending upon their destined cell lineage.

Thus, the germ plasm had a dual role in inheritance and development. The ability to reproduce an organism as a whole was held only by cells with reserve germ plasm that was unchanged from the unification of the parental gametes in the zygote. Once the germ plasm was broken up, the resulting idioplasm only had the potential to generate cell types corresponding to the types of determinants the idioplasm retained, but not the entire organism [5]. Any perturbations that caused changes in the idioplasm could cause variations in cells produced from that idioplasm's lineage. However, according to Weismann, idioplasm could never be reconstructed into germ plasm and such "somatogenic" variations could not be transmitted to subsequent generations via the germ plasm. Thus, any changes that arose in an organism's body during development were independent from what would be transmitted to progeny. Only direct changes to the composition of the reserve germ plasm were heritable. Such changes might result from changes in growth rates of the vital units due to differential effects of nutrition or temperature on the different molecular components. However, Weismann argued that such changes would be imperceptibly small within a single generation.

Weismann explicitly framed his formalized theory of the germ plasm as a rebuttal to Charles Darwin's pangenesis hypothesis [5] and a general rejection of the idea of inheritance of acquired characteristics (IAC). Jean-Baptiste de Lamarck's theory of IAC, where phenotypic changes arising from patterns of use or disuse in an organism's body are transmissible to their offspring was a widely held view throughout the 19th century and informed Darwin's thinking [6]. According to the pangenesis hypothesis, all cells continually release self-replicating units called "gemmules" that freely circulate throughout the body, but concentrate in the sexual organs [7]. Darwin proposed that pangenesis was the mechanism that generated heritable variation upon which natural selection could act. While Weismann

was an early supporter of Darwin's idea of natural selection and also initially accepted IAC [8], his model of the germ plasm rendered any somatic influence on inheritance impossible.

3. Origins of the Weismann Barrier

The inability of the parental body to influence the characteristics of the offspring and the direct refutation of IAC was a key implication and the main thesis that later scientists retained from Weismann's work [9]. However, while Weismann's argument against IAC was firmly derived from the hypothetical structure and function of the germ plasm, the theory of the germ plasm itself was almost universally rejected by other scientists and is largely inconsistent with contemporary understanding of biology. Weismann was most widely criticized for the speculative nature of his theory and the lack of clear experimental or cytological evidence supporting it [10]. He himself acknowledged that it was not possible to discern the structure of the germ plasm with available technology and that the structure he proposed was based on deductive reasoning. Consequently, scientists who wanted to accept Weismann's conclusion against IAC provided reinterpretations that maintained the separation of the body from inheritance while eliminating the germ plasm as a factor.

In the earliest and most basic reinterpretation of Weismann's theory, the cell biologist Edmund Beecher Wilson [11] replaced the germ plasm with the germ cell. According to Wilson, the germ cells, through division, give rise to both soma and new germ cells, which separate from the soma and repeat the process in each successive generation [11]. It was the germ cell that offspring inherited from the parents, not their body, and the characteristics from the germ cell were retained independent from the body via the line of intergenerational germ cell descent. In his influential textbook *The Cell in Development and Inheritance*, Wilson represented this idea with a diagram that is now widely attributed to Weismann [12]. The diagram depicts the line of inheritance running from the parental germ cells through the germ cells of each new generation, with the somatic cells of each individual branching away from the germ cells and outside the realm of inheritance. This diagram ostensibly representing Weismann's idea was adopted and refined, in particular in another influential biology textbook written by Simpson et al. in 1957. In this text, the authors shifted the argument for the separation of germ cells from the somatic cells: they reasoned that "undifferentiated" germ cells could not be derived from differentiated cells of the body but instead must come directly from the lineage of undifferentiated cells that remain such across generations [13]. In both Wilson's and Simpson et al.'s books, the perpetual separation of the germ cells from the somatic cells, as well as the exclusive generation of the latter from the former, made IAC impossible.

An alternative means of reinterpreting Weismann's theory to reject IAC was the replacement of the germ plasm with the genotype [10]. Weismann's notion that hereditary units contained within the gametes of the parents were causal in determining the physical qualities of the resulting offspring, without being determined by the qualities of the parents themselves, was bolstered by the rediscovery of Gregor Mendel's 1866 work on heredity in 1900 [14,15]. Weismann himself was unaware of Mendel's work when he was developing the germ plasm theory in the 1880s and 1890s. This rediscovery led to the emergence of genetics as a distinct field of science. In a parallel way to Weismann, many early geneticists emphasized the idea of continuity of the hereditary substance between generations, and that this hereditary substance was borne by the chromosomes [14]. Weismann was also credited with helping to initiate the "genotype concept", in which the totality of the inherited genes determines an organism's phenotype but the phenotype has no bearing on what is inherited [10]. The unification of neo-Darwinian evolution and Mendelian genetics with the modern synthesis of the 1930s and 1940s [16] further promoted the idea that a genetic program selected over evolutionary time ultimately determined phenotype [17]. Evidence of control of the phenotype by an inherited genetic program was seen by some as a "final blow" to IAC.

Aside from bolstering the rejection of IAC, these reinterpretations of Weismann's work converge on a notion of unidirectionality. Cellular transformations and information conveyance can proceed in a one-way direction only, in effect creating a barrier to reversion. In a developmental version of the barrier,

somatic cells differentiated from parental gametes can neither revert to germ cells [4] nor influence what is inherited via the germ cell lineage [18]. In a genetic version of the barrier, the phenotype cannot recreate the genetic information from which it was derived [19], nor can it influence the heritable genetic information conveyed across generations [20]. Although these ideas diverge from Weismann's original germ plasm theory to varying degrees, they have all been used to define the term "Weismann barrier." While the Weismann barrier presents a relatively simple framework for development and inheritance, it is questionable as to whether this framework is particularly useful going forward.

4. What Is Lost in the Weismann Barrier?

The barrier concept presents germ cells and the inherited phenotype as fixed states while offering no explanation for the processes by which these states came to be. To some extent, it implies that no such explanation is necessary, for the inherent properties of germ cells and the genome alone appear sufficient to determine the course of development and inheritance. However, the barrier concept stands in stark contrast to what we know about the myriad processes regulating germ cell fate and function as well as their interactions with surrounding somatic cells and the external environment (Figure 3). The regulatory processes and interactions that the barrier concept relegates to obscurity are precisely what need to be elucidated to further our understanding of germ cell biology, inheritance, and human health.

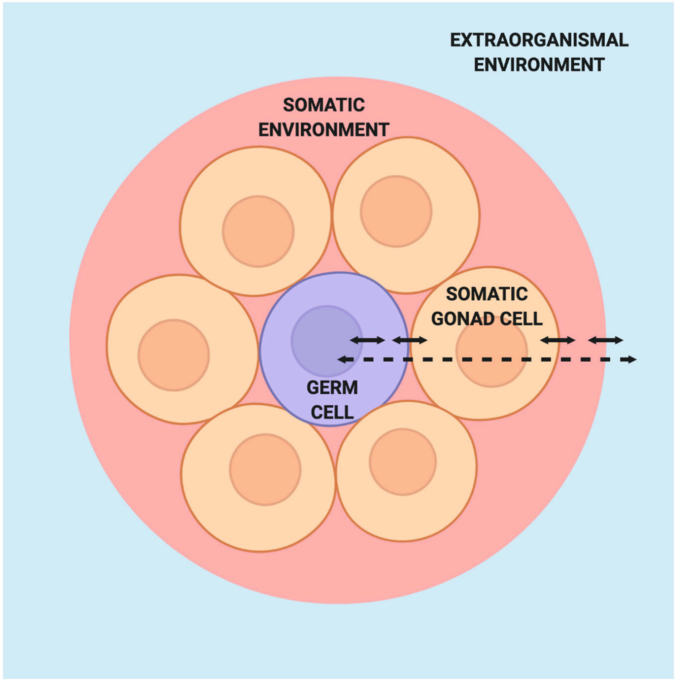

Figure 3. Model of germ cells in the absence of the Weismann barrier. Within the germ cell (purple), interactions between the nuclear and cytoplasmic components integrate signals from the surrounding somatic gonad cells, which themselves are responsive to signals from the germ cell. The germ cells and somatic gonad cells are situated within a larger somatic environment that is in turn influenced by the extraorganismal environment.

4.1. Emergent Germ Cell Fate and Function through Interaction with Somatic Cells

Rather than being perpetually independent from somatic cells, germ cells across many different species are intimately linked with somatic cells throughout development. In mammals, primordial germ cells (PGCs) are induced by a combination of bone morphogenic proteins (BMPs), Wnt proteins, and other signaling molecules provided by the surrounding embryonic tissues [21]. During migration from the site of induction to the gonadal ridge, PGC chromatin is dramatically remodeled, exhibiting a dramatic loss in DNA methylation and an increase in repressive histone marks [22]. It is thought that this epigenomic reprogramming is necessary for PGCs to attain germ cell competence. While the precise mechanisms regulating these epigenomic changes, are not fully known, in vitro models of primordial germ cell-like cells (PGCLCs) suggest that these changes are at least in part due to interactions with somatic cells. PGCLCs can be specified from embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs) derived from somatic tissues [23] using condition media to drive expression of germ cell-specific transcription factors. However, once specified, PGCLCs do not substantially proliferate, survive for extended periods of time, or progress through epigenomic programming without using somatic feeder cells in the culture [24,25]. Furthermore, PGCLCs cannot develop further and ultimately produce functional gametes unless they are either co-cultured with somatic gonad cells [26] or transplanted into a gonad in vivo [24].

Across species, the somatic gonad is necessary for regulating meiosis [27,28] and supporting germ cell function through later development and maturation. Within the gonad, germ cells and somatic cells directly communicate with one another through gap junction connections [29–33], endocytosis [34], and extracellular vesicles [35]. Through these means, both male and female germ cells receive nutrients, small non-coding RNAs (sncRNAs), proteins, metabolites, hormones, and other signaling molecules from the surrounding somatic cells. Somatic cell interactions with germ cells appear to be well conserved from invertebrates to mammals, suggesting their importance in germ cell function. In *Caenorhabditis elegans*, mutations in the innexin proteins that form the gap junctions between germ cells and the somatic gonad result in loss of germ cell proliferation, embryonic lethality, and sterility [32]. Laser ablation of gonad sheath cells results in failure of oocytes to progress through meiosis I, leading to infertility [36]. In both male and female mice, knockout of the connexins forming the gap junctions between germ cells and somatic cells impedes meiotic progression and germ cell maturation [29,31]. The inability of aged granulosa cells to efficiently generate new gap junctions with oocytes may contribute to increasing infertility with maternal age [30]. In male mammals, extracellular vesicles called epididymosomes deliver somatically-derived sncRNAs to maturing spermatozoa and are necessary to support pre-implantation embryonic development [37,38]. Thus, available evidence indicates that somatic cells are not strictly separated from germ cells but essential to germ cell maturation and reproductive competence.

4.2. A Broader View of Heritable Information

Although the barrier concept indicates that the inherited genome sequence alone is sufficient to directly determine phenotype, a substantial degree of phenotypic variation can be generated through layered regulatory processes that control how, when, and where the genome may actually be expressed. From the start of metazoan development, the single-celled zygote differentiates into a wide range of cell types from a single genome. It is not genes per se but the multitudinous ways in which they can be modified, activated with others, and/or silenced that allow organisms to change throughout their life course and in response to a changing environment. The flexibility of genome expression is necessary for the act of living as a multicellular organism and for perpetuating life through reproduction. Increasing evidence indicates that factors inherited from parental germ cells via influences from the somatic cells and/or the larger environment can modulate genome expression and alter phenotype in ways comparable to a change in genetic sequence.

The direct influence of heritable information flow from soma to the germline is particularly well understood in rodents. A recent and rapidly growing body of work has shown that the sncRNA

content of sperm, particularly tRNA fragments, is highly influenceable by environmental changes such as diet and stress [39]. As shown by zygote microinjection experiments in mice, such modified tRNA fragments extracted from sperm are sufficient to modify the offspring's phenotypes, including metabolic dysfunction in the case of paternal high-fat diet [40]. Other types of sncRNA and long non-coding RNAs (lncRNA) can also convey heritable information that alters the phenotype of offspring whose fathers were subjected to chronic stress or trauma [41,42]. Based on available evidence, it appears that alterations in sperm sncRNA may be attributable at least in part to the composition of epididymosomes taken up by the sperm from the epididymal cells. As noted previously, the contents of epididymosomes are necessary for sperm to support the regulation of early embryonic development and embryonic viability [37,43]. Therefore, environmental or other influences that alter the contents of the epididymosomes and thereby the paternal germ cells may have a direct impact on the phenotype of their offspring.

Increasing evidence indicates that environmental influences on non-genetic heritable information can alter offspring phenotype for more than one generation. The last few years have seen a rapid expansion of the field of transgenerational epigenetic inheritance (TEI) in a wide variety of animal models, including *C. elegans*, zebrafish, *Drosophila*, and mouse [44]. Work in the nematode has demonstrated that the impact of numerous environmental cues can be inherited for several generations and provided a molecular understanding of the mechanisms of soma-to-germline and germline-to-germline communication that direct TEI. A recent study demonstrated that in *C. elegans*, small RNAs synthesized in neurons through the RDE-4 pathway alter the expression of 124 mRNAs and 1287 sncRNAs and trigger transgenerational changes in the offspring's transcriptome and chemotaxis behavior [45]. How the neuronal RDE-4 derived sncRNAs impact the germline transcriptome is not fully elucidated but appears to be largely independent of the activity of the siRNA membrane transporter SID-1 [45,46]. It is possible that a non-SID-1 mechanism of transport of sncRNAs or a relay mechanism involving other signaling molecules such as hormones may be at play. This work highlights the ability for factors that are uniquely produced by neurons to impact the germline and the phenotype of the resulting progeny.

A recent study in mice suggests another mechanism of TEI may involve changes to the genome's physical structure [47]. The eukaryotic genome is non-randomly arranged within the nucleus and, at the highest level, is organized into spatially segregated heterochromatin and euchromatin compartments [48]. Although partly influenced by DNA base pair sequence [47], chromatin compartmentalization may largely be driven by phase separation [49]. Phase separation of chromatin is a physicochemical process dependent upon factors affecting intra-/intermolecular forces, such as solute concentration and temperature, as well as features of the genome affecting charge and binding affinity, such as histone post-translational modifications [50]. The local chromatin environment within the phase boundary concentrates proteins and other biomolecules that in turn can affect transcription and reinforce or alter chromatin compartmentalization by modifying DNA and histones [51]. It appears that perturbations to chromatin compartmentalization in response to tributyltin (TBT) exposure during early embryonic development could shift genomic regulatory circuits and impact metabolic phenotype [47]. Furthermore, the authors hypothesize that the resulting changes expression of chromatin organization-related genes could be carried through the adult's germ cells and be "reconstructed" to repeat the cycle in the next generation. These effects appeared to persist in the fourth-generation male offspring. Although changes in DNA methylation patterns were also observed in this generation of offspring ancestrally exposed to TBT, these changes did not correlate with patterns of mRNA expression. These data suggest that the three-dimensional organization of the genome, in addition to sequence, may in effect be heritable across generations.

The debate regarding what information is heritable between generations has mainly been framed in terms of evolutionary relevance. Over evolutionary time, only information that may be faithfully replicated in perpetuity is generally considered to be truly heritable, and thus relevant [19]. However, at the scale of the human lifespan, the perpetual transmissibility of non-genetic heritable information, or lack thereof, becomes less relevant. As long as the phenotype, or more importantly the health

status, of at least one generation can be negatively affected by environmentally-induced changes in heritable information transmitted via the germline, it warrants further study to better understand the mechanisms by which this happens and pursue interventions that may improve human health.

