TOXICITIES OF LEGACY AND CURRENT USE PFAS IN AN ANURAN: DO LARVAL EXPOSURES INFLUENCE RESPONSES TO A TERRESTRIAL PATHOGEN CHALLENGE?

by

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Dedicated to my familia

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GLOSSARY AND LIST OF ABBREVIATIONS

AFFF-Aqueous Firefighting foams

BD- *Batrachochytrium dendrobatidis* is an amphibian chytrid fungus

Compound - Any of the chemicals utilized in this study: perfluorooctane sulfonate (PFOS), perfluorooctanoic acid (PFOA), and hexafluoropropylene oxide (HFPO) dimer acid ammonium salt (GEN-X NH_4^+). These compounds will be referred to as poly- and perfluoroalkyl substances (PFAS)

EEL- Entomology Environmental Lab

Gosner Stage (GS) - A defined developmental stage for frogs based on morphological external changes

HFPO-DA/GenX- ammonium salt of hexafluoropropylene oxide dimer acid

OECD - Organization for Economic Co-operation and Development

PFOA- perfluorooctanoic acid

PFOS- perfluorooctane sulfonate

PWA - Purdue Wildlife Area

SVL - Snout-to-vent length

ABSTRACT

Per- and polyfluoroalkyl substances (PFAS) are a large group of emerging contaminants that include a strong carbon-flourine bond that makes the compounds resistant to physical, chemical and biological degradation. They are found in drinking water supplies, daily human products, manufacturing facilities, and in areas where aqueous film-forming foam (AFFF) was used to extinguish fires. Toxicity levels of these chemicals can vary depending on the characteristics of the specific chemical; longer carbon chain has shown to be more bioaccumulative and toxic than shorter chain length PFAS. Many studies have recognized perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) to be a substantial concern due to their known toxicity to wildlife. For example, studies show strong evidence that PFOA and PFOS suppress the antibody response from animals. Due to adverse health effects and public concern, the U.S stopped perfluorooctanoic acid (PFOA) manufacturing and switched to the production of an alternative fluorinated compound known as hexafluoropropylene oxide (HFPO) dimer acid or GenX, which is thought to be less bioaccumulative and therefore, potentially less toxic. These anthropogenic pollutants are one of many stressors acting on aquatic organisms like anurans. Natural stressors such as the devastating fungal pathogen *Batrachocytrium dendrobatidis* (*Bd*) is another stressor impacting amphibian populations. Despite the co-occurrence of these stressors, no studies have examined interactive effects of the fungal pathogen *Bd* and PFAS, or whether PFAS effects carry over into the terrestrial environment after a larvae exposure. This study tested the growth and developmental effects of PFOS, PFOA, and GenX, on gray treefrog *(Hyla versicolor*) tadpoles, followed by a *Bd* challenge in metamorphs. Our results demonstrate that a PFAS larval exposure interacted with a terrestrial *Bd* challenge to influence growth and development. *Bd* exposed animals were significantly shorter (smaller snout vent length) and had a significant increase in body condition and mass. This is the first study to report effects on amphibian terrestrial life stages after larval exposure to PFAS and to report an increased sensitivity to *Bd*. The environmentally relevant concentrations tested in this study (<10 parts per billion) lend ecological significance to these results however, additional studies are needed to understand the mechanisms behind these effects.

CHAPTER 1. INTRODUCTION

1.1 Multiple Environmental Stressors

Wildlife must cope with a variety of natural stressors including predation, temperature, and disease concurrently with anthropogenic stressors like habitat destruction and contaminant exposures. It is likely that these stressors are not acting alone but in combination, which can negatively influence individuals, populations, and communities. Moreover, the interaction of multiple stressors is complex. Amphibian populations are drastically declining and human activities continue, so there is a need to better understand how organisms respond to exposures of natural and anthropogenic stressors as well as their interactions.

1.2 General Overview of PFAS and its Fluorinated Alternatives

Per- and polyfluoroalkyl substances (PFAS) are a large group of man–made chemicals that were created in the 1930s, but were not environmentally documented until the 1980s. PFAS are pervasive pollutants affecting aquatic ecosystems worldwide and their occurrence has been extensively documented (Jarvis et al., 2021; Sun et al., 2016). Public awareness and attention to their adverse health effects triggered a voluntary phase-out plan in 2009 of the two most common PFAS: perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) (US EPA, 2017). This has led to the development of shorter chain PFAS, which do not bioaccumulate and therefore, are potentially less toxic. A good example of this is hexafluoropropylene oxide dimer (HFPO-DA), also known as GenX, which has been used as a short-chain replacement for PFOA since 2010 by Dupont for the production of Teflon (Sun et al., 2016; US EPA 2021b). In the U.S., detection of GenX was first reported in the Cape Fear River, North Carolina, in association to discharges from Chemours Company (Hopkins et al., 2018). Studies have quantified concentrations of GenX in drinking water downstream from this manufacturing facility and reported average concentrations of 631 ng/L for GenX, compared to 44 ng/L and 46 ng/L for PFOS and PFOA, respectively (Nakayama et al., 2007; Sun et al., 2016). Furthermore, GenX and other alternative short-chain replacements have become dominant PFAS globally, found in air, water, soil, vegetation, and biota across the planet (Heydebreck et al., 2015; Wang et al., 2019). Although GenX was proposed to be a safer alternative due to its low bioaccumulation potential, in 2019, it was added to the list of

"Substances of Very High Concern" by the European Chemicals Agency. Despite this emerging concern, Michigan, Ohio and North Carolina are the only states that have implemented GenX regulations in their drinking water in the U.S. (Kindschuh et al., 2022).

1.3 Summary of PFOS, PFOA and GenX Immune Toxicity

There are few toxicity assessments of these novel fluorinated alternatives. Studies testing the overall sublethal effects of GenX show similar adverse effects on organisms as its predecessor PFOA, which raises concern for the health and safety of wildlife and humans. However, the extent of toxicity and underlying mechanisms are still primarily unknown for GenX (Gebbink et al., 2020; Munoz et al., 2019; Wang et al., 2019; Xie et al., 2021).

Review studies on recent PFAS alternatives have focused on their emission sources and detection in water systems while few have studied toxicity to humans and wildlife (Cui et al., 2018; Heydebreck et al., 2015; Liu et al., 2018; Wang et al., 2019). Wang et al. (2019) provides a review of PFAS alternatives toxicity potential in relation to PFOA and PFOS. The studies described show that bioaccumulation factors (BCF) for chlorinated polyfluoroalkyl ether sulfonic acid (Cl-PFESA, trade name: F-53B) and HFPO-TA in black-spotted frogs (*Pelophylax nigromaculatus*) were comparable with PFOS. The mean BAF for 6:2 Cl-PFESA (1304 L/kg) was higher than that for PFOS (1050 L/kg), and BAF for HFPO-TA (0.76 L/kg) was higher than BAF for PFOA (0.37 L/kg) (Cui et al., 2018). Studies have also shown PFAS alternatives can have slower elimination kinetics, increasing toxicity potential (Shi et al., 2016).

There is evidence that PFOS, PFOA, and GenX can be immunotoxic to humans and laboratory animals. For example, PFOA and PFOS can suppress adaptive immune function in mice. In a mice study, exposure to PFOA and PFOS through diet at concentrations of 0.02% of their body weight (22-28 g) for up to 10 days and fed 3.5 g of food daily resulted in a decrease in body weight and atrophy of the spleen and thymus, two major immune organs (Shane et al., 2020; Qazi et al., 2009; Yang et al., 2000, 2001, 2002). The handful of studies evaluating the immunotoxicity of GenX in mice have reported a suppression of T-cell-dependent antibody response (TDAR), an increase in liver weight, a decrease in spleen weight, increased number of T lymphocytes, and an increase in inflammation with an imbalance of gut microbiota (Rushing et al., 2017; Xie et al., 2021). Thus, GenX at sub-chronic exposures suppresses the ability to generate antibodies. Overall, the limited studies available support the notion that GenX can affect the immune system.

1.4 *Batrachochytrium dendrobatidis:* A Major Amphibian Pathogen

Chytridiomycosis is a fungal disease caused by the chytrid *Batrachochytrium dendrobatidis* (*Bd*). *Bd* is a zoophoric fungus abundant in aquatic habitats and soils and is responsible for ongoing global amphibian declines and extinctions (Lips, 2016; Longcore et al., 1999; Scheele et al., 2019). Its life cycle consists of two stages: 1) a free-swimming aquatic flagellated zoospore stage that infects tadpoles by colonizing the stratum corneum; and 2) a thallus stage which produces zoospores that infect host cells, mature and develop into zoosporangia; the zoospores are then released back into the environment ready to reinfect other hosts (Berger et al., 2005).

Chytridiomycosis occurs only in keratinized tissues, which are restricted to the oral region (jaw sheaths and teeth) of tadpoles, and the skin (epidermis) of metamorphs and adults (Drake et al., 2007; Piotrowski et al., 2004). The disease causes oral deformities in tadpoles, resulting in difficulty feeding, which can lead to death. Infected adults can exhibit hyperkeratosis, ulceration and sloughing of the skin, abnormal posture (extended hind limbs and lack of righting reflex), anorexia and lethargy.

The zoology of *Bd* has been well studied. Some frog species that lack the aquatic reproductive stage have still been found infected with *Bd*, which suggests metamorph-metamorph transmission (Walker et al., 2007). In some cases, the host becomes reinfected in the terrestrial stages after infection during its larvae stage. There is a large range of sensitivities to this pathogen across amphibian species. For example, the African clawed frog, *Xenopus laevis*, can clear an infection quickly but can become a vector for other more sensitive species (Ohmer et al., 2017). *Bd* can also have varying effects such that some species are highly susceptible during larval stages, while others are more susceptible in the metamorphic stage (Fernández-Beaskoetxea et al., 2016).

1.5 Co-exposure of Amphibians to Contaminants and *Batrachochytrium dendrobatidis*

Amphibians are thought to be a highly sensitive taxa to xenobiotics due to their permeable skin, utilization of both aquatic and terrestrial habitats and reconstruction of immune system during metamorphosis (Quaranta et al., 2009; Rowe et al., 2003). During metamorphosis, amphibians undergo loss and reconstruction of many tissues and organs, including the immune system, so it is hypothesized that environmental stressors affect metamorphosis. This process could be highly

impacted by pathogens and contaminant stressors due to their lack of immunocompetence during or right after metamorphosis.

In 2011, a study looked at the combined effects of the pesticide carbaryl and *Bd* on larval Pacific treefrogs (*Pseudacris regilla*) and Cascades frogs (*Rana cascadae*) (Buck et al., 2012). Co-exposures began at Gosner Stage (GS) 35-37 and continued for 7 weeks. This study concluded that carbaryl did not affect *Bd* infection levels or cause any other interactions, except carbaryl increased the growth rate in *P. regilla* larvae. Another study examined the interactive effects of carbaryl and the fungicide copper sulfate and *Bd* on Cope's gray treefrog (*Hyla chrysoscelis*) tadpoles (Gaietto et al., 2014). This study concluded that there was no significant interaction between carbaryl and *Bd*. The researchers acknowledged that the lack of significant effects from *Bd* could be due to a lack of infection in the tadpoles. However, this conclusion is unknown due to no verification of infection with qPCR. Another study found that larval atrazine exposures increased mortality in Cuban tree frogs (*Osteopilus septentrionalis*) after post-metamorphic exposure to *Bd* (Rohr et al., 2013) Overall, studies suggest contaminants affect virulence and susceptibility to *Bd*, but the underlying mechanisms are still largely unknown and vary depending on species and stage of exposure.

1.6 Thesis Objectives, Hypotheses and Predictions

The central objective of this thesis was to investigate the effects of co-exposure to a pathogen (*Bd*) and a contaminant (PFAS) on amphibian larvae and juvenile survival, growth, and development. I hypothesized that a larval exposure to PFAS would (1) negatively affect larvae growth and development in a dose dependent manner for GenX, with PFOA and PFOS having stronger effects than GenX; (2) have carry over effects into metamorph (i.e., juvenile) stages reducing growth and survival in the terrestrial environment and with PFOS and PFOA having a stronger effect than GenX, and (3) influence *Bd* infections as well as growth and survival relative to non-infected animals. To achieve this goal, I first exposed grey tree frogs (*Hyla versicolor*) larvae to different PFAS and quantified effects on growth and development. After metamorphosis, a subset of these animals was also infected with *Bd* zoospores, and their survival and growth monitored for 50 days post- metamorphosis.

CHAPTER 2. METHODS AND MATERIALS

2.1 Animal Collection and Larval Rearing

All methods involving animal use were conducted in accordance with relevant guidelines and regulations; methodological protocols have been approved by the Purdue Institutional Animal Care and Use Committee (IACUC Protocol # 1601001355A012).

A total of 16 amplexed pairs of gray treefrogs were collected from ponds located at the Purdue Wildlife Area (PWA), West Lafayette, Indiana, USA in June 2020. Each pair was placed in individual 7.5 L plastic containers with ~ 5 cm pond water overnight to allow oviposition. Pairs were then returned to ponds from which they were collected the next day. Egg masses and tadpole rearing prior to exposure initiation occurred indoors at the PWA laboratory at 22℃. Egg masses were placed in individual 15-L sterilite plastic containers filled with approximately 12 L aged well water with daily water changes until hatching. Upon hatching, tadpoles were fed TetraMin® Tropical Flakes *ad libitum* and daily water changes were done until reaching the free-swimming stage Gosner 25 (Gosner 1960). Tadpoles from different clutches were combined to increase genetic variation and were then randomly transferred to 36 outdoor cattle tanks (6 treatments replicated 6 times each) filled with ~50 L of well water, n = 50/ tank (**Figure 1**).

2.2 Experimental Design and Larval Exposures to PFAS

Exposures consisted of six PFAS treatments: control (0 ppb PFAS), PFOS 10 ppb, PFOA 10 ppb, and GenX (HFPO-DA) at 10 ppb, 1 ppb, or 0.1 ppb. PFOS and PFOA concentrations are reflective of concentrations found in surface waters at AFFF sites (Anderson et al., 2016; East et al., 2021). For GenX, we selected concentrations encompassing ranges currently being reported from surface waters in the U.S. (Cahoon, 2020; Sun et al., 2016). Experimental units consisted of 150-L cattle tanks with weighted PVC/mesh lids that were prepared with 100 L of well water, 100 g of leaf litter (collected from the PWA), and 5 g of rabbit chow (Country Road® Rabbit Pellets) to provide a nutrient base. In addition, to mimic a natural system, phytoplankton, periphyton, and zooplankton were collected from a PWA pond to establish a self-sustaining aquatic community (Foguth et al., 2020). A single spike of PFAS was added to each of the mesocosms and left for at

least 5 days prior to addition of larvae to ensure time for chemical compounds to partition throughout the components of the tank. All tadpoles at GS 25 were combined into a single container then counted out into sets of 5 and placed in small cups; 10 cups (n= 50) were haphazardly selected and added to each mesocosm. Initial sampling (n=6/tank) on day 14 was done to assess short term, chronic effects of PFAS exposure at which time individuals were measured for mass (to the nearest 0.001 g using a digital scale) and snout-vent-length (SVL, to the nearest 0.001 mm using digital calipers) and assessed for GS. Tanks were checked daily for mortalities and once the first animal reached GS 42 (emergence of forelimbs), daily searches were performed to capture all metamorphs. Each day, metamorphs from every mesocosm were placed in individual ~350 mL plastic deli containers with ventilated lids filled with water from their respective tanks and brought back to the PWA laboratory and the same organismal responses quantified. Metamorphs were subsequently housed in individual deli containers and were raised indoors at the PWA laboratory until reaching GS 46 (tail resorption) at which point they were once again measured for mass, SVL, and staged.

2.3 Terrestrial Exposures to Bd

Post-metamorphosis, a random block design was employed to assign PFAS exposed animals from each of the 6 treatments to one of two pathogen treatments (*Bd* - exposed, NBd - nonexposed), for a total of 12 treatments. One mesocosm from the GenX 10 ppb treatment was excluded from the study due to a notable growth delay in the animals compared to other replicates. At GS 46, 6 individuals from each mesocosm were transferred into individual terraria and reared in the Entomology Environmental Laboratory (EEL) at Purdue University. Terrariums consisted of ~2 cm of pea gravel, ~5 cm of perlite-free topsoil mixed with sphagnum moss, a 60 mL polypropylene cup filled with dechlorinated tap water, and a mesh lid. Terrariums were held at a 12 hr light/dark cycle at a temperature of 22°C ± 2. Animals were checked daily for survival and fed live 1/16'' crickets dusted with calcium and vitamin D_3 powder (Rep-Cal Ultrafine) (Ghann's Cricket Farm, Augusta, GA) equivalent to ~15% of the average mass of the animals three times weekly throughout the experimental period.

The *Bd* strain isolated from an infected *Lithobates* sp. from Ohio (JSOH-1), was obtained from Dr. Searle's aquatic disease ecology laboratory at Purdue University. Zoospores were

incubated in two 1000 mL tryptone broth culture screw capped flasks and were harvested by aliquoting *Bd* inoculates from tryptone broth to 1% tryptone agar plates for 10 days. The plates were then flooded with 3 mL of sterile water and collected zoospores were quantified using a hemocytometer (Hausser Scientific, Horsham, PA). Prior to *Bd* exposure, individuals were measured for mass and SVL to collect experimental day 0 measurements prior to exposure. Animals were held in individual terrariums between 24 and 59 days before *Bd* exposure. To initiate exposure, individuals were placed in ventilated 10 cm plastic petri dishes with 7 mL of dechlorinated tap water. We exposed 103 individuals by adding *Bd* solution at 320,000 zoospores/mL. The control group was composed of 102 individuals (lost one animal during the process) designated as no *Bd* exposure and exposed with an equal volume of dechlorinated tap water (Searle et al., 2011). After a 24-hr exposure period, animals were returned to their individual terrarium. Individuals were monitored daily for survival and mass and SVL measured every 10 days until termination on experimental day 50. Individuals were swabbed at experimental day 20 to measure *Bd* infection prevalence and to make sure no infected animals were found in the control group. We used a standardized swabbing technique that involves swabbing along the frog's ventral area 10x, inner thighs 5x, and under hind limbs toes 5x (Hyatt et al. 2007). Swabs were stored at -20℃ until DNA extraction.

2.3.1 DNA extractions and qPCR

Bd DNA was extracted from swabs using 250 µL of ThermoFisher Scientific PrepMan Ultra and ~100 mg of 0.5 mm zirconium silica beads (Fisher Scientific, Waltham, MA). The swabs were homogenized for 1 min and 30 sec then centrifuged for 30 sec at 13000 x g. The homogenization and centrifugation processes were repeated, and the second time samples were homogenized for 1 min then sample tubes placed in a heat block set to 100℃ for 10 min, cooled for 2 min, and centrifuged at 13000 x g for 3 min (Boyle et al., 2004). A total of 60 µL of the resulting supernatant was divided into two separate tubes to avoid freeze-thaw cycles of the extracted DNA and stored at -–80°C. The extracted DNA was quantified using quantitative polymerase chain reaction (qPCR) following the United States Geological Survey National Wildlife Health Center protocol (NWHC, 2017). Infection load was quantified using manufactured gene fragments (gBlocks) (Integrated Technologies, Coraville, IA, USA) of the targeted *Bd* DNA sequence. The gBlocks were used to create a standard curve that serves as a reference for *Bd* load

quantification, which included five serial dilutions (10^3, 10^2, 10^1, 10^0, 10^{-1}) also run in triplicate along with samples.

2.4 Statistical Analysis

All analyses were carried out using either SigmaPlot13 (Systat Software, Palo Alto, CA, USA, 2014) or R version 4.1.2 (R Core Development Team 2021).

2.4.1 Larval Stage

All analyses were run for a comparative toxicity assessment at 10 ppb (control, PFOS, PFOA, and GenX) and across a GenX dose-response (0, 0.1, 1, 10 ppb). Effects of PFAS treatments on life-history traits (SVL, mass, and SMI) at start of and during metamorphosis; time to metamorphosis, and length of metamorphosis were analyzed using ANCOVA. We assessed body condition using mass, SVL and the mass (M) and length (L) was taken to compute scaled mass index, SMI $= M_i \left[\frac{L_o}{L_i}\right]^{bSMA}$ where M*i* and L*i* are the mass and length measurement of individual *i* respectively; Lo is the mean of the entire study population and bSMA is the scaling exponent estimated by the SMA regression calculated as a slope of reduced major axis regression of log-transformed body length on log-transformed body mass (Peig and Green, 2009). This endpoint takes into account mass and SVL variability and accounts for the interdependence between the two variables and confounding effects of growth, making this index useful for a better understanding of overall species fitness. ANOVA was used to test for effects of the chemical treatment on survival to metamorphosis, survival through metamorphosis, and GS at exposure day 14. All analyses were conducted with a fiducial level of significance of $p \leq 0.05$.

2.4.2 Terrestrial Stage

Effects of PFAS treatments and *Bd* infection on mass, SVL, and SMI over time were analyzed using a linear mixed-effects model (i.e., lmer in lme4 package) (Bates et al., 2015). Analyses were split into two, as described previously for larval analysis, to synthesize GenX dose-response effects on development and growth in comparison with its predecessors PFOA and PFOS. All models included one random effect (sampleid) and five fixed effect variables (chemical

treatment, *Bd*, days, block, and time in captivity). The days refers to the length of the experiment (total of 50 days). Block accounted for shelf level on racks where a random block design was used to organize treatments and time in captivity was the total amount of time an individual spent before experimental day 1 or the initiation of the *Bd* exposure. Animals spent between 24 and 59 days in captivity. Block and time in captivity were treated as fixed effects with time in captivity treated as a continuous variable and Block as a categorical variable to account for any confounding effects. We had a total of 6 animals showing signs of edema, swelling due to excess fluid accumulation in the body, so, all models excluded edemic animals to not influence SMI values. The function "plot _model" was used to check for normality and homoscedasticity. The data was cleaned using the function "outlierTest" to remove outliers and refit the data. All final models included log-transformed life history traits (SVL, mass and SMI). Model means and 95% confidence intervals were extracted directly from models for plotting using lsmeansLT package. When significant main effects were found, post-hoc tests were performed to determine between-treatment differences on dependent variables (SVL, mass, SMI). All multiple comparisons tests were conducted using the emmeans package in RStudio.