5. Conclusions

The writings of August Weismann over 100 years ago helped instill a modern concept of a barrier according to which the germ cells and their genome function independently from the body of an organism. Remarkably, Weismann himself acknowledged that his germ plasm theory, which led to the barrier concept, was a product of deductions from available information and not a proven fact: "It is nevertheless possible that continuity of the germ plasm does not exist in the manner in which I imagine that it takes place, for no one can at present decide whether all the ascertained facts agree with and can be explained by it. Moreover, the ceaseless activity of research brings to light new facts every day, and I am far from maintaining that my theory may not be disproved by some of these. But even if it should have to be abandoned at a later period, it seems to me that, at the present time, it is a necessary stage in the advancement of our knowledge, and one which must be brought forward and passed through, whether it prove right or wrong, in the future" [52]. We now know that it is not the contents of the chromosomes that differs between germ and somatic cells but rather the manner in which they regulate and express the genome. The barrier concept has gained so much prominence over the 20th century because it facilitated scientific progress in some respects, in particular genetic and evolutionary understandings of inheritance. Without dismissing the historical contributions Weismann made to the advancement of knowledge, it may be time to accept that utilization of the barrier concept is a stage through which we have fully passed.

Continued adherence to the barrier concept is extremely limiting in furthering our understanding of germ cell biology and inheritance. Germ cells and their genomes are not abstract conveyors of information but physical structures that can change form and function in numerous ways in response to a combination of cell-autonomous and non-autonomous cues. It is this ability to change as a part of an integrated organismal system that determines both physiological and pathophysiological reproductive outcomes in an individual. The capacity to reproduce is itself a phenotype facilitated by concerted functions and inter-relationships between germ and somatic cells and the germ cell genome is not entirely separable from the larger context of the body in which it is located. Whereas the barrier concept takes as a starting point the germ cells and the resulting phenotype produced through inheritance, discarding the barrier allows us to unboundedly examine the interactive processes and their response to environmental context that generate germ cells in the first place, determine the entirety of what is inherited through them, and set the trajectory for the health status of the progeny they bear.

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CHAPTER 3:

Characterization of Effects of PFAS on Germ Cell Development and Reproduction in *Caenorhabditis elegans*

INTRODUCTION

PFOS and F-53B are both PFAS that have been used as mist suppressants in the chrome plating industry. Despite being voluntarily phased out of US production in 2009, PFOS is still ubiquitously detected in the American population (CDC 2017). Structurally similar to PFOS, F-53B continues to be produced and used mainly in China and, along with PFOS, is ubiquitously detected in the Chinese population (Shi et al. 2016). In addition to being detected in human serum, PFOS has been detected in human follicular fluid at concentrations similar to serum concentrations (Petro et al. 2014; Heffernan et al. 2018), while both PFOS and F-53B have been found to cross the placenta into the fetal compartment (Stein et al. 2012; Mamsen et al. 2019; Pan et al. 2017; Chen et al. 2017). These data indicate that human germ cells may be subject to direct PFOS and F-53B exposure at all stages of development.

In its 2018 draft toxicological profile for PFAS, the Agency for Toxic Substances and Disease Registry (ATSDR) stated: "Overall, the reproductive system does not seem to be a sensitive target of PFOS toxicity." However, almost all of the referenced animal studies were generated by either the former PFOS manufacturer 3M (Seacat et al. 2003; Luebker et al. 2005; Butenhoff et al. 2012) or a contract research organization (Thomford et al. 2002). The one animal study referenced by ATSDR regarding PFOS reproductive toxicity that wasn't generated either directly or indirectly by the manufacturer reported reduced sperm counts in response to PFOS exposure in rats (Wan et al. 2011). A more recent study by Tian et al. (2019) found increased infertility incidence in rats exposed to PFOS with morphological effects on the testes and impaired spermiogenesis in male F1 offspring. Another study similarly found a decrease in the number of mature ovarian follicles with PFOS exposure in female mice (Feng et al. 2015). Thus,

rodent studies do appear to indicate that reproductive endpoints are relevant targets of PFOS toxicity.

Though epidemiological data are limited, some studies have found increases in infertility with increased PFOS serum concentrations (Fei et al. 2009; Whitworth et al. 2012). While other studies did not identify such effects (Jørgensen et al. 2014; Vélez et al. 2015), it should be noted that almost all people have detectable levels of PFOS in their serum. In these studies, individuals placed in the first quartiles used as control groups had serum PFOS concentrations exceeding 10ng/mL, a level beyond which the German Environment Agency has determined may lead to health impairment in women of child-bearing age (Schümann et al. 2021). The inability to obtain truly unexposed control populations in epidemiological studies may limit the ability to detect effects of PFOS exposure on infertility and other reproductive endpoints.

Far fewer studies have been published on the reproductive effects of F-53B exposure. Zebrafish exposed to F-53B at concentrations as low as 5μg/L exhibited a significant decrease in the number of eggs produced and in the number of surviving F1 offspring (Shi et al. 2018). It does not appear that any epidemiological studies on F-53B and infertility have been reported. However, two studies have found a significant decrease in progressive sperm with increased F-53B serum concentrations (Pan et al. 2019; Luo et al. 2022).

While available data point to negative reproductive effects of PFOS and F-53B exposures, little is known about how these substances directly affect germ cells. Data are particularly lacking for oogenic germ cells, which, in mammals, undergo most of meiosis I while *in utero*. The nematode *C. elegans* is a valuable model for examining toxicant effects on oogenic germ cells since the adult hermaphrodite gonad continually generates oogenic germ cells that progress linearly through meiotic prophase and are easily visualized through the transparent body

of the animal. The purpose of the subject work was to characterize the effects of PFOS and F-53B on oogenic germ cells in *C. elegans* with continuous exposure from embryonic development through adulthood.

METHODS

Worm culture and population synchronization

Wild-type N2 Bristol strain *C. elegans* were used for all experiments except the reproductive development staging assay, in which the PGL-1::GFP reporter strain JH3269 (pgl-1(ax3122[pgl-1::gfp]) IV) was used. Worms were cultured on nematode growth medium (NGM) agar plates seeded with OP50 *Escherichia coli* lawns and maintained at 20°C for both culturing and experimental procedures. To obtain synchronized populations, stage L4 larval worms were picked from standard culture plates onto new NGM plates and allowed to reach adulthood (approximately 24 hours). Synchronized adults were used to produce the embryos subjected to PFAS exposure.

PFAS solution preparation and exposure

900μM stock solutions of PFOS (Synquest Laboratories, CAS number 1763-23-1, >99.5%) or F-53B (BOC Sciences, CAS number 73606-19-6, technical grade) were prepared freshly from solid powders dissolved directly in double-deionized water that was filtered through a 0.22 micron membrane filter. PFOS or F-53B stock solutions were further diluted as necessary in filtered double-deionized water. PFAS solutions or filtered double-deionized water (control) were applied to the surface of NGM agar plates with OP50 *E. coli* lawns and the plates rotated gently to spread the solutions evenly. Treatments were applied to the plates at a ratio of 1:45 on a solution:NGM agar volume-for-volume basis (e.g., 0.4mL of PFAS solution/water applied to

18mL of NGM agar). PFAS treatment solutions at concentrations of 225µM, 450µM, or 900µM were applied to NGM plates, resulting in estimated exposure concentrations of 5µM, 10µM, and 20µM based on the volume-for-volume dilution factor. PFAS concentrations reported herein refer to that of the solution applied to the NGM plate and not the estimated exposure concentrations.

Synchronized adult *C. elegans* were transferred to the PFAS- or water-treated NGM plates and allowed to lay embryos for approximately 2-3 hours. After this time, the adult *C. elegans* were removed and the progeny remaining on the plates were used for experimental assays.

Reproductive development staging assay

C. elegans hermaphrodites progress through four defined larval stages prior to becoming adults capable of producing embryos. L1 larval worms have only two germ cells, which begin to proliferate during the L2 larval stage. In L3 larval worms, germ cells continue to proliferate and the gonad arms form bends as the gonad grows. At the L4 larval stage, germ cells begin undergoing meiosis and produce spermatogonia prior to switching to oocyte production as young adults. At 20°C, progression to adulthood is typically completed by 72 hours post-embryo. To evaluate delays in reproductive development, PGL-1::GFP worms exposed to NGM plates treated with water, 450µM PFOS or F-53B, or 900µM PFOS or F-53B were collected at 72 hours post-embryo and imaged using a Nikon H600L epifluorescence microscope at 40X magnification with a FITC filter. PGL-1::GFP labels *C. elegans* hermaphrodite germ cells at all developmental stages, except mature spermatogonia, and reproductive development staging was evaluated based upon morphology of GFP-labeled cells in each worm.

Brood size and lifespan assays

When the control *C. elegans* reached the L4 larval stage (approximately 48 hours after embryos were laid), worms from each condition were transferred to new individual NGM plates with the same water or PFAS treatment. It should be noted that PFAS-exposed worms developed more slowly than control worms so exposed worms were at earlier developmental stages at the time of transfer. The worms were transferred to new treated NGM plates every 12 hours for the duration of their reproductive lifespans (approximately 5 days), after which time the worms were maintained on the same NGM plate and monitored for death every 24 hours. Following each worm transfer to a new NGM plate, the number of embryos on the previous plate were counted under a dissecting microscope. The number of late larval/adult worms on the same plate were counted approximately 72-96 hours later. Brood size was tabulated as the total number of surviving late larval/adults produced per worm. Embryonic lethality was calculated as the percentage of surviving late larval/adults out of the total number of embryos laid per worm.

Gonad dissection, fixation, and imaging

Adult worms from each treatment were collected into a drop of M9 minimal salts solution approximately 96 hours after being laid and the gonads extruded by decapitation using a 25 gauge needle. The worms with extruded gonads were fixed in 3.7% formaldehyde at room temperature for 15 minutes followed by post-fixation in 100% methanol at -20°C overnight. After washing in 0.1% PBST, the fixed worms were resuspended in Fluoroshield mounting medium containing DAPI. Gonad nuclei were imaged using a Nikon H600L epifluorescence microscope at 40X magnification with a DAPI filter. Images captured a single flattened layer of nuclei surrounding the gonad core. Mitotic and meiotic transition zone, pachytene, and diplotene/

diakinetic nuclei were identified based upon chromosome morphology. Lengths of each gonad region were quantified as numbers of nuclei rows.

Acridine orange germ cell apoptosis assay

Approximately 24 hours after the L4 larval stage, worms were removed from their treated NGM plates and incubated for 1 hour in a 25 μg/mL solution of acridine orange diluted in M9 minimal salts solution supplemented with OP50 *E. coli*. Immediately following acridine orange incubation, live worms were imaged using the FITC filter of a Nikon H600L epifluorescence microscope at 40X magnification. Acridine orange emits green fluorescence under acidic conditions created by cells undergoing apoptosis and apoptotic germ cell nuclei were scored as FITC-positive foci in the late pachytene region of the gonad.

Statistical analysis

Unless otherwise noted, Welch's ANOVA with Games-Howell post-hoc test were used for statistical analyses to account for heterogeneous variances and unequal sample sizes. Statistical analyses were performed using R statistical software.

RESULTS

PFOS and F-53B delay development and decrease brood size

To determine if PFOS or F-53B impact development, the gonads of PGL-1::GFP worms were examined under an epifluorescence microscope to determine developmental stage based on germ cell and gonad morphology. As in wild-type N2 worms, PGL-1::GFP worms grown on water-treated NGM plates reached adulthood by 72 hours post-embryo. However, only approximately 10% of PGL-1::GFP worms exposed to 450μM F-53B reached adulthood by this

time (Figure 1A). Furthermore, no worms exposed to 900μM F-53B or either concentration of PFOS reached adulthood by this time and most worms subject to these treatments reached only the L3 larval stage. In wild-type N2 worms subjected to the brood size assay, embryos were not observed on the plates of worms exposed to the highest concentrations of PFOS or F-53B until approximately 12-24 later than water control worms (Figures 1B-C). Thus, both PFOS and F-53B appear to cause delays in development in *C. elegans*.

Based on brood size assay results, in addition to delaying reproductive maturity, exposure to PFOS and F-53B also shifted the time of maximal reproductive output later in life and decreased the maximum rate of offspring produced per day (Figures 2A-B) without substantially affecting the worms' reproductive lifespan (Figures 3A-B). Consequently, *C. elegans* exposed to PFOS and F-53B exhibited dose-dependent decreases in total live offspring produced (Figures 2C-D) with significant decreases of approximately 20-30% observed at the highest exposure concentration for both PFAS compared to controls. Although the incidence of embryonic lethality was not significantly increased in the PFOS or F-53B exposed worms, elevated levels were observed at the highest concentrations (Figures 2E-F). No significant differences in survival time were observed in the PFOS or F-53B exposed worms, nor were any particular concentration-dependent trends evident for this endpoint (Figures 3C-D).

Although PFOS and F-53B exposures delayed development, since the reproductive lifespans of the worms were not altered but rather shifted later in life, it does not appear that the developmental delay is responsible for the observed decrease in brood size. Therefore, germ cell nuclei from adult worms were examined next to determine if effects of PFAS exposure were evident.

Meiotic germ cells are more sensitive to PFAS exposure than mitotic germ cells

Gonads dissected from adult worms exposed to PFOS and F-53 were visually smaller than those from control worms, particularly at the highest concentrations (Figures 4A-B). Based on imaging of DAPI-stained dissected gonads, PFAS-exposed worms also exhibited fewer oogenic germ cells. For both PFOS and F-53B exposures, the length of the mitotic zone located in the distal end of the gonad was slightly reduced, with no clear dose response in PFOS-exposed worms (Figures 5A & 5C). However, meiotic regions were much more strongly affected by PFAS exposure than the mitotic region with a reduction of up to almost 50% at the highest concentrations relative to controls (Figures 5B &5D; Supplemental Figures 1-2). Germ cells additionally exhibited a significant increase in pachytene exit (Pex) defect incidence with increasing PFAS concentration (Figures 5E-F). Altogether, these findings suggest that the reductions in gonad size and number of germ cells observed in PFAS-exposed worms could be primarily attributable to defects in meiotic progression.

PFAS-induced germ cell reductions do not appear to be caused by increased apoptosis

To determine if increased apoptosis contributed significant to the decrease in meiotic cells observed in PFAS-exposed worms, young adult gonads were imaged after incubation with acridine orange. Relatively low levels of physiological apoptosis normally occurs in the mid- to late-pachytene region of the adult *C. elegans* gonad, but apoptosis can be increased in response to various stressors. Based upon the acridine orange assay, neither PFOS nor F-53B exposure significantly increased the number of apoptotic germ cell nuclei at the concentration tested (450μM) (Figure 6). Since elevated apoptosis levels were not observed, it supports the inference that PFAS exposure reduces meiotic germ cell numbers in the *C. elegans* gonad primarily by impairing meiotic progression.

DISCUSSION

Results of the subject study indicate that both PFOS and F-53B have significant impacts on decreasing germ cell numbers and total reproductive output in *C. elegans*. While exposed worms exhibit developmental delays, they also exhibit a later shift in the end of their reproductive lifespans such that their overall reproductive lifespans are not significantly different from control worms. These data indicate that reduction in total reproductive output was not caused by the developmental delays in the PFAS-exposed worms. Although, previous studies have generally found a tradeoff between reproductive output and parental survivorship in *C. elegans*, with genetic mutants that produce few or no offspring exhibiting longer lifespans (Mukhopadhyay & Tissenbaum 2006), PFAS-exposed worms did not live longer than controls and no such tradeoff was observed.

Further examination of the germ cells pointed to minimal effects on mitotic cells but substantial effects on meiotic cell populations. Because apoptosis was not significantly elevated in PFAS-treated worms, and because a dose-dependent increase in Pex defect was observed with both PFOS and F-53B treatment, it appears that meiotic progression may be particularly susceptible to PFAS exposure. The MAP kinase signaling pathway is known to be an important regulator of meiotic progression in *C. elegans*, as well as in other organisms (Lopez et al. 2013). Specifically, the MAPK1/ERK2 ortholog MPK-1 must be activated by diphosphorylation in the mid-pachytene region via an insulin signaling pathway initiated by DAF-2 (an IGF1R ortholog) (Lopez et al. 2013). One function of MPK-1 activation in this region is to initiate phospholipid membrane reorganization to transition from syncytial to cellularized oocytes (Arur et al. 2011).

Another function of MPK-1 activation in mid-pachytene is to phosphorylate the synaptonemal complex (SC) protein SYP-2, which coincides with crossover designation

(Nadarajan et al. 2016). Once crossover designation is detected, MPK-1 is inactivated and SYP-2 in the SC is either passively dephosphorylated or replaced with unphosphorylated SYP-2. This change in phosphorylation of SYP-2 promotes disassembly of the SC from the long arm of the bivalent, which facilitates chromosome reorganization in the diplotene stage (Nadarajan et al. 2016). Persistent phosphorylation of SYP-2 prevents SC disassembly and produces Pex defect (Nadarajan et al. 2016). Loss-of-function mutations in any of the genes in the MPK-1 signaling pathway (*let-60*, *lin-45*, *mek-1*, and *mpk-1*) similarly produce Pex defect (Achache et al. 2019). In addition to aberrant MPK-1 pathway signaling, Pex defect has also been observed with somatic gonad sheath cell ablation (McCarter et al. 1997), loss-of-function mutations in mitofusin gene *fzo-1* that prevent mitochondrial maturation (Charmpilas & Tavernarakis 2020), and knockout or knock down of the gonad-specific miRNA miR-35 and miR61 (Minogue et al. 2018). Data from dpMPK-1 immunofluorescence performed on PFAS-exposed worms have not yet been quantified and further research is needed to determine the cause of Pex defect observed in these worms.

While it does not appear that exposure to F-53B has previously been tested in *C. elegans*, the effects of PFOS exposure on reproductive output in *C. elegans* has been examined by others (Guo et al. 2016; Chen et al. 2018; Kim et al. 2020; Chowdhury et al. 2022). Previous studies have found signifiant reductions in reproductive output at PFOS concentrations as low as 0.01µM. However, all of these exposures were performed with the worms suspended in a liquid medium, which has been shown to significantly alter the transcriptome of *C. elegans* even in the absence of an added toxicant (Çelen et al. 2018). Specifically in the context of PFOS, Stylianou et al. (2019) found that worms exposed to 25µM PFOS in liquid medium exhibited significant upregulation of transcripts related to oxidative stress, heavy metal response, and heat shock

proteins. However, worms exposed to PFOS via pretreated bacteria on solid agar plates (comparable to the exposure scenario in the present study) exhibited decreased expression in transcripts for heat shock proteins with minimal impacts on oxidative stress transcripts (Stylianou et al. 2019). Thus, direct comparisons cannot be made between studies with different modes of toxicant exposure in *C. elegans*.

One limitation from the present study is that the internal dose of PFOS and F-53B received by the exposed worms is not known. Based upon the fact that the PFAS solutions were applied to the surface of solid NGM plates with bacterial lawns, and given the fact that *C. elegans* have a proteinaceous cuticle that would likely prevent substantial absorption of the chemicals, the most likely route of exposure is ingestion of bacteria with sorbed and/or internalized PFAS. Thus, the internal dose administered to the worms would be limited by their ingestion rate and the amount of PFAS retained by the bacteria. Others have found that worms exposed to PFOS on NGM plates by feeding L1 larval worms with bacteria treated with 25μM PFOS for 48 hours exhibited an internal concentration of approximately 0.01μM (Stylianou et al. 2019). Extrapolating to the subject study, assuming the bacteria on the NGM plates was treated with the maximal PFAS solution concentration (900μM), it would suggest a potential internal dose of 0.4μM (roughly 200μg/L) in the exposed worms. This estimated internal exposure concentration is comparable to that detected in serum of highly exposed humans.

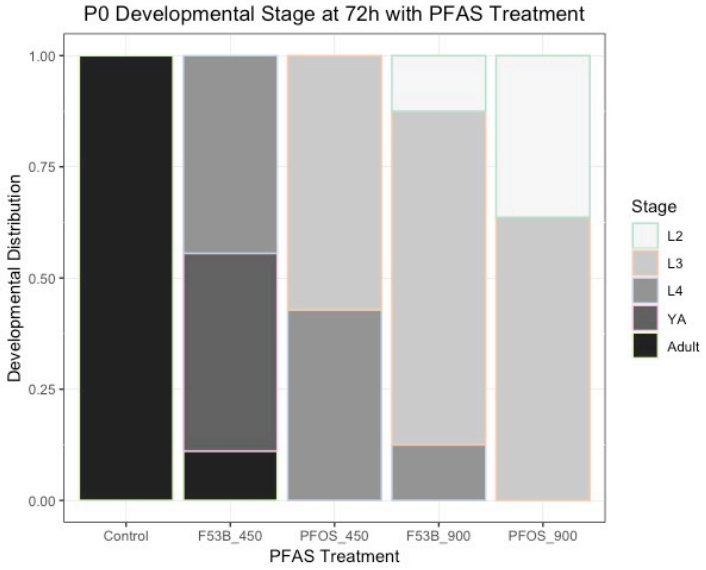
CONCLUSION

PFOS and F-53B exposure is extremely prevalent in the human population. Given the long elimination half lives and environmental stability of these compounds, human exposures can be expected to continue for decades into the future even if production and use were

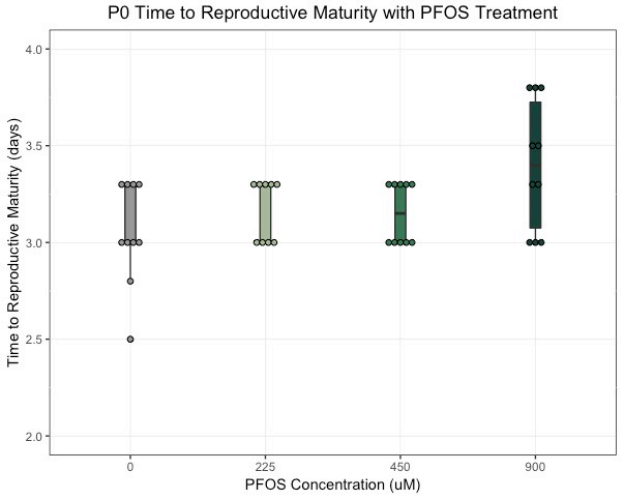
discontinued today. Therefore, having a clear understanding of their reproductive health effects is important for informing public health and policy decisions. Results of the subject study indicate that both PFOS and F-53B have significant impacts on decreasing germ cell number and total reproductive output in *C. elegans*. Examination of the worms' gonads points to particular effects on meiotic cell development and progression with limited effects of apoptosis. Further understanding of the molecular mechanisms underlying the effects of PFOS and F-53B on germ cells in *C. elegans* could identify conserved pathways that have translational relevance to human health. Chapter 4 explores biomolecular condensates as a proposed target contributing to germ cell toxicity from PFOS and F-53B exposure *C. elegans* with potential implications for humans.

FIGURE 1

1A



1B



1C

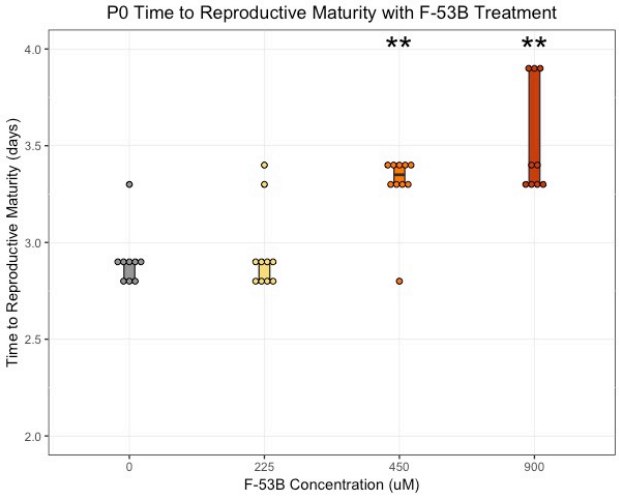
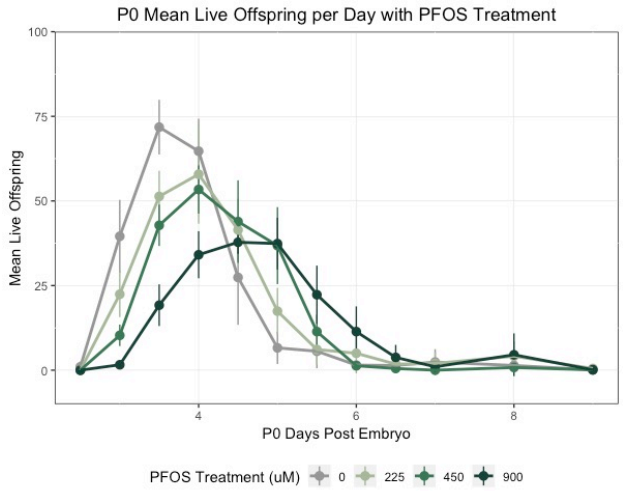


Figure 1. Exposure to PFOS and F-53B causes delays in reproductive development. *(A)* Control PGL1::GFP worms reach adulthood by 72 hours post-embryo. Most PGL-1::GFP worms exposed to plates treated with 450µM or 900µM PFOS or F-53B are still at young adult or earlier larval stages at 72 hours with more severe delays with PFOS and at higher concentrations. n=8 worms per treatment, N=1 experimental repeat. *(B)* N2 worms exposed to 900µM PFOS exhibit a non-significant increase in time to reproductive maturity while *(C)* N2 worms exposed to

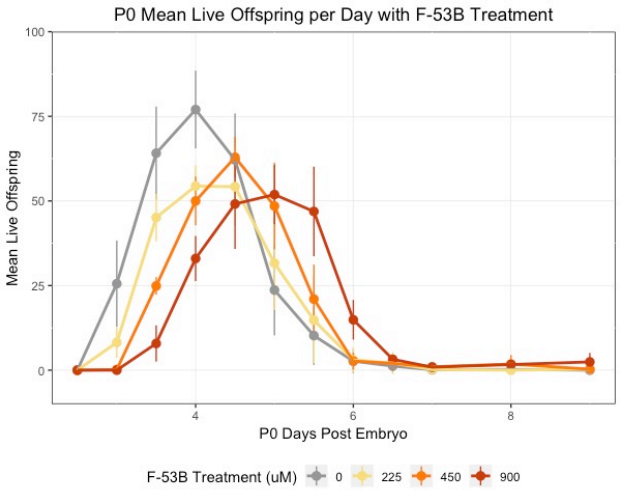
450μM and 900μM F-53B exhibit significant increases in time to reproductive maturity. n=10 worms per treatment, N=2 experimental repeats. Boxes represent the interquartile range while whiskers extend 1.5 times the interquartile range. Circles represent data for individual worms. **p<0.01.

FIGURE 2

2A



2B



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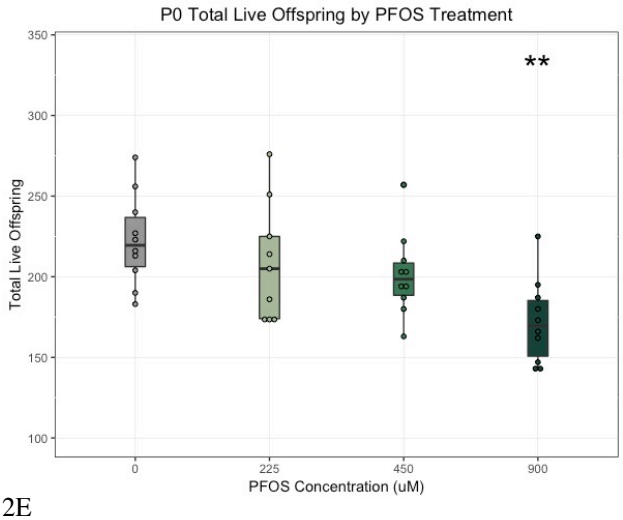
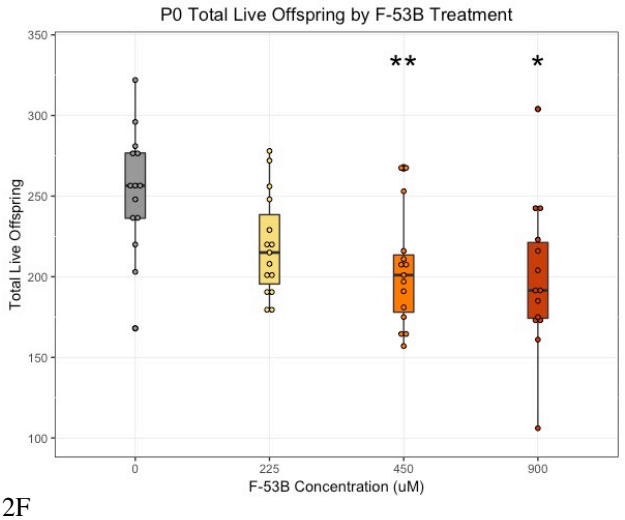


2D



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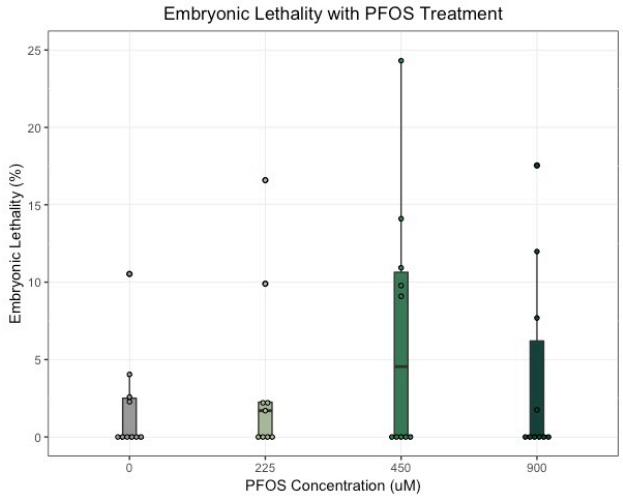


2F

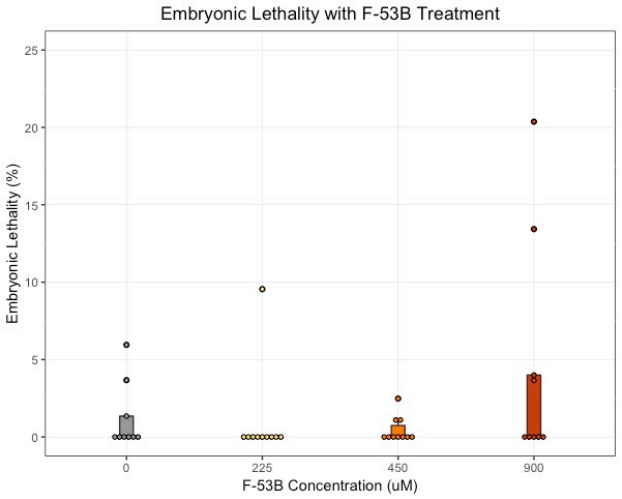
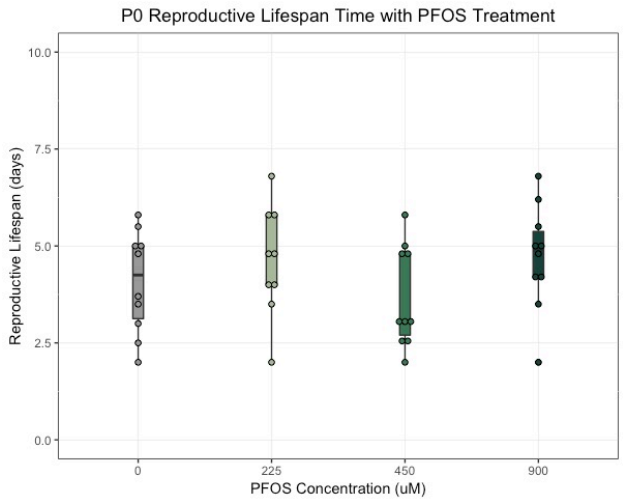


Figure 2. PFOS and F-53B exposure significantly decreases reproductive output in a dose-dependent manner. Exposure to both *(A)* PFOS and *(B)* F-53B shifts maximal reproductive output later and lower compared to controls. Circles represent mean live offspring produced per day and error bars represent 95% confidence intervals. Furthermore, exposure to both *(C)* PFOS and *(D)* F-53B results in a dose-dependent decrease in brood size as quantified by total offspring surviving to adulthood. However, embryonic lethality is not significantly affected by either PFAS *(E,F)*. Boxes represent the interquartile range while whiskers extend 1.5 times the interquartile range. Circles represent data for individual worms. n=10 worms per treatment, N=2 experimental repeats. *$p<0.05$, **$p<0.01$.