A generalized linear model (ie glmer in lme4 package) with a logit link function and a binomial error distribution was used to determine whether treatments influenced survival, limp legs and edema. The model included fixed effects of Bd, Chemical, and their interaction and included block and time in captivity as covariates.

CHAPTER 3. RESULTS

3.1 Water Quality Parameters and PFAS Concentrations

Water quality parameters were taken from control mesocosms (N = 6) and averaged (± SEM) 26.8 ± 0.12 °C for temperature; 8.47 ± 0.55 mg/L for DO; and 8.19 ± 0.08 for pH. (**Supplemental Table 1B**). Average daily temperatures across the larval and terrestrial stage exposures can be found in **Supplemental Figure 1 A & B**, respectively.

Measured water concentrations were close to nominal for PFOA and PFOS (**Table 1**). However, GenX concentrations were overall about half of the target. All measured water PFAS concentrations are listed in **Supplemental Table 1**.

3.2 Effects of PFAS on Survival, Growth and Development

3.2.1 Larvae Stage

PFAS exposure during the larval stage did not alter gray treefrog survival to GS 42 or GS 46 and the average survival rate across all treatments at initial capture and GS 46 were 93% and 87%, respectively. Similarly, there were no significant effects for any of the treatments on GS, SVL, mass, and SMI relative to controls at day 14. However, SVL was significantly decreased at metamorphic climax in the GenX 10 ppb treatment compared to PFOA and controls ($p < 0.05$ and $p = 0.026$, respectively) (**Figure 2A**). Moreover, GenX 1 ppb and 10 ppb significantly decreased SVL in treefrog metamorphs compared to controls ($p = 0.015$ and $p = 0.016$, respectively) (**Figure 2B**). We found no evidence for chemical effects on mass or change in SVL and mass during metamorphosis. Time to metamorphosis with GS as a covariate as well as length of metamorphosis and the interaction between treatments and GS did not vary significantly by treatment. Means ± SEM, minimum, and maximum values for all endpoints measured across the larval stage experimental period can be found in **Supplemental Table 2.** Statistical summaries of all tests run on larval stage data can be found in **Supplemental Table 3**.

3.2.2 Terrestrial Stage

Survival of controls and PFAS exposed animals during the larval stage and raised into post metamorphosis averaged 80 ± 0.04 % at the end of the 50-day terrestrial study. However, the longer an individual was held in captivity, the less likely it survived and the more likely it was to develop signs of limp legs and edema regardless of chemical.

A subset of swabs (n=3 per treatment) were randomly selected from the first time point (Day 20) to confirm the presence or absence of infection in exposed and nonexposed animals. No control animals tested positive for *Bd* infection (**Table 2**). The standard curve regression was y= -3.488x + 23.996 with a total efficiency of 93%. We did omit a single standard replicate due to its skewed amplification. Although there was no effect of chemical treatment on SVL of frogs exposed to 10 ppb PFOS, PFOA, or GenX (**Figure 3A**), all frogs exposed to *Bd*, irrespective of treatment, had lower SVL compared to non-exposed animals ($p < 0.001$) (**Figures 3B & 3C**). SVL of frogs exposed to both 10 ppb GenX and *Bd* was significantly lower than that of control frogs exposed to *Bd* ($p = 0.005$) and lower than that of frogs exposed to both 10 ppb PFOA and *Bd* ($p = 0.042$) (**Figure 3C)**. At day 20 and regardless of chemical, SVL was shorter in exposed frogs than controls (**Figure 3D)**. This significant reduction in SVL in *Bd* exposed frogs was consistent across time (**Figure 3E)**. PFOS, PFOA, and GenX at 10 ppb had an interactive effect with *Bd* over the 50-day period such that PFAS exposed animals that were exposed to *Bd* grew slower in SVL than animals from the same treatments that were not exposed to the pathogen (**Figure 3F**).

Although there was no effect of PFAS treatment on body mass of frogs (**Figure 4A**), exposure to *Bd* resulted in a significant increase in body mass ($p = 0.02$) (**Figure 4B**). Body mass was increased in frogs exposed to *Bd* and 10 ppb PFOS resulted in significant increases in mass of control frogs and those exposed to 10 ppb PFOS (**Figure 4C**). However, exposure to *Bd* and 10 ppb PFOA or 10 ppb GenX did not result in significant changes in mass relative to frogs exposed to the same chemical but not exposed to *Bd.* Additionally, exposure to both *Bd* and either 10 ppb PFOA or 10 ppb GenX resulted in smaller masses relative to control *Bd* exposed frogs and those exposed to both *Bd* and 10 ppb PFOS (**Figure 4C**). Mass was transiently decreased at day 20 for all chemical exposures irrespective of *Bd* exposure (**Figure 4D**). At day 50, frogs exposed to 10 ppb GenX had significantly reduced masses compared to controls and PFOA exposed frogs (**Figure 4D)**. Mass of frogs exposed to *Bd* was significantly greater than those not exposed on days 10, 20, and 30 of the experiment (**Figure 4E**). Overall trends show a divergence in mass over

time with *Bd* exposed frogs gaining more mass than their non-*Bd* exposed counterparts of the same chemical treatment (**Figure 4F).**

There was no effect of chemical treatment on SMI of frogs exposed to 10 ppb PFOS, PFOA, or GenX (**Figure 5A**). SMI was significantly greater in individuals exposed to *Bd* than those not exposed regardless of chemical treatment ($p < 0.001$) (**Figures 5B & 5C)**. At day 0, SMI of PFOA and GenX individuals were significantly larger than controls, however this difference disappeared by day 10 **(Figure 4D).** SMI was greater in animals exposed to *Bd* on days 10, 20 and 30 ($p = 0.015$, $p = 0.06$, $p < 0.001$) of the experiment (**Figure 5E**). Overall trends show a divergence in SMI driven by *Bd* exposure that occurs by day 10 and remained throughout the experiment (**Figure 5F).**

There was no effect of GenX dose on SVL of frogs exposed to 0.1 ppb, 1 ppb, or 10 ppb GenX (**Figure 6A**). All frogs exposed to *Bd*, irrespective of treatment, had lower SVL compared to non-exposed animals ($p <0.001$) (**Figures 6B & 6C**). Co-exposure to 10 ppb GenX and *Bd* resulted in a significant decrease in SVL compared to *Bd* exposed control animals ($p = 0.010$) (**Figure 6C**). By chance, all GenX exposed individuals had significantly smaller SVL than control individuals at day 0, however by day 10 all differences in SVL had disappeared and SVL remained the same across treatments for the remainder of the experiment (**Figure 6D)**. There was an effect of *Bd* over time, such that animals exposed to the pathogen were statistically shorter compared to non-exposed during all timepoints except for experimental Day 0 (measured immediately prior to infection) (**Figure 6E**). This trend is also apparent when assessing GenX and *Bd* over time – where exposure to *Bd* generally reduces SVL regardless of GenX treatment (**Figure 6F**).

There was no effect of GenX dose on mass of frogs exposed to 0.1 ppb, 1 ppb, or 10 ppb GenX (**Figure 7A**). *Bd* infection had no effect on body mass (**Figure 7B)**. A difference in mass between *Bd* exposed and non-*Bd* exposed individuals of the same chemical treatment was only observed in controls ($p = 0.003$) (**Figure 7C)**. However, a chemical by *Bd* interaction was observed with a decrease in mass corresponding with any GenX exposure in *Bd* exposed individuals (**Figure 7C**) A significant, transient decrease in mass was observed at day 20 for all GenX treatments that disappeared by day 30 but reappeared again at day 50 for 1 ppb and 10 ppb GenX treatments **(Figure 7D)**. Exposure to *Bd* resulted in a transient increase in mass on days 10 and 20 of exposure regardless of chemical treatment. This difference was abated by day 30 and on days 40 and 50, the mass of *Bd* exposed individuals were significantly lower than non-*Bd*

exposed individuals **(Figure 7E**). No clear interactions between GenX dose and *Bd* exposure appeared over time (**Figure 7F**).

There was no effect of GenX dose on SMI of frogs exposed to 0.1 ppb, 1 ppb, or 10 ppb GenX (**Figure 8A**). All frogs exposed to *Bd*, irrespective of treatment, had higher SMI compared to non-exposed animals ($p < 0.001$) (**Figures 8B & 8C**). Non-*Bd* exposed animals exposed to PFOS exhibited significantly higher SMI than those of non-*Bd* controls (**Figure 8C**). By chance, the SMI of all GenX treatments were significantly higher than controls on day 0; this difference disappears by day 10 and SMI remains the same across treatments until day 40 at which 0.1 ppb and 1 ppb GenX treatments again exhibit significantly higher SMI than controls and day 50 where 0.1 ppb and 1 ppb GenX treatments exhibit significantly lower SMI relative to controls (**Figure 8D)**. Across all timepoints, *Bd* exposed individuals exhibited significantly higher SMI than non-*Bd* exposed individuals (**Figure 8E)**. Generally, trends in SMI over time appear to be driven by *Bd* exposure and not GenX treatment with *Bd* exposed individuals exhibiting higher SMI than non-*Bd* exposed individuals (**Figure 8F**).

Statistical summaries of all tests run on terrestrial stage data can be found in **Supplemental Table 4.**

CHAPTER 4. DISCUSSION

Amphibians are exposed to anthropogenic and natural stressors either alone or in combination; however, little is known about their consequences on survival and growth and underlying mechanisms that drive these effects. Studies have shown contaminants like pesticides can impact *Bd* exposure effects that results in greater reduced fitness from interactive effects than individual pressures alone (Boone et al. 2007; Brown et al. 2021; Gaietto et al. 2014; Hanlon et al, 2013; Parris and Beaudoin, 2004; Parris and Cornelius, 2004). Exposure to the pathogen, *Bd* has been well documented as a devastating pathogen bringing some amphibian species to extinction. Studies looking at development found that *Bd* exposure has negative impacts on mass and the few that measured SVL have also seen negative effects on amphibians (Davidson et al., 2007; Relyea and Mill, 2001). In contrast to our hypothesis and predictions, we saw PFOS and PFOA acting in a similar manner to control animals, even though these PFAS are more bioaccumulative than GenX. Researchers have suggested bioaccumulation is positively correlated with negative health effects (Ankley et al., 2004), but our results show that may no longer stand true. It is possible that GenX is affecting health of amphibians through other underlying mechanisms.