FIGURE 3

3A



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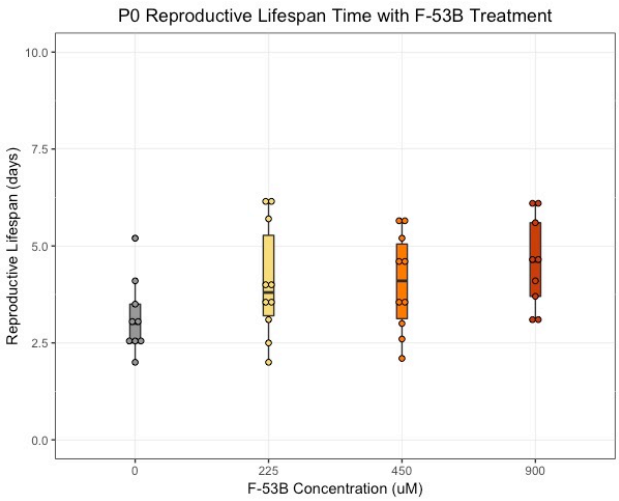
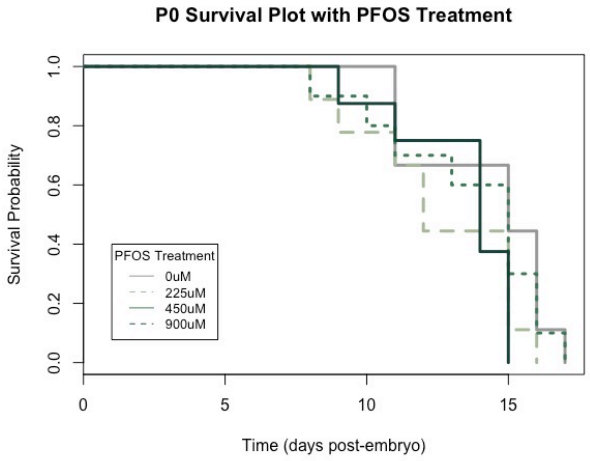


3C



3D

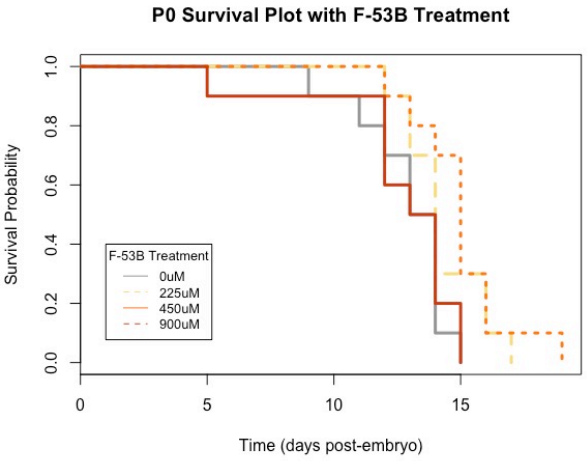


Figure 3. Neither PFOS nor F-53B significantly affect reproductive lifespan or survival time of N2 worms. Reproductive lifespans of N2 worms exposed to *(A)* PFOS and *(B)* F-53B are not significantly different from controls. It should be noted that the slight increase in reproductive lifespan evident at the highest PFAS concentrations is attributable to these worms producing a few viable offspring late in life after their main reproductive period has ended (see Figures 2A-B). Boxes represent the interquartile range while whiskers extend 1.5 times the interquartile range. Circles represent data for individual worms. Survival time is also unaffected by *(C)* PFOS and *(D)* F-53B exposure based upon logrank testing. n=10 worms per treatment, N=2 experimental repeats.

FIGURE 4

4A 4B 4C

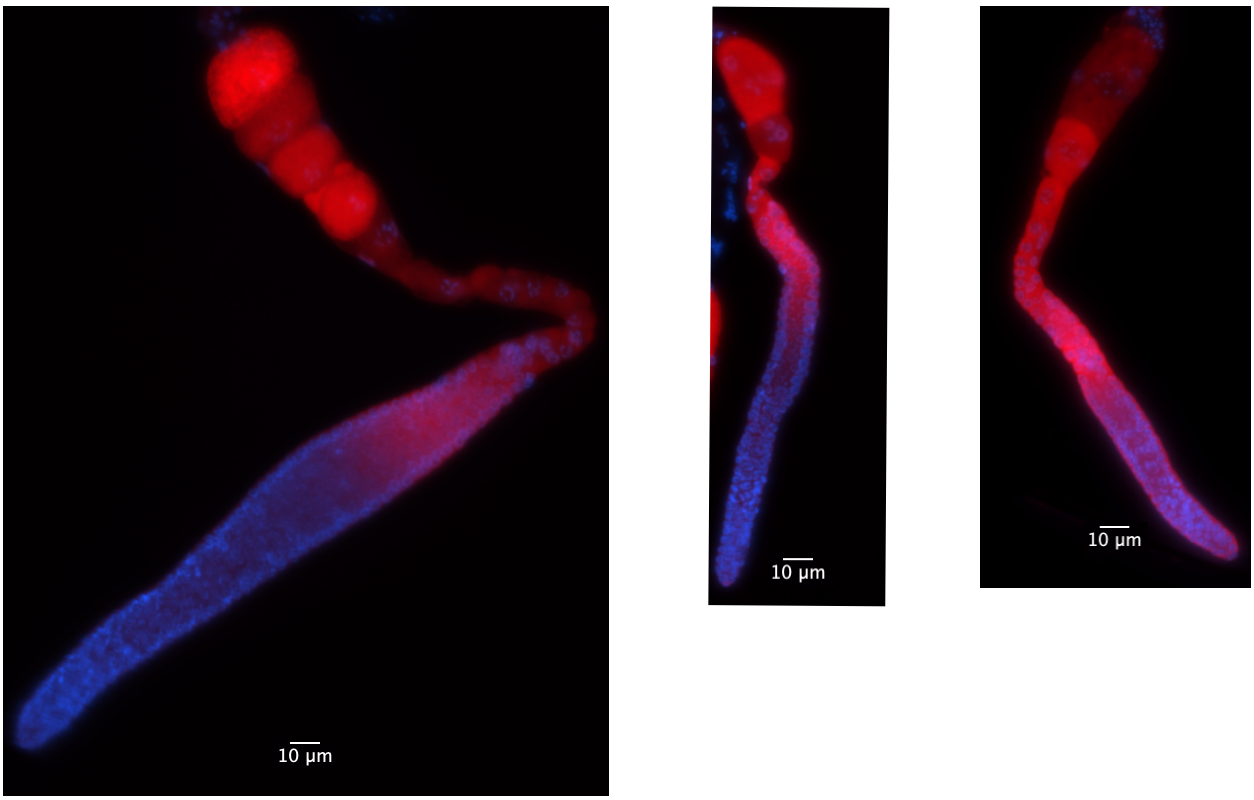
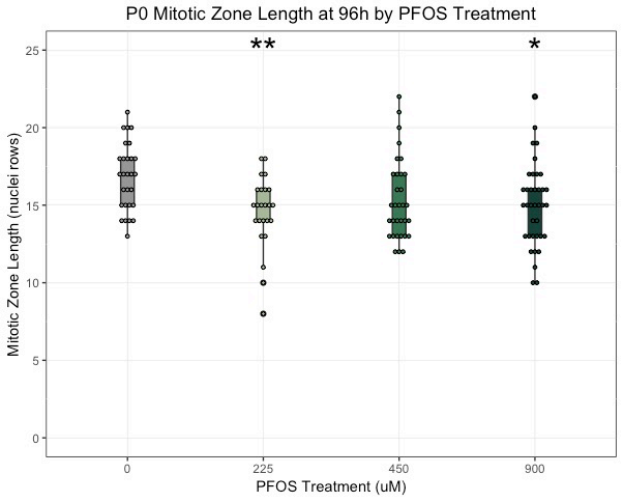


Figure 4. Gonads exposed to PFOS and F-53B are visually smaller than control gonads. A representative gonad dissected from *(A)* an adult control worm is compared to a representative gonad dissected from *(B)* an adult worm exposed to an NGM plate treated with 900µM PFOS or *(C)* and adult worm exposed to an NGM plate treated with 900µM F-53B. Gonads are oriented with the distal ends (mitotic zone) towards the bottom and proximal ends (mature oocytes) towards the top. Blue represents DAPI-stained DNA while red represents diphosphorylated MPK-1.

FIGURE 5

5A



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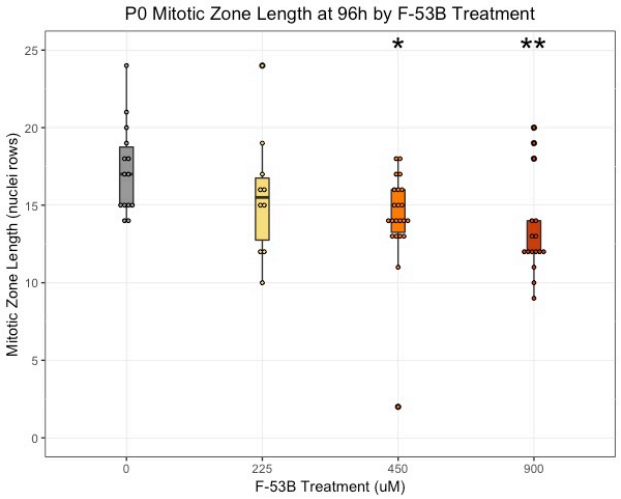

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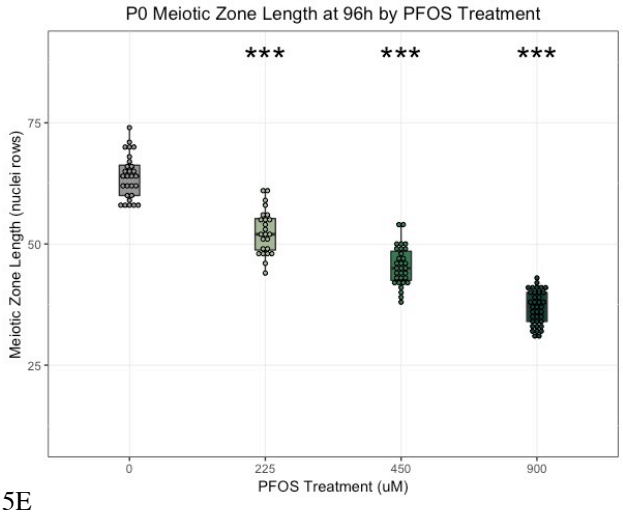
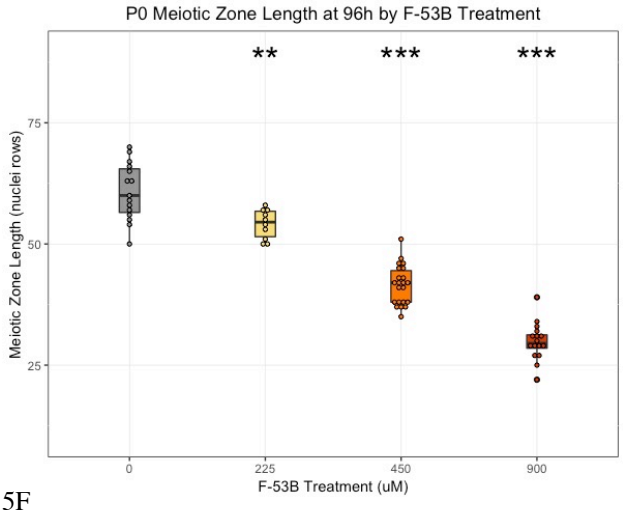


5D



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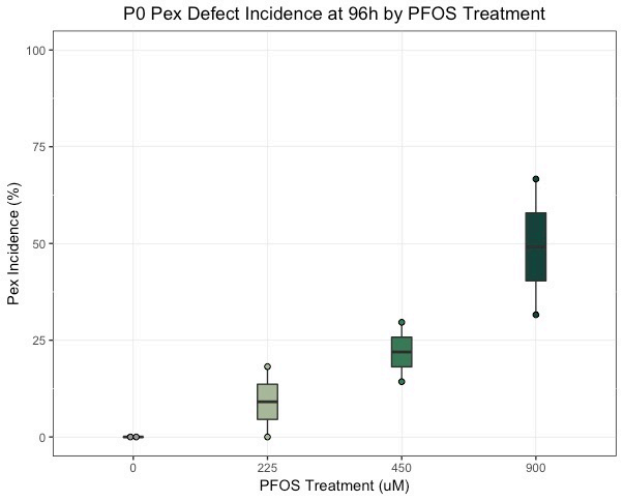


5F

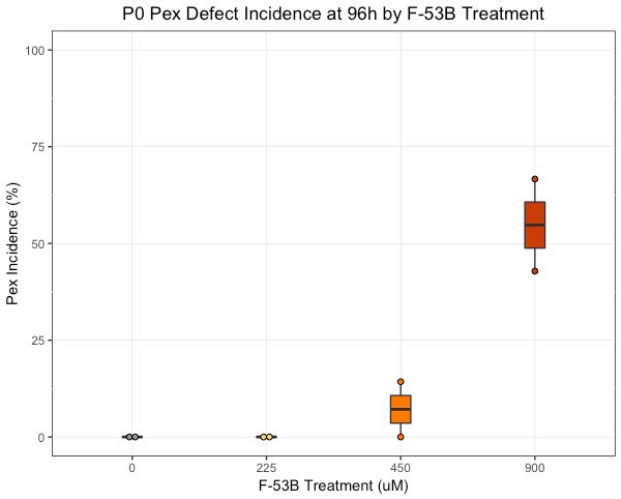


Figure 5. PFOS and F-53B exposure has more substantial impacts on meiotic germ cell numbers compared to mitotic germ cells and impairs pachytene exit. *(A)* PFOS exposure leads to inconsistent significant reductions in mitotic zone germ cells (up to approximately 15%) located at the distal end of the *C. elegans* gonad. However, *(B)* germ cells undergoing meiotic prophase appear to be impacted to a greater extent (approximately 40% maximal reduction) with a clear dose response evident. n=26-41 gonads scored per treatment, N=2 experimental repeats. *p<0.05, **p<0.01, ***p<0.001. *(C)* F-53B exposure leads to a small dose-dependent decrease in mitotic zone cells (up to approximately 25%), but similar to PFOS, *(D)* effects on meiotic cells are more pronounced (nearly 50% maximal reduction). n=14-23 gonads scored per treatment, N=2 experimental repeats. *p<0.05, **p<0.01, ***p<0.001. Boxes represent the interquartile range while whiskers extend 1.5 times the interquartile range. Circles represent data for individual worms. Within meiotic prophase, both *(E)* PFOS and *(F)* F-53B significantly increase the incidence of pachytene exit (Pex) defect, suggesting potential effects on synaptonemal complex disassembly. Significance determined using binomial logistic regression (PFOS p<0.001; F-53B p=0.002). Boxes represent the interquartile range while whiskers extend 1.5 times the interquartile range. Circles represent Pex incidence for each experimental repeat. n=14-23 gonads scored per treatment, N=2 experimental repeats.

FIGURE 6

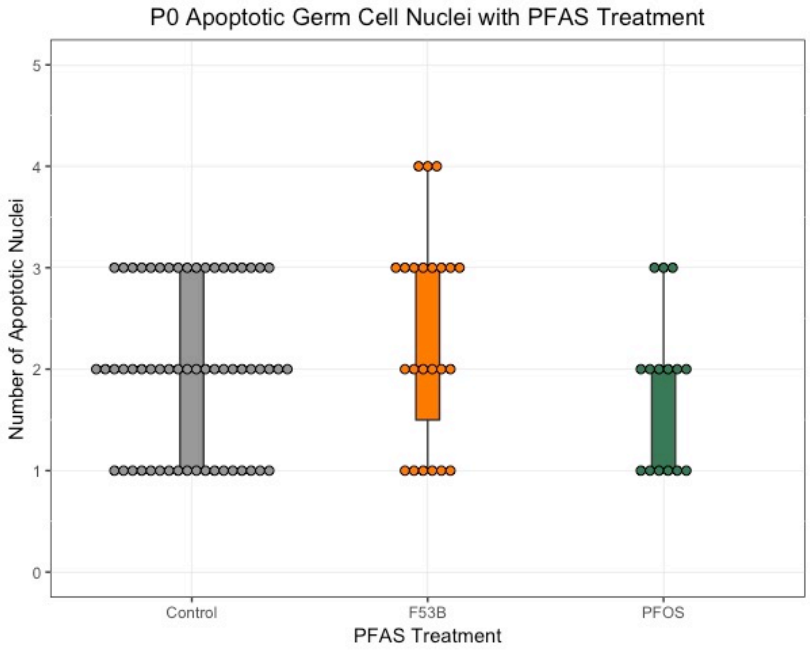
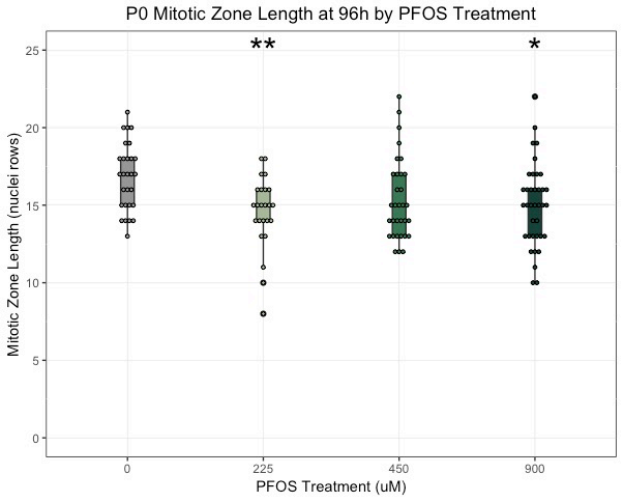


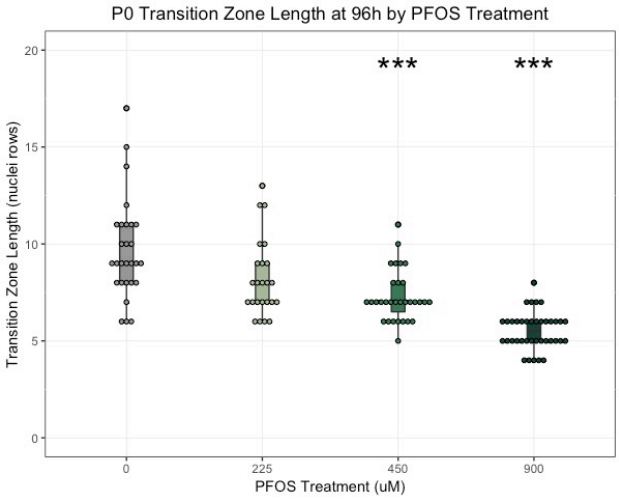
Figure 6. Apoptosis does not appear to substantially contribute to reductions in meiotic germ cells in worms exposed to PFOS and F-53B. Worms exposed to NGM plates treated with 450μM PFOS or F-53B did not exhibit statistically significant differences in the number of apoptotic germ cells compared to worms exposed to water-treated plates based upon acridine orange staining. Boxes represent the interquartile range while whiskers extend 1.5 times the interquartile range. Circles represent data for individual worms. n=15-58 worms scored per treatment. N=2 experimental repeats.

SUPPLEMENTAL FIGURE 1

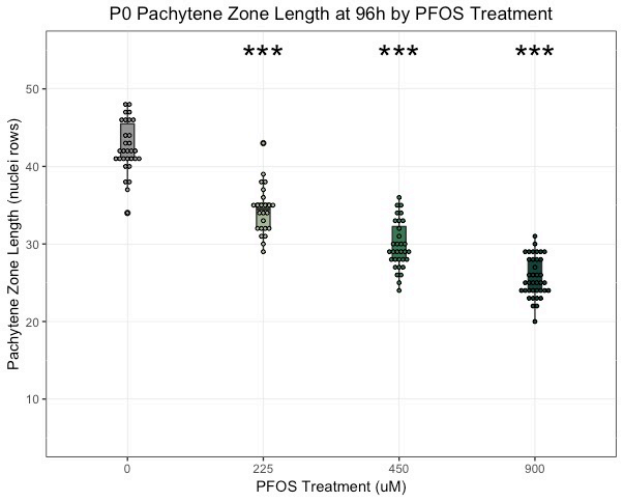
S1A



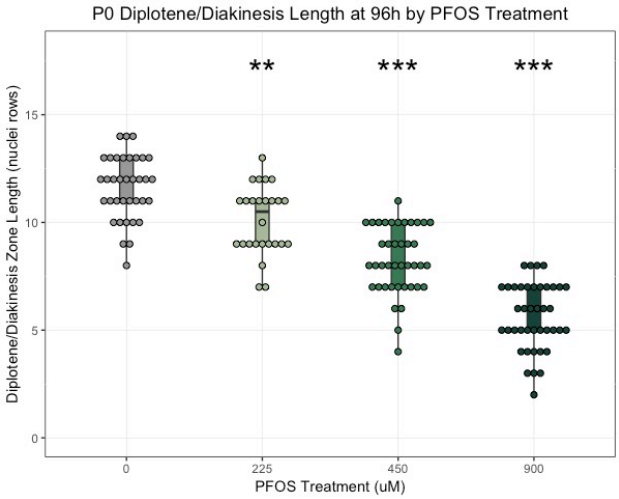
S1B



S1C



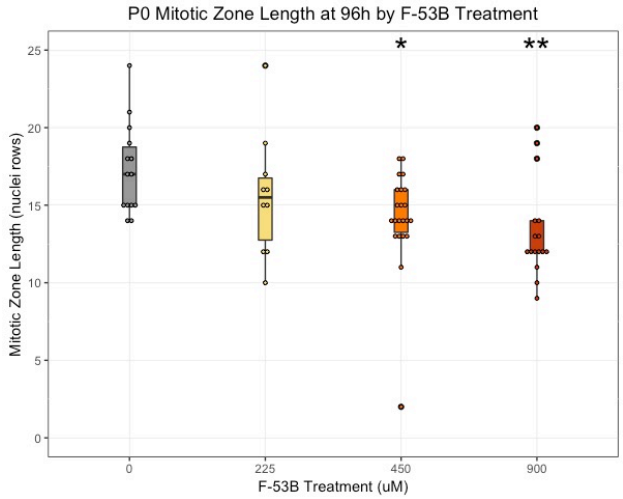
S1D



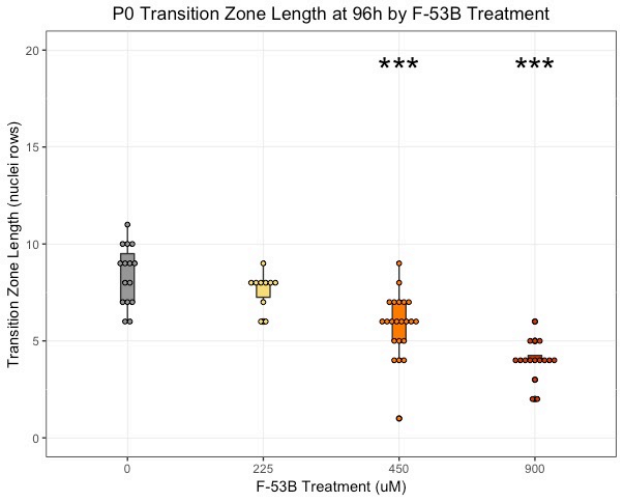
Supplemental Figure 1. Germ cells in different stages of meiotic prophase exhibit significant decreases from PFOS exposure. Germ cells in the *(A)* mitotic zone of the gonad appear to be less affected by PFOS exposure than germ cells in all stages of meiotic prophase, including *(B)* leptotene/zygotene (collectively termed the transition zone), *(C)* pachytene, and *(D)* diplotene and diakinesis, where individual oocytes cellularize. Boxes represent the interquartile range while whiskers extend 1.5 times the interquartile range. Circles represent data for individual worms. n=26-41 gonads scored per treatment, N=2 experimental repeats. *p<0.05, **p<0.01, ***p<0.001.

SUPPLEMENTAL FIGURE 2

S2A



S2B



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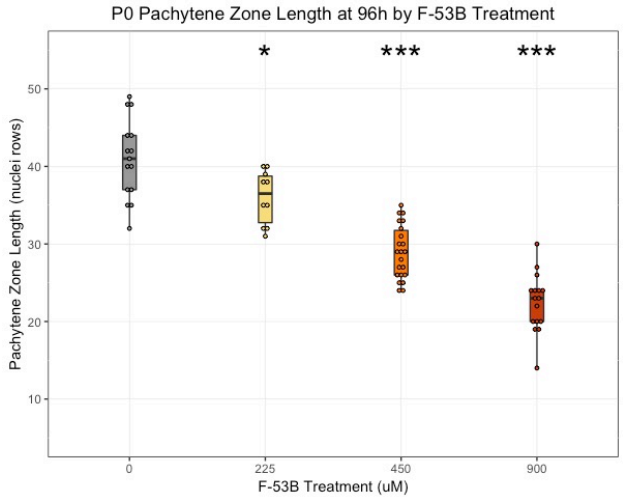
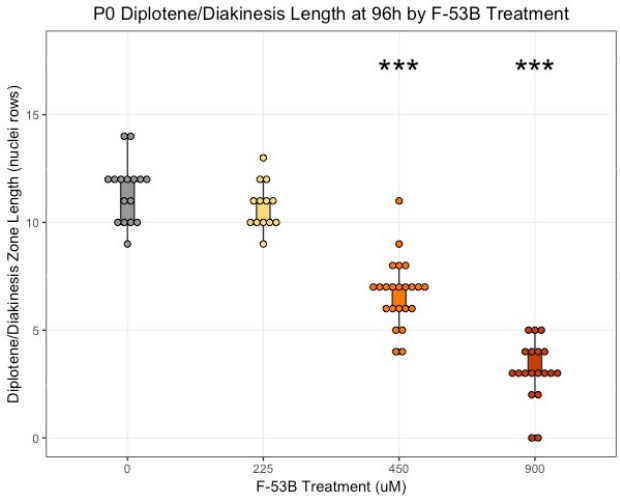


S2D



Supplemental Figure 2. Germ cells in different stages of meiotic prophase exhibit significant decreases from F-53B exposure. Germ cells in the *(A)* mitotic zone of the gonad appear to be less affected by F-53B exposure than germ cells in all stages of meiotic prophase, including *(B)* leptotene/zygotene (collectively termed the transition zone), *(C)* pachytene, and *(D)* diplotene and diakinesis, where individual oocytes cellularize. Boxes represent the interquartile range while whiskers extend 1.5 times the interquartile range. Circles represent data for individual worms. n=14-23 gonads scored per treatment, N=2 experimental repeats. *p<0.05, **p<0.01, ***p<0.001.

CHAPTER 4:

Biomolecular Condensates as Potential Mediators of Reproductive Toxicity from PFAS Exposure

INTRODUCTION

Toxicity data are limited or lacking entirely for most of the thousands of per- and polyfluoroalkyl substances (PFAS) known to exist (Fenton et al. 2020). To efficiently tackle this large data gap, one approach is to consider a broadly shared physicochemical property of PFAS and determine if this property may produce a common biological effect. Specifically, many PFAS have been used because they are particularly effective surfactants, compounds that preferentially partition to phase boundaries and reduce surface tension (ITRC 2021). Within a cell, BCs have the potential to be especially sensitive to the effects of surfactants since their assemblage and stability are dependent in part upon relatively weak intermolecular forces that can be altered by surfactants, such as electrostatic and hydrophobic interactions (Aguirre-Ramírez et al. 2021).

C. elegans is a valuable model to use in examining the effects of PFAS on BCs since several BCs have been well characterized in this organism and several fluorescent reporter strains and mutants in core BC proteins are available from the Caenorhabditis Genetics Center. Among others, germ cells of *C. elegans* include two BCs important for fertility: P granules and the synaptonemal complex (SC). P granules are dynamic, cytoplasmic assemblages of RNA and proteins that associate with the nuclear pores of germ cells until cellularization of oocytes in the adult hermaphrodite *C. elegans* gonad. P granules contain RNA and proteins essential for maintaining germ cell identity, including components of the piRNA, siRNA, and miRNA pathways (Lee et al. 2020; Chen et al. 2020; Price et al. 2021; Sundby et al. 2021). Research to date indicates that P granules act as post-transcriptional regulatory hubs that license germ cell-specific transcripts, suppress somatic transcripts (and sperm transcripts in the adult hermaphrodite), and modulate small RNA silencing activity (Voronina et al. 2012; Updike et al. 2014; Campbell & Updike 2015; Knutson et al. 2017; Dodson & Kennedy 2020; Ouyang &

Seydoux 2022). Based upon studies utilizing mutations or knockdown of P granule components, the proper functioning of P granules is dependent upon their molecular composition, localization, and structure; manipulations that alter P granule structure alter the transcriptome and can lead to a range of phenotypes, including defects in germ cell proliferation and progression, germ-to-somatic cell conversion, germ cell tumors, impaired RNA interference response, embryonic lethality, and/or sterility (Knutson et al. 2017; Marnik et al. 2019; Lev et al. 2019; Aoki et al. 2021).

While P granules are structures specific to nematodes, the SC is a broadly conserved structure essential for meiosis. The SC serves to align homologous chromosomes and regulate crossover events during meiosis (Libuda et al. 2013; Gordon et al. 2021). Exhibiting a characteristic ladder-like appearance, the SC has historically been thought of as a rigid structure functioning purely to stabilize paired chromosomes. However, increasing studies indicate that the SC is a labile structure (Pattabiraman et al. 2017) that actively recruits pro-crossover factors and regulates crossover interference (Gordon et al. 2021). Perturbation of SC structure by genetic mutation or knockdown of SC proteins impairs its crossover interference function and leads to an increase in the number of crossover sites (Libuda et al. 2013; Gordon et al. 2021), which can lead to aneuploid gametes (Zhang et al. 2021).

Common to both P granules and the SC is that they exhibit liquid-like properties (Brangwynne et al. 2009; Rog et al. 2021). Furthermore, stabilization of certain components of both P granules and the SC are dependent upon hydrophobic interactions that can be disrupted in the presence of 1,6-hexanediol, an aliphatic alcohol surfactant (Updike et al. 2011; Rog et al. 2017; Alleva et al. 2019). If P granule and SC structure can be disrupted by an aliphatic alcohol surfactant, it is possible that they may also be disrupted by PFAS surfactants. The purpose of the

subject work was to test the hypothesis that PFAS exposure can disrupt the structure of BCs in germ cells, leading to functional consequences for germ cell development and reproduction.

METHODS

PFAS solution preparation and exposure

900μM stock solutions of PFOS (Synquest Laboratories, CAS number 1763-23-1, >99.5%) or F-53B (BOC Sciences, CAS number 73606-19-6, technical grade) were prepared freshly from solid powders dissolved directly in double-deionized water that was filtered through a 0.22 micron membrane filter. PFOS or F-53B stock solutions were further diluted as necessary in filtered double-deionized water. PFAS solutions or filtered double-deionized water (control) were applied to the surface of NGM agar plates with OP50 *E. coli* lawns and the plates rotated gently to spread the solutions evenly. Treatments were applied to the plates at a ratio of 1:45 on a solution:NGM agar volume-for-volume basis (e.g., 0.4mL of PFAS solution/water applied to 18mL of NGM agar). PFAS treatment solutions at concentrations of 225μM, 450μM, or 900μM were applied to NGM plates, resulting in estimated exposure concentrations of 5μM, 10μM, and 20μM based on the volume-for-volume dilution factor. PFAS concentrations reported herein refer to that of the solution applied to the NGM plate and not the estimated exposure concentrations.