Interestingly, many studies looking at combined effects of pesticides and *Bd* on grey tree frogs have found positive effects and mitigating effects, resulting in increased survival rate and increases in mass. Researchers believe this is due to the chemical exposure mitigating *Bd* effects by affecting virulence of the *Bd* fungus (Gaietto et al., 2014; Hanlon et al., 2013). However, contaminant effects on grey tree frogs have been shown to be highly variable (Relyea and Mill, 2001) depending on the timing of exposure, chemical type, feeding rate, and other abiotic and biotic factors. This highlights the complexity that exists in nature and the need to investigate the combined effects of multiple stressors and their underlying mechanisms. We found that *Bd* exposure reduces SVL in gray treefrogs over time regardless of PFAS treatment, which is consistent with other studies (Hanlon et al., 2013; Parris and Baud, 2004). However, mass and SMI significantly increased in almost all *Bd* treatments, regardless of chemical, indicating that *Bd* was the main driver of these effects. We believe there are underlying metabolic responses induced by the innate immune system that are inducing mass gain and that effects of early PFAS exposure carry over through metamorphosis and contribute to additive effects to the *Bd* exposure observed here, however this is unclear. Understanding the links between host-pathogen interactions with

other stressors can further help identify the extent of *Bd's* virulence, persistence, and heterogeneous distribution in freshwater ecosystems.

Studies examining amphibian responses to *Bd* infection describe the first stages involving chemotaxis and hyperplasia, which involve zoosporangia containing zoospores on the surface of the skin, associated with hyperkeratosis, a dermal condition that involves the increase in thickness of the stratum corneum (Grogan et al., 2018). Furthermore, the stratum corneum is where many defenses are found that act as immune surveillance. Researchers have found fungi secrete protein effectors that work as immunosuppressants and interact with the hosts metabolism (Brutyn et al., 2012; Grogan et al., 2018). We believe these mechanisms could explain the *Bd* exposure response we saw in grey treefrogs. While we postulate that these mechanisms could be driving the effects observed, additional studies are needed that confirm these findings.

We found that GenX at sublethal concentrations poses a greater effect on growth and development in grey tree frogs post metamorphosis than its predecessor PFOA and has a synergistic effect with a pathogen exposure. Overall, effects after GenX exposures resulted in dose-dependent responses with GenX 10 ppb having the greatest effect on SVL, mass, and SMI. However, in contrast to predictions, PFOA and PFOS had little to no effects throughout the duration of the study, with PFOS more closely reflecting control treatments followed by PFOA in the terrestrial phase. Because shorter chain PFAS such as GenX are supposedly safer alternatives to PFOA and PFOS due to their shorter half-life and less bioaccumulative potential, we expected PFOA and PFOS to have the strongest effects on survival, development, and susceptibility to *Bd*. Our results deviate from the assumption that greater bioaccumulation leads to greater effects, and are supported by other studies that have shown similar findings in fish. For instance, a study examining fish bioaccumulation and kinetics of short-chain PFAS found high levels of GenX in Striped Bass serum and liver indicating bioaccumulative properties in fish species and aquatic organisms (Guillette et al., 2020).

Although we found no strong evidence for carry-over effects, PFOS, PFOA, and GenX 10 ppb showed significant effects in SVL and mass against controls by day 20 post-exposure. These differences were gone by day 50, except for effects on mass in GenX treatments indicating possible compensatory growth in PFAS treatments over time after a *Bd* challenge, and GenX possibly posing greater long-term effects than PFOS and PFOA. To date, this is the first study to have examined interactive effects of *Bd* and PFAS and whether PFAS effects carry over into the

terrestrial environment after larval exposure. An interesting and paradoxical result from these studies was the increase in body weight in *Bd* infected animals. A review on the immune system at metamorphosis synthesized that amphibians lose most of their immune defense cells, such as lymphocytes after undergoing metamorphosis and they are replaced by a new set (Rollins-Smith, 1998). Because animals were in captivity for an extended period of time (up to 59 days) before the *Bd* exposure, we believe they may have been more immunocompetent and may have been capable of better clearing the infection, resulting in less chronic health effects. Previous studies have seen age-dependent sensitivities to infection where exposure to *Bd* immediately following tail absorption resulted in a lower survival rate than a later exposure time (Bancroft et al., 2011; Jones et al., 2016) supporting the idea that the animals from the study had more time to recuperate their immune system to combat a pathogen. Another consideration of our results is that grey treefrogs are known to be less susceptible to *Bd* (Searle et al., 2011), which brings up a need for a deeper understanding of the mechanisms to understand why *Bd* exposed animals were significantly affected during the terrestrial stage. Studies have demonstrated that more susceptible amphibian species have a greater immunological effect than least susceptible individuals (Ellison et al., 2014; Grogan et al., 2018). Furthermore, Grogan et al. (2018) reviews amphibian responses to *Bd* infection and suggests a disruption in homeostatic mechanisms involved in the stratum corneum that involve water and ion transport, epithelial stability, and musculoskeletal functions. For example, the innate immune response to *Bd* exposure involves the zoospores encyst upon the keratinized tissue. Germination tubes then encyst through one or more skin layers, including the stratum spinosum and stratum granulosum. This process allows *Bd* evasion of host immune surveillance. However, there is still a lot unknown about the detection response after a *Bd* exposure. It is important to note that these studies were conducted in susceptible anurans, emphasizing the importance of further research determining the mechanisms for these interactive effects in less susceptible species, as they may be experiencing additive effects from multiple stressors which could negatively impact immune function resulting in increased rates of infection, increased energy consumption, and decreased survival (Grogan et al., 2018). The immune responses described by Grogan et al. (2018) may explain the anurans response to *Bd* by disturbing epithelial stability from hyperplasia and inducing a disruption in metabolism. This could be an explanation for the increase in mass we saw in *Bd* exposed animals.

CHAPTER 5: CONCLUSIONS

Our results suggest that PFAS growth and developmental effects carryover into the terrestrial environment and that *Bd* exposure after metamorphosis greatly affects growth and development in grey treefrogs despite their relative tolerance to *Bd* (Searle et al., 2011). Additionally, because of later exposure, animals were able to recover from metamorphosis and combat the *Bd* challenge differently; rather than a typical immune response, animals go through clearing the infection through their first line of defense that then presumptively causes a metabolic response inducing animals to gain mass. Our data illustrates the importance of investigating the long-term growth and developmental effects of PFAS and its interactive effects of a pathogen on amphibians, specifically the effects of contaminants on host-pathogen interactions. Although we see PFAS and *Bd* acting in a 'neutral' manner on survival/growth of grey treefrogs, it is important to recognize they may serve as a vector for other species that are more susceptible and are at a higher risk of infection and have more trouble clearing the infection. Moreover, this paper recognizes that the immune system of amphibians is poorly understood and finding the key mechanisms for these great immune system differences is important to finding the best conservation plan. Furthermore, understanding how multiple stressors are acting on amphibians during aquatic and terrestrial phases of their life can bring light to species in need for better conservation plans. Lastly but most importantly, collecting data for SVL and mass to calculate SMI would be a good start to getting a better understanding of how amphibians' fitness is affected by abiotic and biotic stressors in the environment.

REFERENCES

Anderson, R. H., Long, G. C., Porter, R. C., & Anderson, J. K. (2016). Occurrence of select perfluoroalkyl substances at U.S. Air Force aqueous film-forming foam release sites other than fire-training areas: Field-validation of critical fate and transport properties. Chemosphere, 150, 678–685. https://doi.org/10.1016/j.chemosphere.2016.01.014

Ankley, G. T., Kuehl, D. W., Kahl, M. D., Jensen, K. M., Butterworth, B. C., & Nichols, J. W. (2004). Partial life-cycle toxicity and biocentration modeling of perfluorooctanesulfonate in the northern leopard frog (*Rana pipiens*). Environmental Toxicology and Chemistry, 23(11), 2745. https://doi.org/10.1897/03-667

Bancroft, B. A., Han, B. A., Searle, C. L., Biga, L. M., Olson, D. H., Kats, L. B., Lawler, J. J., & Blaustein, A. R. (2011). Species-level correlates of susceptibility to the pathogenic amphibian fungus *Batrachochytrium dendrobatidis* in the United States. Biodiversity and Conservation, 20(9), 1911–1920. https://doi.org/10.1007/s10531-011-0066-4

Bates, D., Maechler, M., Bolker, B., Walker, S., Christensen, R. H. B., Singmann, H., & Grothendieck, G. (2011). Package 'lme4'. Linear mixed-effects models using S4 classes. R package version, 1(6).

Berger, L., Hyatt, A., Speare, R., & Longcore, J. (2005). Life cycle stages of the amphibian chytrid *Batrachochytrium dendrobatidis*. Diseases of Aquatic Organisms, 68, 51–63. https://doi.org/10.3354/dao068051

Boone, M. D., Semlitsch, R. D., Little, E. E., & Doyle, M. C. (2007). Multiple stressors in amphibian communities: effects of chemical contamination, bullfrogs, and fish. Ecological Applications, 17(1), 291–301. https://doi.org/10.1890/1051

Boyle, D., Boyle, D., Olsen, V., Morgan, J., & Hyatt, A. (2004). Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. Diseases of Aquatic Organisms, 60, 141–148. https://doi.org/10.3354/dao060141

Brown, S. R., Flynn, R. W., & Hoverman, J. T. (2020). Perfluoroalkyl Substances Increase Susceptibility of Northern Leopard Frog Tadpoles to Trematode Infection. Environmental Toxicology and Chemistry, 40(3), 689–694. https://doi.org/10.1002/etc.4678

Brutyn, M., D'Herde, K., Dhaenens, M., Rooij, P. V., Verbrugghe, E., Hyatt, A. D., Croubels, S., Deforce, D., Ducatelle, R., Haesebrouck, F., Martel, A., & Pasmans, F. (2012). *Batrachochytrium dendrobatidis* zoospore secretions rapidly disturb intercellular junctions in frog skin. Fungal Genetics and Biology, 49(10), 830–837. https://doi.org/10.1016/j.fgb.2012.07.002

Buck, J. C., Scheessehe, E. A., Relyea, R. A., & Blaustein, A. R. (2011). The effects of multiple stressors on wetland communities: pesticides, pathogens and competing amphibians. Freshwater Biology, 57(1), 61–73. https://doi.org/10.1111/j.1365-2427.2011.02695.x

Cahoon, L. (2020). GenX in Cape Fear River Water Was Only One Part of the PFAS Story in North Carolina. 10.1021/bk-2020-1352.ch005.