C. elegans strains used

The PGL-1::GFP reporter strain JH3269 (pgl-1(ax3122[pgl-1::gfp])) and the PGL-1::RFP reporter strain YY969 (znfx-1(gg544[3xflag::gfp::znfx-1]) II; pgl-1(gg547 [pgl-1::3xflag::tagRFP]) IV) obtained from the University of Minnesota Caenorhabditis Genetics Center (CGC) were used to visualize P granule structure and localization. In these strains, GFP or

RFP is inserted into the endogenous *pgl-1* locus. The *pgl-1(bn102)* mutant strain SS580 obtained from the CGC was used to examine synthetic sterility with PFAS exposure. *pgl-1(bn102)* is likely a null allele (Bilgir et al. 2013) and these worms form abnormally small P granules with altered structure and function. *pgl-1(bn102)* worms exhibit a sterility incidence of approximately 7-19% at 20°C, compared to near 0% sterility in wild type worms. The SYP-3::GFP reporter strain CA1218 (syp-3(ok758) I; ieSi11 [syp-3p::EmeraldGFP::syp-3::syp-3 3'UTR + Cbr-unc-119(+)] II; unc-119(ed3) III) was used both to visualize synaptonemal complex structure and localization and to examine synthetic embryonic lethality with PFAS exposure. This strain was generated using a *syp-3(ok758)* mutant background, which exhibits nearly 100% embryonic lethality. The transgenic *syp-3* expression in the SYP-3::GFP strain partially rescues this phenotype and embryonic lethality is reduced to approximately 15%. The SYP-3::GFP strain also produces male progeny more frequently than wild type worms due to increased nondisjunction of meiotic chromosomes.

P granule structure and localization with ex vivo or in vivo PFAS exposure

For *ex vivo* exposures, PGL-1::GFP worms were synchronized either from embryos or at the L4 larval stage to obtain an age-matched adult population. Synchronized worms were maintained on standard, untreated NGM agar plates until reaching adulthood. Early adult worms were picked into an 8µL drop of M9 minimal salts solution placed on a microscope slide. The gonads were extruded from the worms by decapitating with a 25 gauge needle. 8µL of a PFOS or F-53B solution prepared in filtered double-deionized water or filtered double-deionized water alone (control) was added to the drop of M9 and gently mixed using a plastic pipette tip. After covering with a cover slip, the gonads were imaged on Nikon H600L epifluorescence microscope at 40X magnification using a FITC filter. Specifically, the pachytene region of a different worm

was imaged every 2-4 minutes for 20 minutes on each slide to monitor time-dependent effects of PFAS exposure on P granules.

For *in vivo* exposures, synchronized adult PGL-1::GFP or PGL-1::RFP worms were transferred to PFAS- or water-treated NGM plates and allowed to lay embryos for approximately 2-3 hours. After this time, the adult *C. elegans* were removed and the embryos laid on the plates were allowed grow for 96 hours, reaching adulthood. Adult worms were picked into a 14µL drop of M9 minimal salts solution placed on a microscope slide, to which 1µL of 10mM levamisole hydrochloride was added to immobilize the worms. The pachytene regions of immobilized worms were imaged under Nikon H600L epifluorescence microscope at 100X magnification using a Texas Red filter.

Synthetic sterility with PFAS exposure in a pgl-1 mutant

Synchronized adult *pgl-1(bn102) C. elegans* were transferred to NGM plates treated with water, PFOS, or F-53B (225µM, 450µM, or 900µM) and allowed to lay embryos for approximately 3-4 hours. After this time, the adults were removed and the embryos allowed to reach adulthood (approximately 96 hours). The exposed adults were imaged with differential interference contrast (DIC) microscopy at 10-40X. Worms exhibiting one or more embryos in their uteri were scored as fertile. Worms lacking embryos and exhibiting a degenerated and/or malformed gonad were scored as sterile. Worms that lacked embryos but appeared to be young adult or larval stage based upon gonad morphology were discounted since the lack of embryos could have been due to developmental delay rather than sterility.

Synaptonemal complex structure and localization with ex vivo PFAS exposure

SYP-3::GFP were synchronized either from embryos or at the L4 larval stage to obtain an age-matched adult population. Early adult worms were picked into an 8µL drop of M9 minimal salts solution placed on a microscope slide. The gonads were extruded from the worms by decapitating with a 25 gauge needle. 8µL of either a PFOS or F-53B solution prepared in filtered double-deionized water was added to the drop of M9 and gently mixed using a plastic pipette tip. After covering with a cover slip, the gonads were imaged on Nikon H600L epifluorescence microscope at 100X magnification using a FITC filter. Specifically, the pachytene region of a different worm was imaged every 2-4 minutes for 20 minutes on each slide to monitor time-dependent effects of PFAS exposure on the SC.

Synthetic embryonic lethality with PFAS exposure in a syp-3 partial rescue mutant

Synchronized adult SYP-3::GFP *C. elegans* were transferred to NGM plates treated with water, PFOS, or F-53B (225µM, 450µM, or 900µM) and allowed to lay embryos for approximately 3-4 hours. After this time, the adults were removed and the embryos allowed to reach adulthood (approximately 96 hours). After this time 4-5 worms were transferred to new treated NGM plates and allowed to lay embryos for approximately 6 hours. The adults were then removed from the plates and the number of embryos laid on each plate counted. Approximately 48 hours later, the number of larval stage worms surviving on each plate were counted. Finally, the number of surviving adult worms on each plate were counted at 96 hours after the embryos were laid. Embryonic lethality was calculated as the percentage of embryos laid that failed to hatch or produce viable larvae. Larval lethality was calculated as the percentage of larvae that failed to survive to adulthood.

Statistical analysis

Unless otherwise noted, Welch's ANOVA with Games-Howell post-hoc test were used for statistical analyses to account for heterogeneous variances and unequal sample sizes. Statistical analyses were performed using R statistical software.

RESULTS

PFOS and F-53B ex vivo and in vivo exposures exhibit different effects on P granules

Since others have previously shown that exposing dissected gonads *ex vivo* to 1,6-hexanediol can quickly dissolve P granules (Updike et al. 2011), *ex vivo* gonad exposures were performed with PFOS and F-53B to determine if these PFAS can elicit a similar effect. With *ex vivo* exposure at a concentration of 450μM, both PFOS and F-53B dissolved the vast majority of PGL-1 granules as represented by PGL-1::GFP foci within 20 minutes (Figures 7A-E). At a concentration of 10μM, gross effects on P granules were not observed up to 12 minutes post exposure for either PFAS. While observations were not recorded for PFOS after this time, F-53B exposure did begin to dissolve P granules at 16 minutes with an increased effect at 20 minutes. The time dependency of PFAS exposure effects on P granules likely reflect the kinetics of PFAS migration through the somatic gonad sheath cells and accumulation in the germ cell cytoplasm at a sufficient concentration to destabilize P granule structure. Though effects on the pachytene region were quantified, it should be noted that the earliest effects on P granule structure were observed in the distal mitotic region of the gonad. This observation may reflect the fact that the somatic gonad sheath cell processes exhibit discontinuous coverage of the germ cells in this region (Killian & Hubbard 2005), which could facilitate faster PFAS penetration into this region of the gonad. It is also possible that the composition of P granules in the mitotic compared to meiotic pachytene region makes them more susceptible to disruption by PFAS. However, given

the observation that P granules in mature, fully cellularized oocytes were generally last in the gonad to be affected by PFAS exposure, it appears that differences in chemical uptake kinetics in the different gonad regions likely drive these effects.

To determine if the *ex vivo* effects of PFAS exposure on P granules could be replicated with *in vivo* exposures, adult PGL-1::GFP or PGL-1::RFP worms were imaged using an epifluorescence microscope following 96h of PFOS or F-53B exposure from the embryo stage. Based upon results from both P granule reporter strains, it does not appear that *in vivo* PFOS or F-53B exposure consistently dissolved P granules in adult gonads at the concentrations tested. However, *in vivo* exposure did appear to enlarge P granules, significantly increasing P granule size in a dose-dependent manner when quantified in PGL-1::RFP worms (Figure 8A-C). While P granule size was not quantified in PGL-1::GFP worms, it appeared that there was a similar increase in P granule size comparable to that of PGL-1::RFP worms.

Mutants lacking the P granule protein PGL-1 exhibit increased sterility with PFOS and F-53B exposure

Since it appeared that PFOS and F-53B exposure produced structural alterations to P granules, these PFAS were next tested in a sensitized P granule mutant. Control *pgl-1(bn102)* worms exhibited variability in their sterility rate, ranging from 0-14%, which is generally consistent with the reported range. Sterile worms had visibly atrophied or absent gonads and no embryos (Figures 9A-D). While this sterility rate is generally consistent with the reported range of 7-19%, the variability in control sterility rate hindered direct comparisons between experiments. Therefore, to account for variability between controls, sterility rate was first transformed to 1 + log(% sterility) to account for samples with 0% sterility. For each experimental repeat, the relative sterility rate was calculated as the exponent of the log difference

between each treatment and the control. Based on this comparison, sterility incidence increased with increasing PFAS concentration, with PFOS eliciting a stronger effect than F-53B for each concentration (Figure 9E). Exposure to 900μM PFOS-treated plates elicited the greatest effect with a geometric mean sterility incidence over two times higher than that of the controls. These data suggest that PFAS exposure may enhance the sterility defect in *pgl-1(bn102)* worms.

PFOS and F-53B ex vivo and in vivo exposure disrupts SC structure

Similar to P granules, the SC has previously been shown to be dissolved with *ex vivo* exposure to 1,6-hexanediol (Rog et al. 2017) and PFOS and F-53B were tested to see if they could elicit a a similar effect on the SC. *Ex vivo* exposure to 450μM PFOS caused SYP-3::GFP to lose most of its linear structure along pachytene chromosomes, appearing almost completely diffuse within the nucleus (Figures 10A-B). Similar to P granules, the effect of *ex vivo* PFOS exposure on the SC was time dependent. Up to 12 minutes post treatment with PFOS, only 20% of gonads exhibited diffuse SYP-3::GFP (Figure 10C). However, from 12 to 20 minutes post treatment, 80% of gonads exhibited diffuse SYP-3::GFP. While additional concentrations and F-53B were not tested, these data indicate that at least PFOS is capable of penetrating into germ cell nuclei and disrupting SC structure. When SYP-3::GFP worms were exposed to PFOS or F-53B for 96h *in vivo* at the highest NGM agar treatment concentration (900μM), up to approximately 50% of worms exhibited diffuse SYP-3::GFP in their gonads (Figure 11A). However, this effect was not consistently observed when NGM agar plates were treated with 450μM PFOS or F-53B, suggesting that these treatments resulted in internal PFAS concentrations that were insufficient to disrupt SC structure.

SYP-3 partial rescue mutants exhibit increased embryonic lethality with PFOS and F-53B exposure

Since it appeared that PFOS and F-53B exposure produced structural alterations to the SC, these PFAS were next tested in the sensitized SC mutant to determine if PFAS exposure could enhance their embryonic lethality defect. As noted previously, the SYP-3::GFP strain used was constructed on a *syp-3* loss of function mutant with near complete embryonic lethality and the transgenic *syp-3::gfp* expression partially rescues this effect. Control SYP-3::GFP worms exhibited embryonic lethality rates ranging from 1-10%, which is somewhat lower than the reported rate of 15%. However, exposure to PFOS and F-53B resulted in dramatic dose-dependent increases in embryonic lethality, which reached up to 100% for the two higher exposure concentrations (Figure 11B). These data suggest that the effects of PFAS exposure on SC structure in SYP-3::GFP worms has negative consequences for SC function and impairs proper chromosome segregation. Although fully diffuse SYP-3::GFP was not always observed in exposed worms, it is possible that PFOS and F-53B exposure could cause more subtle effects on SC structure that is nevertheless sufficient to impact its function.

DISCUSSION

Based upon the data presented herein, it appears that both PFOS and F-53B are able to disrupt the structure of germ cell BCs in *C. elegans* with functional consequences. In the case of P granules, *ex vivo* exposures to PFOS and F-53B produced fast, dramatic effects on the condensates with near complete dissolution at the relatively high concentration of 450µM. However, it should be noted that this concentration is lower than the concentration of 1,6-hexanediol used by others in their *ex vivo* experiments, which, at 5%, is equivalent to approximately 1mM. P granule dissolution was also observed with *ex vivo* exposure to F-53B at

a concentration of 10μM, though the effect took longer to produce. These observations are consistent with high surfactant concentrations inhibiting liquid-liquid phase separation, leading to complete mixing with the bulk phase (Sanchez-Burgos et al. 2021). In contrast, *in vivo* exposures to PFOS and F-53B did not consistently produce complete dissolution of P granules in adult gonads, but rather increased condensate size. This result seems counterintuitive because increasing surfactant concentration is generally associated with decreased condensate size since the lowering of surface tension reduces the energetic burden of the increased surface area to volume ratio of smaller condensates (Sanchez-Burgos et al. 2021).

One possible explanation is based on the substantial difference in *ex vivo* compared to in *vivo* concentrations to which the P granules were exposed. At concentrations well below their critical micelle concentrations (CMC), ionic surfactants generally bind to proteins via electrostatic interactions without causing any structural change and may actually promote protein stabilization (Aguirre-Ramírez et al. 2021). As the surfactant concentration increases, hydrophobic interactions between the surfactant tail and hydrophobic regions of the protein increase, producing effects on secondary protein structure (Aguirre-Ramírez et al. 2021). At high enough surfactant concentrations, all protein binding sites are occupied by surfactant molecules and proteins become completely dispersed in the bulk phase (Aguirre-Ramírez et al. 2021). Under this model, the internal PFAS exposure achieved by *in vivo* exposure may result in some PFAS molecules binding to outermost P granule proteins and potentially affect their stability or structure without dispersing them from the P granule. However, the altered properties of the PFAS-bound proteins may lead to changes in their dynamics between the P granule and bulk phase or alter their function within the granule. The core P granule protein GLH-1 (a DEAD-box RNA helicase) may be particularly susceptible to changes in stability or structure because it

normally cycles through a folded and unfolded conformation depending on if it is bound to ATP and RNA or has released ADP and phosphate, respectively (Marnik et al. 2019; Chen et al. 2020). In *glh-1* mutants that have specifically lost their ATP hydrolysis ability, GLH-1 binds RNA more strongly and retains more mRNA in P granules, leading to an increase in P granules size (Marnik et al. 2019; Chen et al. 2020). The preference for mRNA binding in these mutants also shifts from WAGO-1 targets to CSR-1 targets, which are mainly germline transcripts normally licensed by CSR-1 and exported from P granules for translation or storage in the cytoplasm (Dai et al. 2022). Furthermore, the Argonaute proteins PRG-1, CSR-1, and WAGO-1, which normally transiently interact with GLH-1, form more stable interactions with GLH-1 in these *glh-1* mutants. It is possible that PFAS binding to GLH-1 at low concentrations could alter its ATP hydrolysis cycling or otherwise stabilize normally transient interactions with P granule mRNA or proteins, altering P granule composition and function while increasing P granule size. Enlarged P granules are also observed in *csr-1* mutants as well as in mutants in other components of the CSR-1 complex (Andralojc et al. 2017). These mutants exhibited increased RNA content in their P granules, suggesting that their enlargement could at least in part be due to aberrant accumulation of RNA (Andralojc et al. 2017)

An alternative explanation for increased P granule size at low PFOS and F-53B concentrations would be altered P granule composition due to the cellular stress response. In addition to having overlapping constituent proteins, P granules, stress granules, and P bodies can also physically interact in response to cellular stress (Schisa 2019). These different BCs may even fuse and form a hybrid BC that serves to limit translation, preserve mRNA stability, and promote cell survival under stress (Schisa 2019). This possible scenario is supported by the fact that apparent TIAR-1 granules were observed in perinuclear foci in some L4 larval worms

exposed to F-53B for 48 hours (data not shown) and by the fact that PFAS exposure is reportedly able to generate ROS, which can trigger the cellular stress response. The creation of hybrid stress-induced BCs could increase P granule size by increasing the protein and RNA content.

As discussed in Chapter 3, the estimated internal concentration in worms exposed to PFAS *in vivo* is over 10,000 times lower than the *ex vivo* exposure concentrations. In the proposed scenarios, PFOS and F-53B gonad concentrations would be low enough that their surface tension lowering effect on decreasing droplet size would be outweighed by increase in P granule mRNA and protein content, ultimately producing a net increase in P granule size. In contrast, the relatively high PFOS and F-53B concentrations achieved by *ex vivo* exposure would fully destabilize P granules, resulting in PFAS-protein micelles being distributing throughout the cytoplasm (Figure 12).