Cui, Q., Pan, Y., Zhang, H., Sheng, N., Wang, J., Guo, Y., & Dai, J. (2018). Occurrence and Tissue Distribution of Novel Perfluoroether Carboxylic and Sulfonic Acids and Legacy Per/Polyfluoroalkyl Substances in Black-Spotted Frog (*Pelophylax nigromaculatus*). Environmental Science & Technology, 52(3), 982–990. https://doi.org/10.1021/acs.est.7b03662

Davidson, C., Benard, M. F., Shaffer, H. B., Parker, J. M., O'Leary, C., Conlon, J. M., & Rollins-Smith, L. A. (2007). Effects of Chytrid and Carbaryl Exposure on Survival, Growth and Skin Peptide Defenses in Foothill Yellow-legged Frogs. Environmental Science & Technology, 41(5), 1771–1776. https://doi.org/10.1021/es0611947

Drake, D. L., Altig, R., Grace, J. B., & Walls, S. C. (2007). Occurrence of Oral Deformities in Larval Anurans. Copeia, 2007(2), 449–458. https://doi.org/10.1643/0045-8511

East, A., Egeghy, P. P., Hubal, E. A. C., Slover, R., & Vallero, D. A. (2021). Computational estimates of daily aggregate exposure to PFOA/PFOS from 2011 to 2017 using a basic intake model. Journal of Exposure Science & Environmental Epidemiology. https://doi.org/10.1038/s41370-021-00374-w

Ellison, A. R., Savage, A. E., DiRenzo, G. V., Langhammer, P., Lips, K. R., & Zamudio, K. R. (2014b). Fighting a Losing Battle: Vigorous Immune Response Countered by Pathogen Suppression of Host Defenses in the Chytridiomycosis-Susceptible Frog *Atelopus zeteki*. G3 Genes|Genomes|Genetics, 4(7), 1275–1289. https://doi.org/10.1534/g3.114.010744

Fernández-Beaskoetxea, S., Bosch, J., & Bielby, J. (2016). Infection and transmission heterogeneity of a multi-host pathogen (*Batrachochytrium dendrobatidis*) within an amphibian community. Diseases of Aquatic Organisms, 118(1), 11–20. https://doi.org/10.3354/dao02963

Foguth, R., Hoskins, T., Clark, G., Nelson, M., Flynn, R., de Perre, C., Hoverman, J., Lee, L., Sepúlveda, M., & Cannon, J. (2020). Single and mixture per- and polyfluoroalkyl substances accumulate in developing Northern leopard frog brains and produce complex neurotransmission alterations. Neurotoxicology and Teratology, 81, 106907. https://doi.org/10.1016/j.ntt.2020.106907

Gaietto, K. M., Rumschlag, S. L., & Boone, M. D. (2014). Effects of pesticide exposure and the amphibian chytrid fungus on gray treefrog (*Hyla chrysoscelis*) metamorphosis. Environmental Toxicology and Chemistry, 33(10), 2358–2362. https://doi.org/10.1002/etc.2689

Gebbink, W. A., & van Leeuwen, S. P. (2020). Environmental contamination and human exposure to PFASs near a fluorochemical production plant: Review of historic and current PFOA and GenX contamination in the Netherlands. Environment International, 137, 105583. https://doi.org/10.1016/j.envint.2020.105583

Gosner, K. L. (1960). A Simplified Table for Staging Anuran Embryos and Larvae with Notes on Identification. Herpetologica, 16(3), 183–190.

Grogan, L. F., Robert, J., Berger, L., Skerratt, L. F., Scheele, B. C., Castley, J. G., Newell, D. A., & McCallum, H. I. (2018). Review of the Amphibian Immune Response to Chytridiomycosis, and Future Directions. Frontiers in Immunology, 9. https://doi.org/10.3389/fimmu.2018.02536

Grogan, L. F., Skerratt, L. F., Berger, L., Cashins, S. D., Trengove, R. D., & Gummer, J. P. A. (2018). Chytridiomycosis causes catastrophic organism-wide metabolic dysregulation including profound failure of cellular energy pathways. Scientific Reports, 8(1). https://doi.org/10.1038/s41598-018-26427-z

Guillette, T., McCord, J., Guillette, M., Polera, M., Rachels, K. T., Morgeson, C., Kotlarz, N., Knappe, D. R., Reading, B. J., Strynar, M., & Belcher, S. M. (2020). Elevated levels of per- and polyfluoroalkyl substances in Cape Fear River Striped Bass (*Morone saxatilis*) are associated with biomarkers of altered immune and liver function. Environment International, 136, 105358. https://doi.org/10.1016/j.envint.2019.105358

Hanlon, S. M., & Parris, M. J. (2013). The interactive effects of chytrid fungus, pesticides, and exposure timing on gray treefrog (*Hyla versicolor*) larvae. Environmental Toxicology and Chemistry, 33(1), 216–222. https://doi.org/10.1002/etc.2419

houHeydebreck, F., Tang, J., Xie, Z., & Ebinghaus, R. (2015). Alternative and Legacy Perfluoroalkyl Substances: Differences between European and Chinese River/Estuary Systems. Environmental Science & Technology, 49(14), 8386–8395. https://doi.org/10.1021/acs.est.5b01648

Hopkins, Z. R., Sun, M., DeWitt, J. C., & Knappe, D. R. (2018). Recently Detected Drinking Water Contaminants: GenX and Other Per- and Polyfluoroalkyl Ether Acids. Journal AWWA, 110(7), 13–28. https://doi.org/10.1002/awwa.1073

Hyatt, A., Boyle, D., Olsen, V., Boyle, D., Berger, L., Obendorf, D., Dalton, A., Kriger, K., Hero, M., Hines, H., Phillott, R., Campbell, R., Marantelli, G., Gleason, F., & Colling, A. (2007). Diagnostic assays and sampling protocols for the detection of *Batrachochytrium dendrobatidis*. Diseases of Aquatic Organisms, 73, 175–192. https://doi.org/10.3354/dao073175

Jarvis, A. L., Justice, J. R., Elias, M. C., Schnitker, B., & Gallagher, K. (2021). Perfluorooctane Sulfonate in US Ambient Surface Waters: A Review of Occurrence in Aquatic Environments and Comparison to Global Concentrations. Environmental Toxicology and Chemistry, 40(9), 2425–2442. https://doi.org/10.1002/etc.5147

Jones, D. K., Dang, T. D., Urbina, J., Bendis, R. J., Buck, J. C., Cothran, R. D., Blaustein, A. R., & Relyea, R. A. (2016). Effect of Simultaneous Amphibian Exposure to Pesticides and an Emerging Fungal Pathogen, *Batrachochytrium dendrobatidis*. Environmental Science & Technology, 51(1), 671–679. https://doi.org/10.1021/acs.est.6b06055

Kindschuh, J. R., Lee, T. S., & Voyen, E. S. (2022). Pfas update: State-by-state regulation of pfas substances in drinking water - energy and Natural Resources - United States. Welcome to Mondaq.

Lips, K. R. (2016). Overview of chytrid emergence and impacts on amphibians. Philosophical Transactions of the Royal Society B: Biological Sciences, 371(1709), 20150465. https://doi.org/10.1098/rstb.2015.0465

Liu, Y., Qian, M., Ma, X., Zhu, L., & Martin, J. W. (2018). Nontarget Mass Spectrometry Reveals New Perfluoroalkyl Substances in Fish from the Yangtze River and Tangxun Lake, China. Environmental Science & Technology, 52(10), 5830–5840. https://doi.org/10.1021/acs.est.8b00779

Longcore, J. E., Pessier, A. P., & Nichols, D. K. (1999). *Batrachochytrium dendrobatidis* gen. et sp. nov., a Chytrid Pathogenic to Amphibians. Mycologia, 91(2), 219. https://doi.org/10.2307/3761366

Munoz, G., Liu, J., Vo Duy, S., & Sauvé, S. (2019). Analysis of F-53B, Gen-X, ADONA, and emerging fluoroalkylether substances in environmental and biomonitoring samples: A review. Trends in Environmental Analytical Chemistry, 23, e00066. https://doi.org/10.1016/j.teac.2019.e00066

Nakayama, S., Strynar, M. J., Helfant, L., Egeghy, P., Ye, X., & Lindstrom, A. B. (2007). Perfluorinated Compounds in the Cape Fear Drainage Basin in North Carolina. Environmental Science & Technology, 41(15), 5271–5276. https://doi.org/10.1021/es070792y

Ohmer, M. E. B., Cramp, R. L., Russo, C. J. M., White, C. R., & Franklin, C. E. (2017). Skin sloughing in susceptible and resistant amphibians regulates infection with a fungal pathogen. Scientific Reports, 7(1). https://doi.org/10.1038/s41598-017-03605-z

Parris, M. J., & Baud, D. R. (2004). Interactive Effects of a Heavy Metal and Chytridiomycosis on Gray Treefrog Larvae (*Hyla chrysoscelis*). Copeia, 2004(2), 344–350. https://doi.org/10.1643/ce-03-116r1

Parris, M. J., & Beaudoin, J. G. (2004). Chytridiomycosis impacts predator-prey interactions in larval amphibian communities. Oecologia, 140(4), 626–632. https://doi.org/10.1007/s00442-004-1631-2

Parris, M. J., & Cornelius, T. O. (2004). Fungal Pathogen Causes Competitive and Developmental Stress in Larval Amphibian Communities. Ecology, 85(12), 3385–3395. https://doi.org/10.1890/04-0383

Peig, J., & Green, A. J. (2009). New perspectives for estimating body condition from mass/length data: the scaled mass index as an alternative method. Oikos, 118(12), 1883–1891. https://doi.org/10.1111/j.1600-0706.2009.17643.x

Piotrowski, J. S., Annis, S. L., & Longcore, J. E. (2004). Physiology of Batrachochytrium dendrobatidis, a Chytrid Pathogen of Amphibians. Mycologia, 96(1), 9. https://doi.org/10.2307/3761981

Qazi, M. R., Bogdanska, J., Butenhoff, J. L., Nelson, B. D., DePierre, J. W., & Abedi-Valugerdi, M. (2009). High-dose, short-term exposure of mice to perfluorooctanesulfonate (PFOS) or perfluorooctanoate (PFOA) affects the number of circulating neutrophils differently, but enhances the inflammatory responses of macrophages to lipopolysaccharide (LPS) in a similar fashion. Toxicology, 262(3), 207–214. https://doi.org/10.1016/j.tox.2009.06.010

Quaranta, A., Bellantuono, V., Cassano, G., & Lippe, C. (2009). Why Amphibians Are More Sensitive than Mammals to Xenobiotics. PLoS ONE, 4(11), e7699. https://doi.org/10.1371/journal.pone.0007699

R Core Development Team. 2021. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria

Relyea, R. A., & Mills, N. (2001). Predator-induced stress makes the pesticide carbaryl more deadly to gray treefrog tadpoles (Hyla versicolor). Proceedings of the National Academy of Sciences, 98(5), 2491–2496. https://doi.org/10.1073/pnas.031076198

Rohr, J. R., Raffel, T. R., Halstead, N. T., McMahon, T. A., Johnson, S. A., Boughton, R. K., & Martin, L. B. (2013). Early-life exposure to a herbicide has enduring effects on pathogen-induced mortality. Proceedings of the Royal Society B: Biological Sciences, 280(1772), 20131502. https://doi.org/10.1098/rspb.2013.1502