Results of the PFOS and F-53B *in vivo* exposures in *pgl-1* loss-of-function mutant worms further suggest a role for altered P granule composition and RNA regulation in germ cell toxicity. When PGL-1 is either absent or cannot assemble into granules, mRNA is aberrantly de-repressed (Aoki et al. 2021), which likely contributes to the increased sterility observed in *pgl-1(bn102)* worms. Similar to *pgl-1(bn102)*, single mutants in other P granule components, including *csr-1*, *deps-1*, *glh-1*, and *prg-1* exhibit some sterility defects that become fully penetrant in double mutants and/or after several generations (Phillips & Updike 2022). Therefore, increasing the number of disrupted P granule components increases the effects on sterility. Since increased sterility was observed with PFOS and F-53B treatment in the *pgl-1(bn102)* worms, it suggests that these exposures could inhibit localization of other proteins to P granules, further contributing to an aberrant transcriptome that is incompatible with normal germ cell development.

Enhanced defects were also observed in the *syp-3* partial rescue mutant when exposed to PFOS or F-53B. While some worms exposed *in vivo* at the highest concentrations did exhibit near complete dissolution of SYP-3::GFP, significantly increased embryonic lethality was observed at concentrations that did not elicit this effect. Furthermore, oocytes of fixed worms generally exhibited 6 DAPI-stained bodies (data not shown), corresponding to the 6 chromosome bivalents, which indicates that most homologs were properly synapsed. Although complete dissolution of SYP-3::GFP may not be necessary to cause embryonic lethality, PFOS and F-53B exposure could still decrease the strength of self-association between SC subunits that appears to be required for robust crossover interference (Gordon et al. 2021). Even when homologs are properly synapsed, crossover interference is less effective when SC self-association is decreased and can lead to multiple crossovers between homolog pairs (Gordon et al. 2021). Excessive crossovers prevent homologs from properly aligning along the metaphase plate and lead to increases in aneuploid gametes and embryonic lethality (Hollis et al. 2021). Therefore, PFOS and F-53B exposure in SYP-3::GFP worms may cause increased embryonic lethality by inhibiting crossover interference and increasing the number of crossover events.

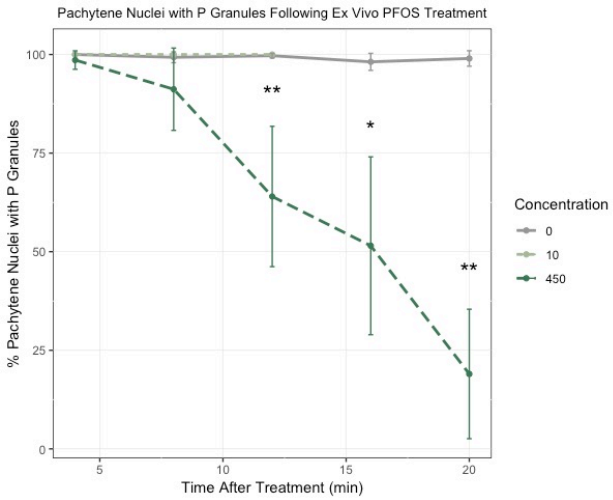
Because only modest effects on embryonic lethality were observed in wild type worms, it is possible that the GFP fusion protein makes SYP-3 more susceptible to disruption from PFOS and F-53B exposure. Similarly, no obvious sterility was observed in wild type worms exposed to PFOS and F-53B despite the increases observed in *pgl-1(bn102)* worms. Loss of PGL-1 may make remaining P granule components more susceptible to disruption by PFOS and F-53B exposure and enhance the baseline sterility observed in the mutant strain. What both of these mutant strain effects highlight is the potential for genetic variants that alter BC composition or dynamics to be more susceptible to toxicity from PFAS exposure.

CONCLUSION

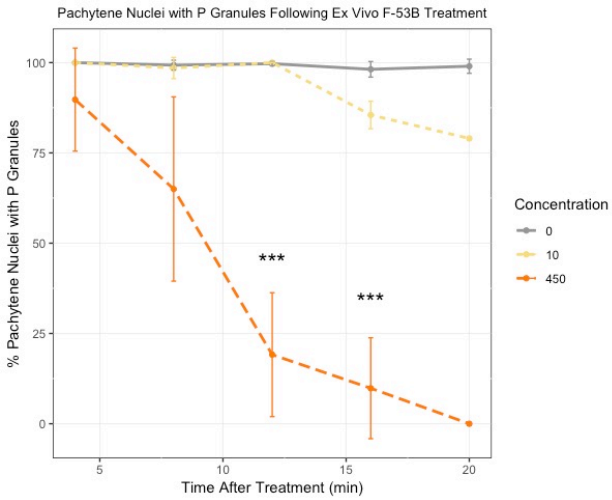
BCs are a novel target to examine in assessing PFAS toxicity. Two types of BCs present in the germ cells of *C. elegans* appear to be susceptible to disruption by PFAS exposure with functional consequences for germ cell development and reproduction. It would be worth examining effects of PFAS exposure on BCs in other organisms and other tissue types to determine if BC disruption occurs more widely. Effects on BCs could be a useful endpoint to use in large-scale PFAS toxicity screening assays since the ability of PFAS to alter BC structure is likely due surfactant activity, which is shared among many PFAS (Glüge et al. 2020).

FIGURE 7

7A



7B



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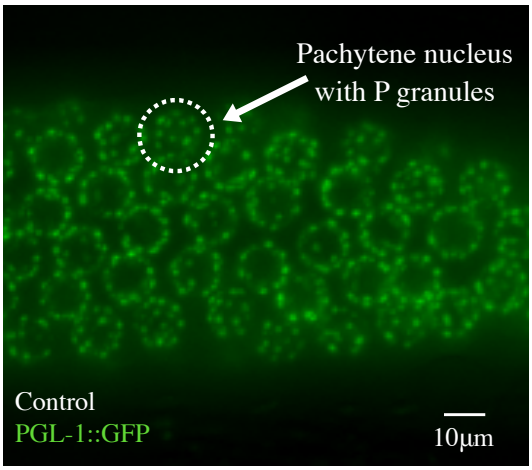

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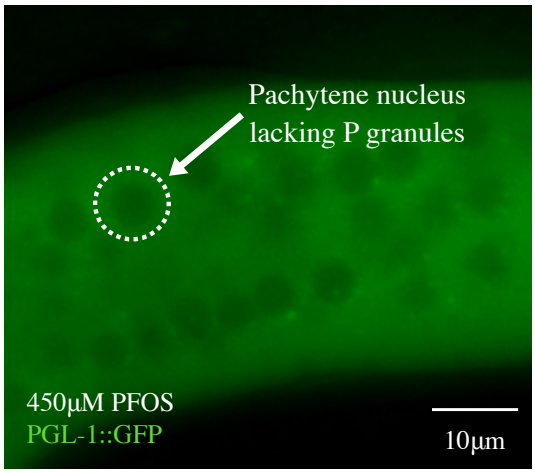
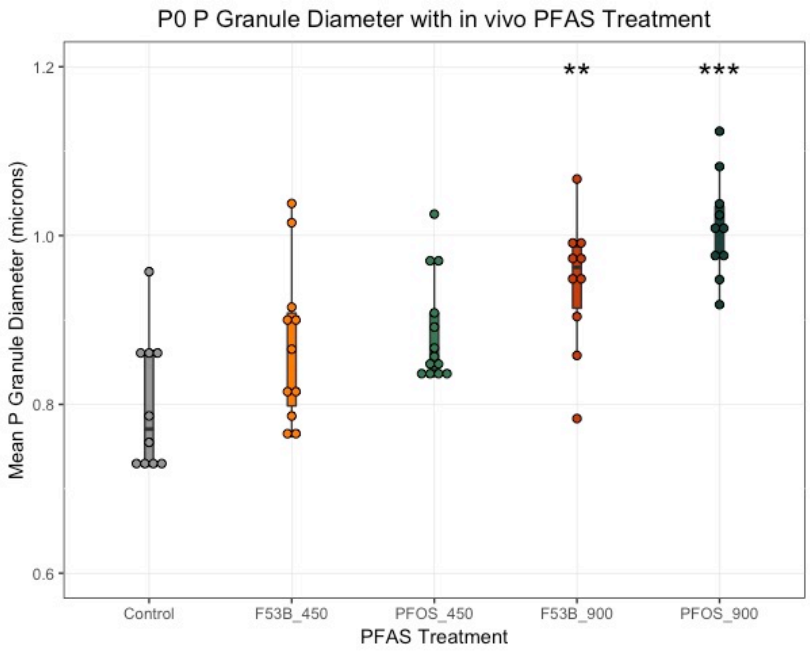


Figure 7. *Ex vivo* exposure to PFOS and F-53B causes a time- and concentration-dependent loss of PGL-1 granules. When applied to dissected gonads *ex vivo*, *(A)* PFOS and *(B)* F-53B exposure at 450µM leads to almost complete loss of P granules in a PGL-1::GFP reporter by 20 minutes post treatment. 10µM PFOS did not dissolve granules by 12 minutes post treatment, the latest time point tested. However, 10µM F-53B exposure did begin to dissolve P granules by 16 minutes post treatment. n=8-10 gonads imaged for each time point, N=2 experimental repeats. Error bars represent 95% confidence interval. *p<0.05, **p<0.01. *(C)* Control gonads treated *ex vivo* with

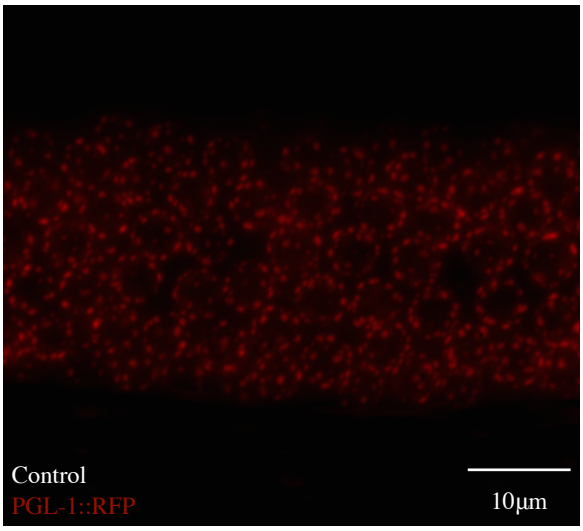
double-deionized water did not exhibit effects on P granule localization for at least 20 minutes post treatment. *(D)* In contrast, most P granules are fully dissolved by 20 minutes post treatment with 450μM PFOS.

FIGURE 8

8A



8B



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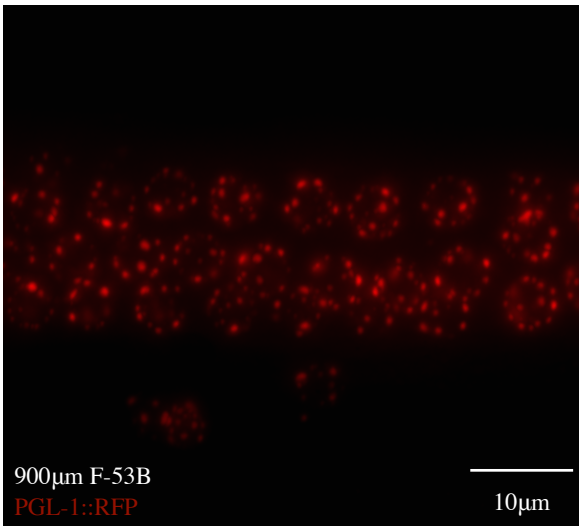
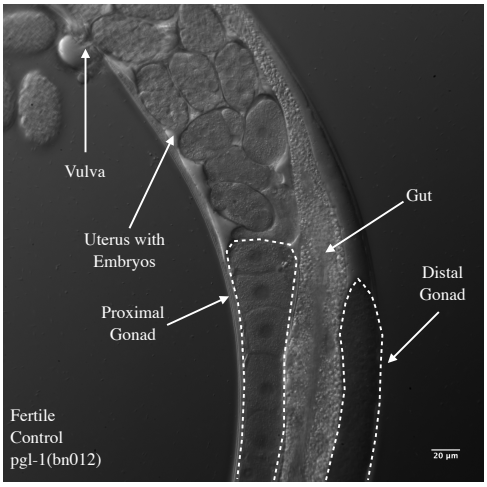


Figure 8. *In vivo* exposure to PFOS and F-53B produces larger P granules. *(A)* Worms exposed in vivo to PFOS or F-53B from embryos through adulthood exhibit an increase in P granule diameter in a PGL-1::RFP reporter strain. n=10-11 worms per treatment, N=1 experimental repeat. **p<0.01, ***p<0.001. *(B)* Representative

pachytene nuclei are surrounded by P granules that are smaller in diameter than *(C)* those exposed to NGM plates treated with 900μM F-53B.

FIGURE 9

9A



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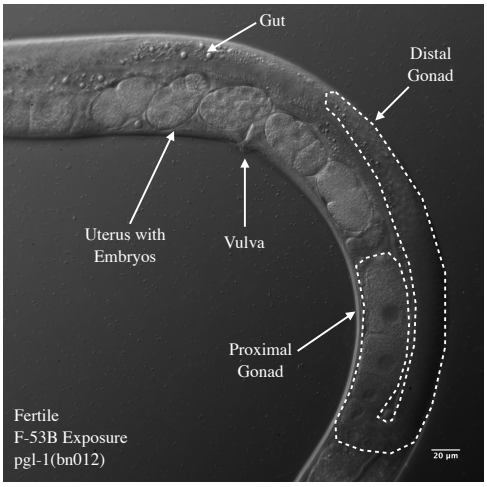

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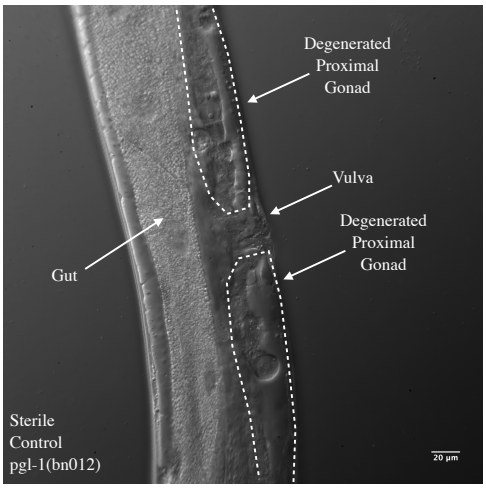

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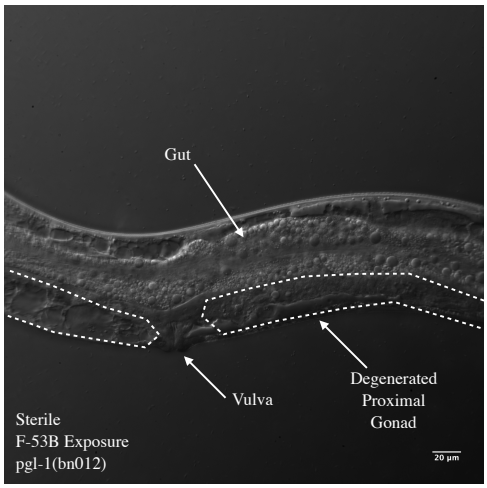

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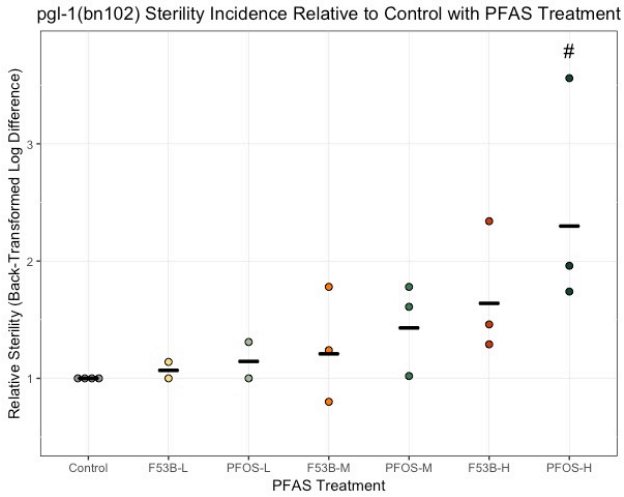
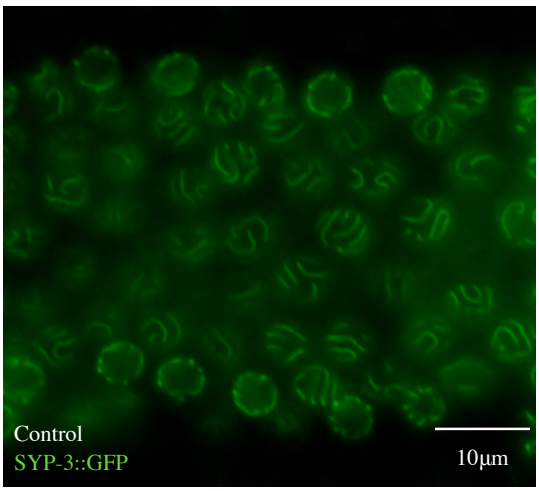


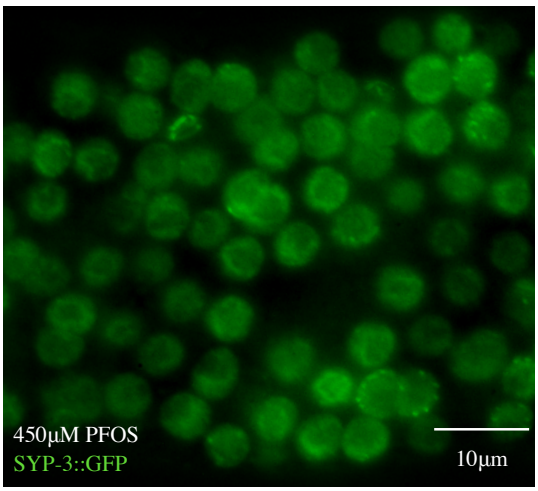
Figure 9. PFOS and F-53B exposure in pgl-1(bn102) mutants increases sterility incidence. Fertile worms exhibited morphologically recognizable gonads and embryos in their uteri and, as in wild type worms, *(A)* controls generally had larger gonads with more embryos compared to *(B)* PFAS-treated worms. Sterile *(C)* control and *(D)* PFAS-treated worms generally exhibited degenerated gonads without normal morphological features and lacked embryos. *(E) pgl-1(bn102)* worms exposed to NGM plates treated with PFOS and F-53B exhibited an increasing trend in sterility incidence relative to controls with increasing concentration. Crossbar marks represent the geometric mean. n=60-110 worms scored per treatment, N=2-3 experimental repeats. #$p<0.1$.

FIGURE 10

10A



10B



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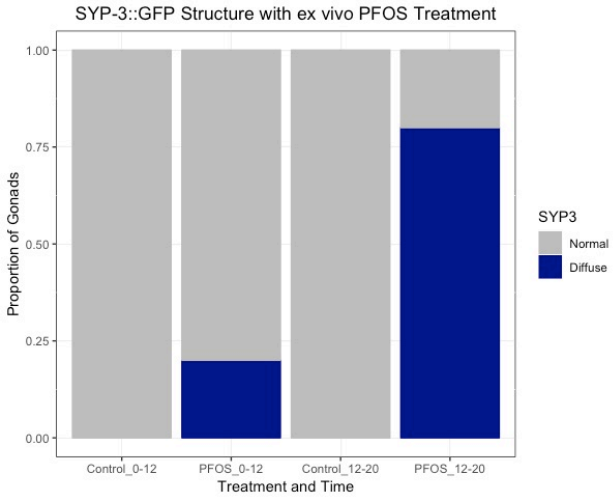
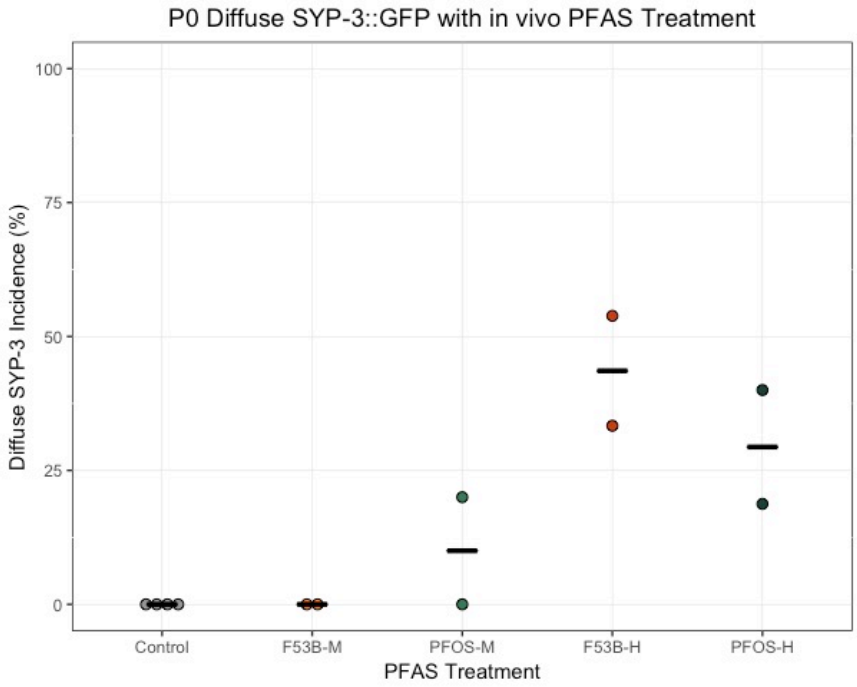


Figure 10. *Ex vivo* exposure to PFOS causes SYP-3::GFP to become diffuse within the nucleus. *(A)* Control worms exhibit SYP-3::GFP as threads, which mark the SC between homologous chromosomes in the nucleus. *(B)* Worms exposed to 450µM PFOS *ex vivo* exhibit mostly diffuse SYP-3::GFP within nuclei, likely indicating disruption of SC structure. *(C)* The effect of *ex vivo* PFOS exposure is time dependent with a higher incidence of diffuse SYP-3::GFP observed at 12-20 minutes post treatment compared to 0-12 minutes post treatment. n=8-10 worms per treatment, N=1 experimental repeat.

FIGURE 11

11A



11B

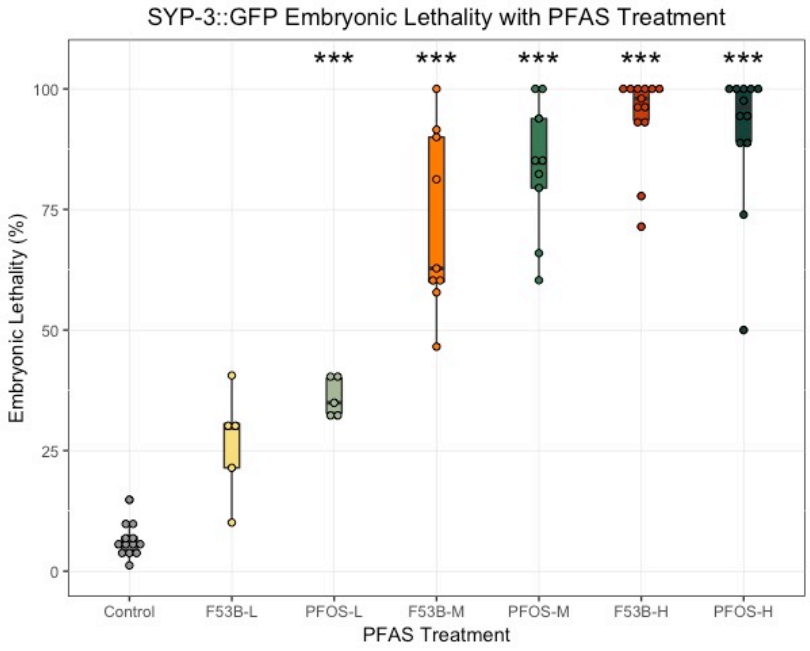
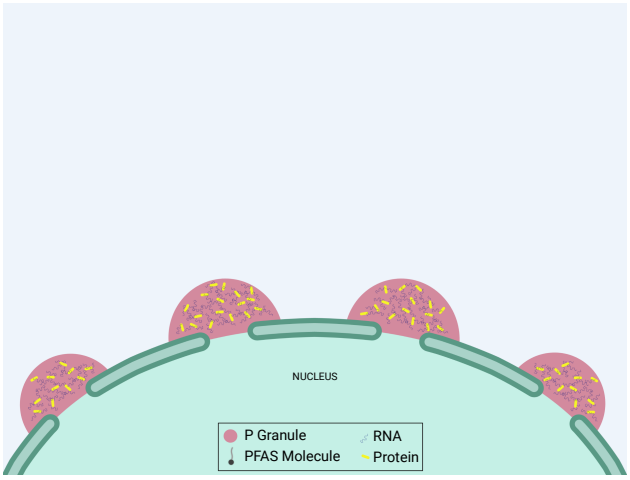


Figure 11. *In vivo* exposure to PFOS and F-53B in SYP-3::GFP worms may disrupt the SC and causes a dramatic increase in embryonic lethality. *(A)* Worms exposed to PFOS and F-53B *in vivo* exhibit inconsistent effects on SYP-3::GFP with some increased incidence of diffuse SYP-3::GFP at the highest tested concentration. n=11-21 worms per treatment, N=2 experimental repeats. Crossbars represent the mean. *(B)* PFOS and F-53B exposure causes a statistically significant, dose-dependent increase in embryonic lethality in SYP-3::GFP worms, with worms treated at the highest concentrations exhibiting nearly 100% embryonic lethality. Boxes represent the interquartile range while whiskers extend 1.5 times the interquartile range. Circles represent data for individual worms. n=5-13 worms per treatment, N=2 experimental repeats. ***$p<0.001$.

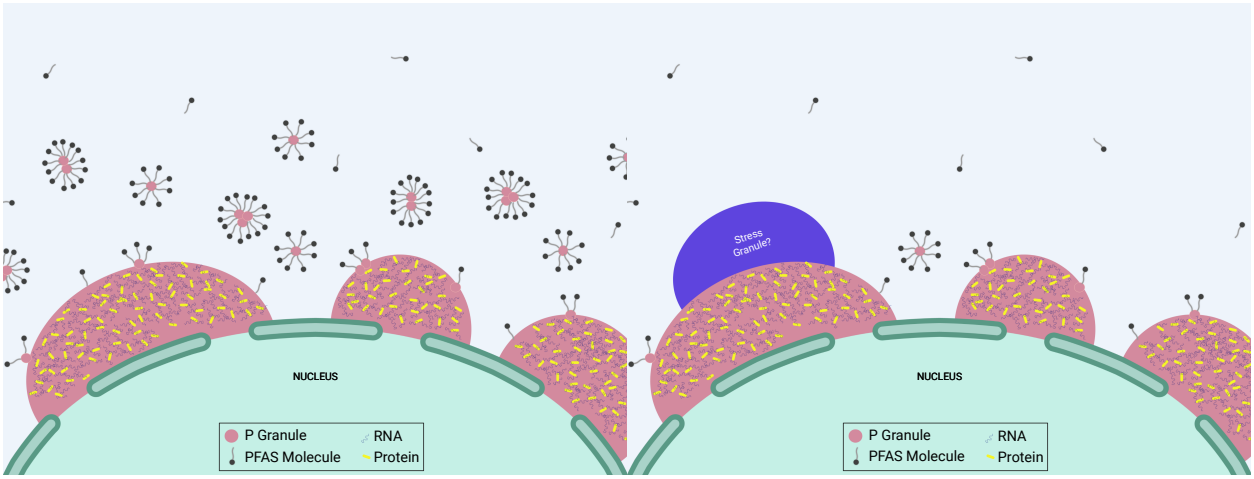
FIGURE 12

12A



12B

12C



12D

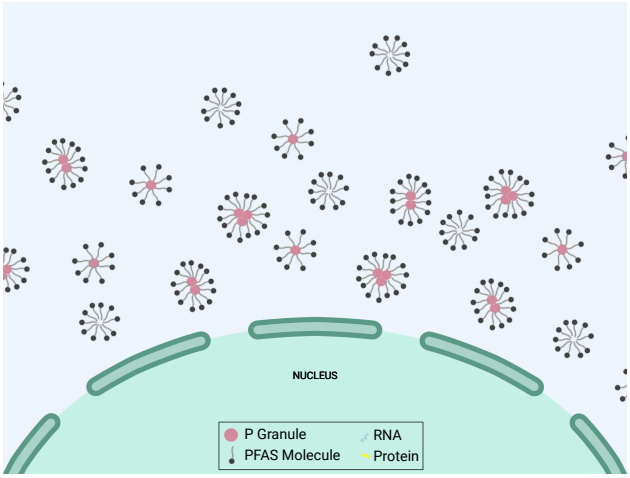


Figure 12. Proposed model for concentration-dependent effects of PFAS exposure on P granule structure. *(A)* In the absence of PFAS, P granules are regularly sized and associate with nuclear pores in the pachytene region of the adult gonad. *(B)* At relatively low concentrations of PFAS resulting from *in vivo* exposure, PFAS may stabilize P granule proteins near the interface and/or alter P granule protein folding and function, limiting the ability of mRNA to be transported out of P granules and leading to an increase in mRNA accumulated within the granules, thus increasing their size. *(C)* Alternatively, relatively low PFAS concentrations resulting from *in vivo* exposure could induce the formation of stress granules, which accumulate RNA from the cytoplasm. Stress granules could merge with P granules to increase their size. *(D)* In the *ex vivo* scenario, PFAS exposure is acute and at a relatively high concentration. As a result, PFAS molecules occupy all binding sites of the P granule constituents, completely disrupting P granule structure and distributing P granule constituents into the bulk phase. The dispersal of P granules would occur too quickly for accumulation of RNA or other constituents to increase granule size. Figures prepared using BioRender.

CHAPTER 5:

Summary and Conclusions

PFAS are among the most prevalent and persistent environmental toxicants globally. Despite the lack of substantial toxicological data for the majority of PFAS, they continue to be manufactured, used in consumer products, and released to the environment with minimal regulatory restrictions. The scale of human exposure to PFAS is vast and available data indicate that they can target most organ systems in the body and produce a wide range of health effects. However, the lack of existing data, the occurrence of PFAS human exposures in mixtures, and the sheer number of PFAS present extreme challenges to deciphering how PFAS elicit their biological effects.

This dissertation presented biomolecular condensates as novel toxicological endpoints to examine with PFAS exposure. BCs exist in all organisms and cell types and are a common means for organizing and regulating dynamic cellular processes. BCs assemble via relatively weak intermolecular forces and their behavior is governed by conditions that modulate phase separation, including interfacial tension. As surfactants, many PFAS have the potential to alter the phase behavior of BCs and change their biological function. Since BCs are involved in an array of important cellular functions, including regulation of transcription, translation, and the cell cycle, effects of PFAS on BCs could be amplified into larger cellular and organismal effects.

In this work, the PFAS surfactants PFOS and F-53B were shown to cause reproductive toxicity in *C. elegans* with particular effects on meiotic progression of germ cells. Based upon examination of P granules and the synaptonemal complex as model BCs, it appears that PFOS and F-53B are capable of altering BC structure. Since reproductive defects in P granule and SC sensitized mutants were exacerbated with PFOS and F-53B exposures, it appears that the structural changes that these PFAS elicit on BCs may have functional consequences. Since P granules play a substantial role in sRNA-mediated post-transcriptional gene regulation, future

research will focus on examining the mRNA and sRNA transcriptomes of *C. elegans* gonads exposed to PFAS. Additionally, since SC defects can impact crossover interference, the ability of PFAS to increase the number of crossover sites in *C. elegans* oocytes will also be examined.

This work also suggests the possibility that BCs in other organisms and tissue types may be susceptible to structural and functional changes in response to PFAS exposure. Therefore, BC structural modulation may be a useful endpoint for examining in other toxicological contexts.

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