Rollins-Smith, L. A. (1998). Metamorphosis and the amphibian immune system. Immunological Reviews, 166(1), 221–230. https://doi.org/10.1111/j.1600-065x.1998.tb01265.x

Rowe, G., & Beebee, T. J. C. (2003). Population on the verge of a mutational meltdown? Fitness costs of genetic load for an amhobian in the wild. Evolution, 57(1), 177. https://doi.org/10.1554/0014-3820

Rushing, B. R., Hu, Q., Franklin, J. N., McMahen, R., Dagnino, S., Higgins, C. P., Strynar, M. J., & DeWitt, J. C. (2017). Evaluation of the immunomodulatory effects of 2,3,3,3-tetrafluoro-2-(heptafluoropropoxy)-propanoate in C57BL/6 mice. Toxicological Sciences, kfw251. https://doi.org/10.1093/toxsci/kfw251

Scheele, B. C., Pasmans, F., Skerratt, L. F., Berger, L., Martel, A., Beukema, W., Acevedo, A. A., Burrowes, P. A., Carvalho, T., Catenazzi, A., de la Riva, I., Fisher, M. C., Flechas, S. V., Foster, C. N., Frías-Álvarez, P., Garner, T. W. J., Gratwicke, B., Guayasamin, J. M., Hirschfeld, M., . . . Canessa, S. (2019). Amphibian fungal panzootic causes catastrophic and ongoing loss of biodiversity. Science, 363(6434), 1459–1463. https://doi.org/10.1126/science.aav0379

Searle, C. L., Gervasi, S. S., Hua, J., Hammond, J. I., Relyea, R. A., Olson, D. H., & Blaustein, A. R. (2011). Differential Host Susceptibility to Batrachochytrium dendrobatidis, an Emerging Amphibian Pathogen. Conservation Biology, 25(5), 965–974. https://doi.org/10.1111/j.1523-1739.2011.01708.x

Shane, H. L., Baur, R., Lukomska, E., Weatherly, L., & Anderson, S. E. (2020). Immunotoxicity and allergenic potential induced by topical application of perfluorooctanoic acid (PFOA) in a murine model. Food and Chemical Toxicology, 136, 111114. https://doi.org/10.1016/j.fct.2020.111114

Shi, Y., Vestergren, R., Xu, L., Zhou, Z., Li, C., Liang, Y., & Cai, Y. (2016). Human Exposure and Elimination Kinetics of Chlorinated Polyfluoroalkyl Ether Sulfonic Acids (Cl-PFESAs). Environmental Science & Technology, 50(5), 2396–2404. https://doi.org/10.1021/acs.est.5b05849

Sun, M., Arevalo, E., Strynar, M., Lindstrom, A., Richardson, M., Kearns, B., Pickett, A., Smith, C., & Knappe, D. R. U. (2016). Legacy and Emerging Perfluoroalkyl Substances Are Important Drinking Water Contaminants in the Cape Fear River Watershed of North Carolina. Environmental Science & Technology Letters, 3(12), 415–419. https://doi.org/10.1021/acs.estlett.6b00398

United Sates EPA (Environmental Protection Agency). (2021a). Drinking Water Health Advisories for PFOA and PFOS: Questions and Answers. https://www.epa.gov/ground-water-and-drinking-water/drinking-water-health-advisories-pfoa-and-pfos-questions-and.

United States EPA (Environmental Protection Agency). (2021b). Technical Fact Sheet: Human Health Toxicity Assessment for GenX Chemicals.Technical Fact Sheet: Human Health Toxicity Assessment for GenX Chemicals (epa.gov)

United States EPA (Environmental Protection Agency). (2017). Technical Fact Sheet – Perfluorooctane Sulfonate (PFOS) and Perfluorooctanoic Acid (PFOA), https://www.epa.gov/sites/production/files/2017-12/documents/ffrrofactsheet_contaminants_pfos_pfoa_11-20-17_508_0.pdf

United States Geological Survey National Wildlife Health Center. (2017). SOP –Quantitative Real-time Duplex PCR for Batrachochytrium salamandrivorans (Bsal) and Batrachochytrium dendrobatidis (Bd).

Walker, S., Baldi Salas, M., Jenkins, D., Garner, T., Cunningham, A., Hyatt, A., Bosch, J., & Fisher, M. (2007). Environmental detection of *Batrachochytrium dendrobatidis* in a temperate climate. Diseases of Aquatic Organisms, 77, 105–112. https://doi.org/10.3354/dao01850

Wang, Y., Chang, W., Wang, L., Zhang, Y., Zhang, Y., Wang, M., Wang, Y., & Li, P. (2019). A review of sources, multimedia distribution and health risks of novel fluorinated alternatives. Ecotoxicology and Environmental Safety, 182, 109402. https://doi.org/10.1016/j.ecoenv.2019.109402

Xie, X., Zhou, J., Hu, L., Shu, R., Zhang, M., Xiong, Z., Wu, F., & Fu, Z. (2021). Exposure to hexafluoropropylene oxide dimer acid (HFPO-DA) disturbs the gut barrier function and gut microbiota in mice. Environmental Pollution, 290, 117934. https://doi.org/10.1016/j.envpol.2021.117934

Yang, Q., Abedi-Valugerdi, M., Xie, Y., Zhao, X. Y., Möller, G., Dean Nelson, B., & DePierre, J. W. (2002). Potent suppression of the adaptive immune response in mice upon dietary exposure to the potent peroxisome proliferator, perfluorooctanoic acid. International Immunopharmacology, 2(2–3), 389–397. https://doi.org/10.1016/s1567-5769(01)00164-3

Yang, Q., Xie, Y., & Depierre, J. W. (2000). Effects of peroxisome proliferators on the thymus and spleen of mice. Clinical and Experimental Immunology, 122(2), 219–226. https://doi.org/10.1046/j.1365-2249.2000.01367.x

Yang, Q., Xie, Y., Eriksson, A. M., Nelson, B., & DePierre, J. W. (2001). Further evidence for the involvement of inhibition of cell proliferation and development in thymic and splenic atrophy induced by the peroxisome proliferator perfluoroctanoic acid in mice33Abbreviations: PFOA, perfluorooctanoic acid; PP, peroxisome proliferator; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; and DEHP, di(2-ethylhexyl)phthalate. Biochemical Pharmacology, 62(8), 1133–1140. https://doi.org/10.1016/s0006-2952(01)00752-3

Table 1: Nominal and mean measured PFAS concentrations for water samples collected at the beginning of the larval exposure and at termination, N=36 for each chemical treatment. Units are parts per billion (ppb) and are reported as mean (SEM). Treatments were listed as NA if the chemical was not tested listed as < Lower Limit of Quantification (LLOQ) if ≥ 2 samples from a treatment were <LLOQ. Mesocosm-specific measurements LLOQs are provided in ppb.

Treatment	**Nominal Concentrations**			**Measured Concentrations**		
	PFOS	**PFOA**	**GenX**	**PFOS**	**PFOA**	**GenX**
Control	0	0	0	NA	NA	<LLOQ
PFOS	10	0	0	9.8 (0.219)	0.1985	NA
PFOA	0	10	0	<LLOQ	10.117 (0.610)	NA
GenX 10ppb	0	0	10	NA	NA	4.325 (0.438)
GenX 1ppb	0	0	1	NA	NA	0.87 (0.089)
GenX 0.1 ppb	0	0	0.1	NA	NA	0.045 (0.012)

Table 2: Mean (± SEM) of qPCR Cycle Threshold (CT) values and zoospore equivalence (ZE) for subset of swab samples taken from Bd-exposed (N = 18) and NBd exposed individuals (N=8). Animals that tested negative were given a CT value of 40.

	CT Value	**ZE**
Bd-Exposed	23.85 (± 2.05)	1.58 (± 0.78)
NBd-Exposed	40	0

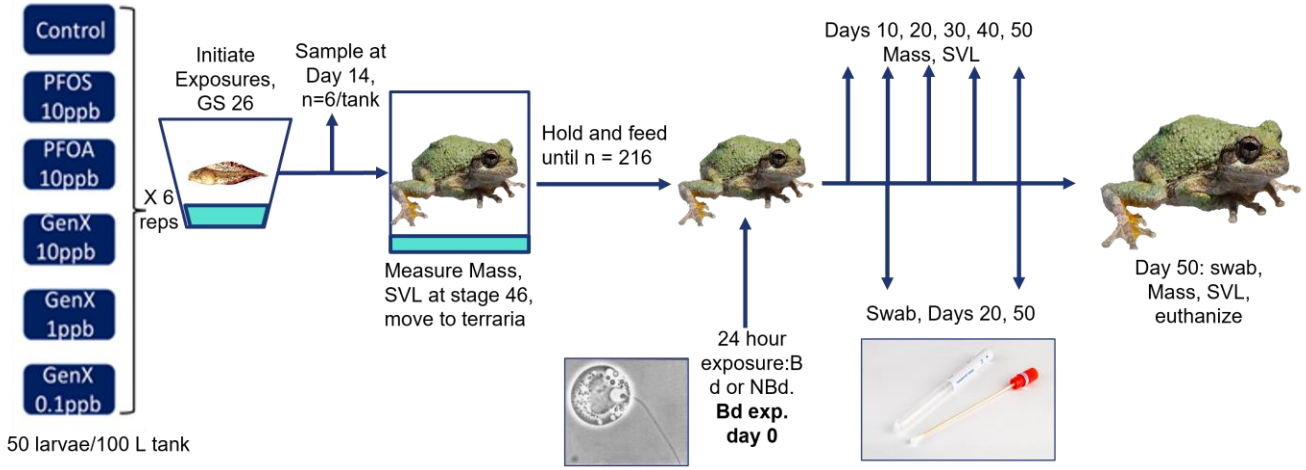


Figure 1: Experimental design of larvae PFAS exposure and terrestrial stage *Bd* exposure.

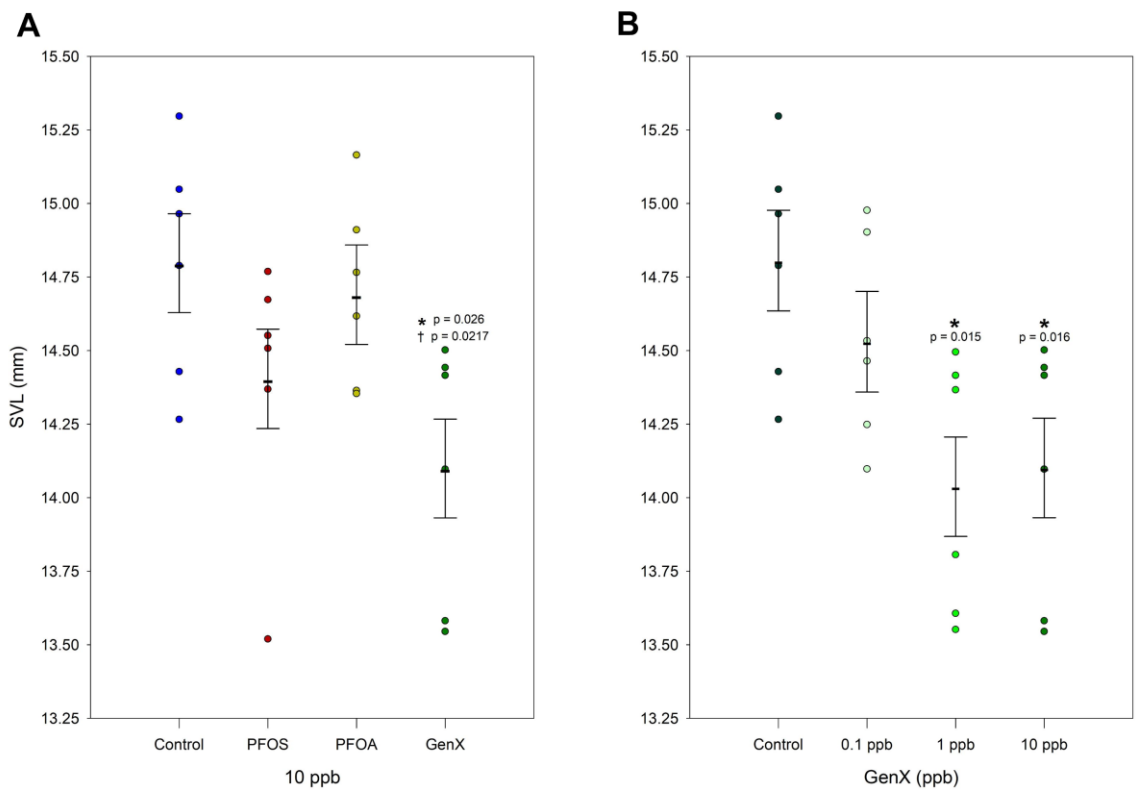


Figure 2: Changes in SVL at metamorphic climax for grey treefrogs exposed to 10 ppb PFOS, PFOA, or GenX **(A)** and at three concentrations of GenX **(B)**. Asterisks (*) denote significant differences from controls, daggers (†) denote significant difference between PFOA and GenX at 10 ppb ($p \leq 0.05$).

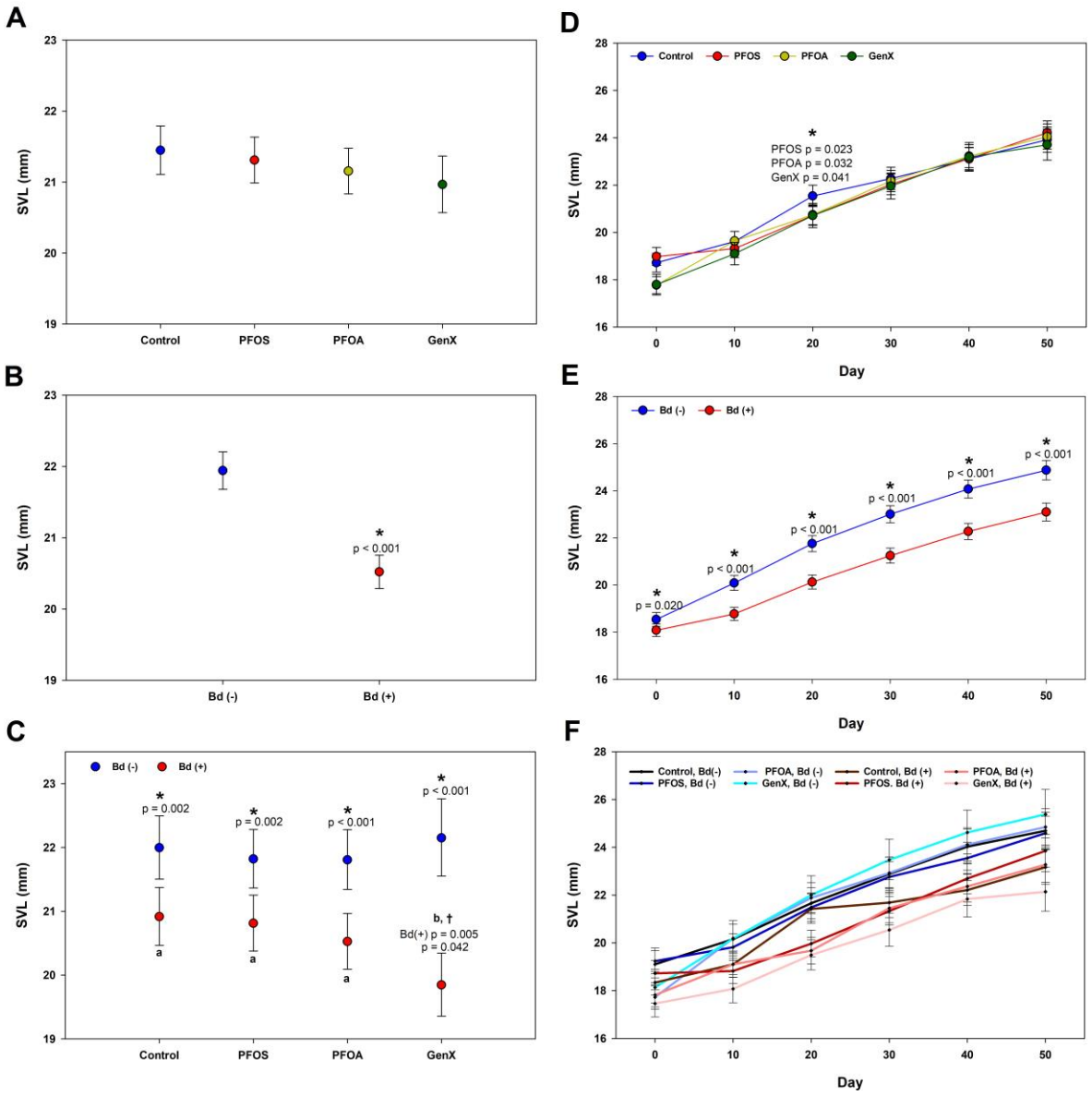


Figure 3: **(A)** Average SVL in grey treefrog metamorphs following larval exposure to 10 ppb PFOS, PFOA, or GenX. **(B)** Average SVL of grey treefrogs after post metamorphic exposure to Bd. **(C)** Combined effects of larval exposure to 10 ppb PFOS, PFOA, or GenX and post metamorphic exposure to Bd on average SVL in grey treefrog metamorphs. (D) Average SVL of grey treefrog metamorphs over time following larval exposure to 10 ppb PFOS, PFOA, or GenX. (E) Average SVL of grey treefrogs over time after post metamorphic exposure to Bd. (F) Combined effects of larval exposure to 10 ppb PFOS, PFOA, or GenX and post metamorphic exposure to Bd over time on average SVL in grey treefrog metamorphs. Across all panels, asterisks (*) denote significant differences from controls ($p \leq 0.05$), single daggers (†) indicates difference between PFOA and GenX ($p \leq 0.05$). In Panel C, letters denote differences within Bd treatment ($p \leq 0.05$), single daggers (†) indicates difference between PFOA and GenX within Bd treatment ($p \leq 0.05$), and double daggers (‡) denote differences between PFOA and GenX within No Bd treatment ($p \leq 0.05$).

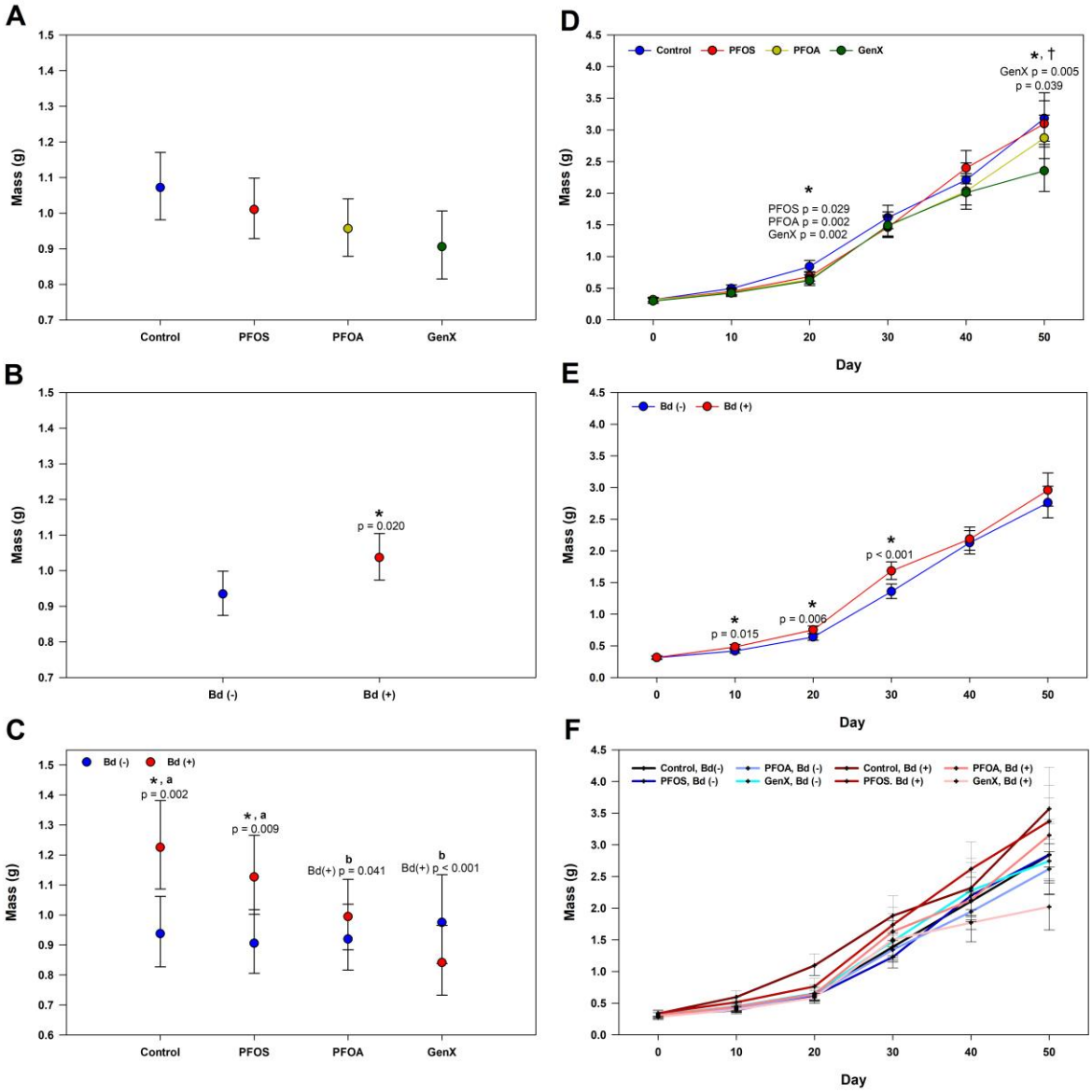


Figure 4: (A) Average mass in grey treefrog metamorphs following larval exposure to 10 ppb PFOS, PFOA, or GenX. (B) Average mass of grey treefrogs after post metamorphic exposure to Bd. (C) Combined effects of larval exposure to 10 ppb PFOS, PFOA, or GenX and post metamorphic exposure to Bd on average mass in grey treefrog metamorphs. (D) Average mass of grey treefrog metamorphs over time following larval exposure to 10 ppb PFOS, PFOA, or GenX. (E) Average mass of grey treefrogs over time after post metamorphic exposure to Bd. (F) Combined effects of larval exposure to 10 ppb PFOS, PFOA, or GenX and post metamorphic exposure to Bd over time on average mass in grey treefrog metamorphs. Across all panels, asterisks (*) denote significant differences from controls ($p \leq 0.05$), single daggers (†) indicates difference between PFOA and GenX ($p \leq 0.05$). In Panel C, letters denote differences within Bd treatment ($p \leq 0.05$), single daggers (†) indicates difference between PFOA and GenX within Bd treatment ($p \leq 0.05$), and double daggers (‡) denote differences between PFOA and GenX within No Bd treatment ($p \leq 0.05$).

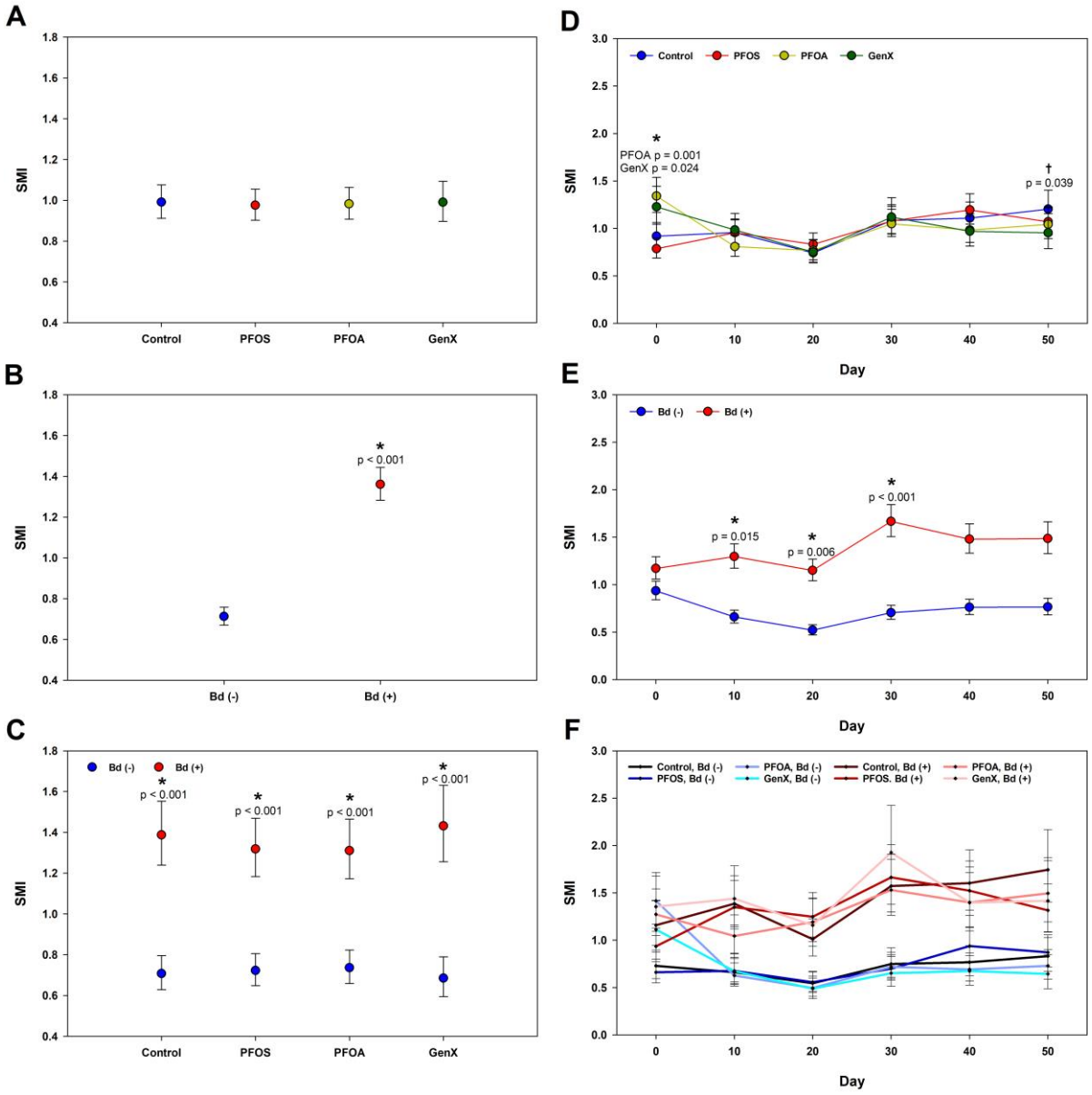


Figure 5: (A) Average SMI in grey treefrog metamorphs following larval exposure to 10 ppb PFOS, PFOA, or GenX. (B) Average SMI of grey treefrogs after post metamorphic exposure to Bd. (C) Combined effects of larval exposure to 10 ppb PFOS, PFOA, or GenX and post metamorphic exposure to Bd on average SMI in grey treefrog metamorphs. (D) Average SMI of grey treefrog metamorphs over time following larval exposure to 10 ppb PFOS, PFOA, or GenX. (E) Average SMI of grey treefrogs over time after post metamorphic exposure to Bd. (F) Combined effects of larval exposure to 10 ppb PFOS, PFOA, or GenX and post metamorphic exposure to Bd over time on average SMI in grey treefrog metamorphs. Across all panels, asterisks (*) denote significant differences from controls ($p \leq 0.05$), single daggers (†) indicates difference between PFOA and GenX ($p \leq 0.05$). In Panel C, letters denote differences within Bd treatment ($p \leq 0.05$), single daggers (†) indicates difference between PFOA and GenX within Bd treatment ($p \leq 0.05$), and double daggers (‡) denote differences between PFOA and GenX within No Bd treatment ($p \leq 0.05$).

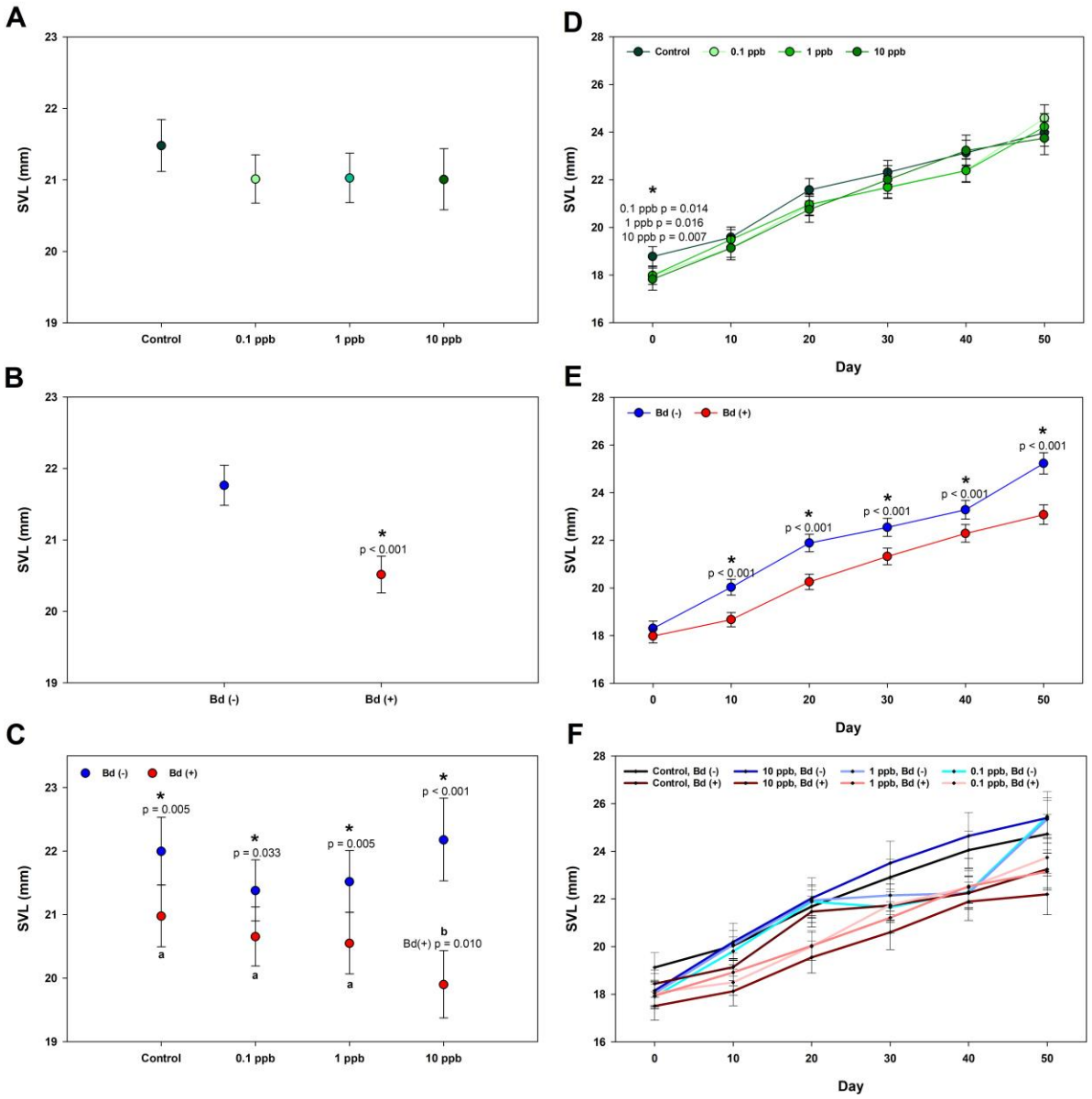


Figure 6: (A) Average SVL in grey treefrog metamorphs following larval exposure to three concentrations of GenX. (B) Average SVL of grey treefrogs after post metamorphic exposure to Bd. (C) Combined effects of larval exposure to three concentrations of GenX and post metamorphic exposure to Bd on average SVL in grey treefrog metamorphs. (D) Average SVL of grey treefrog metamorphs over time following larval exposure to three concentrations of GenX. (E) Average SVL of grey treefrogs over time after post metamorphic exposure to Bd. (F) Combined effects of larval exposure to three concentrations of GenX and post metamorphic exposure to Bd over time on average SVL in grey treefrog metamorphs. Across all panels, asterisks denote significant differences from controls ($p \leq 0.05$), in Panel C, letters denote differences within Bd treatment ($p \leq 0.05$).

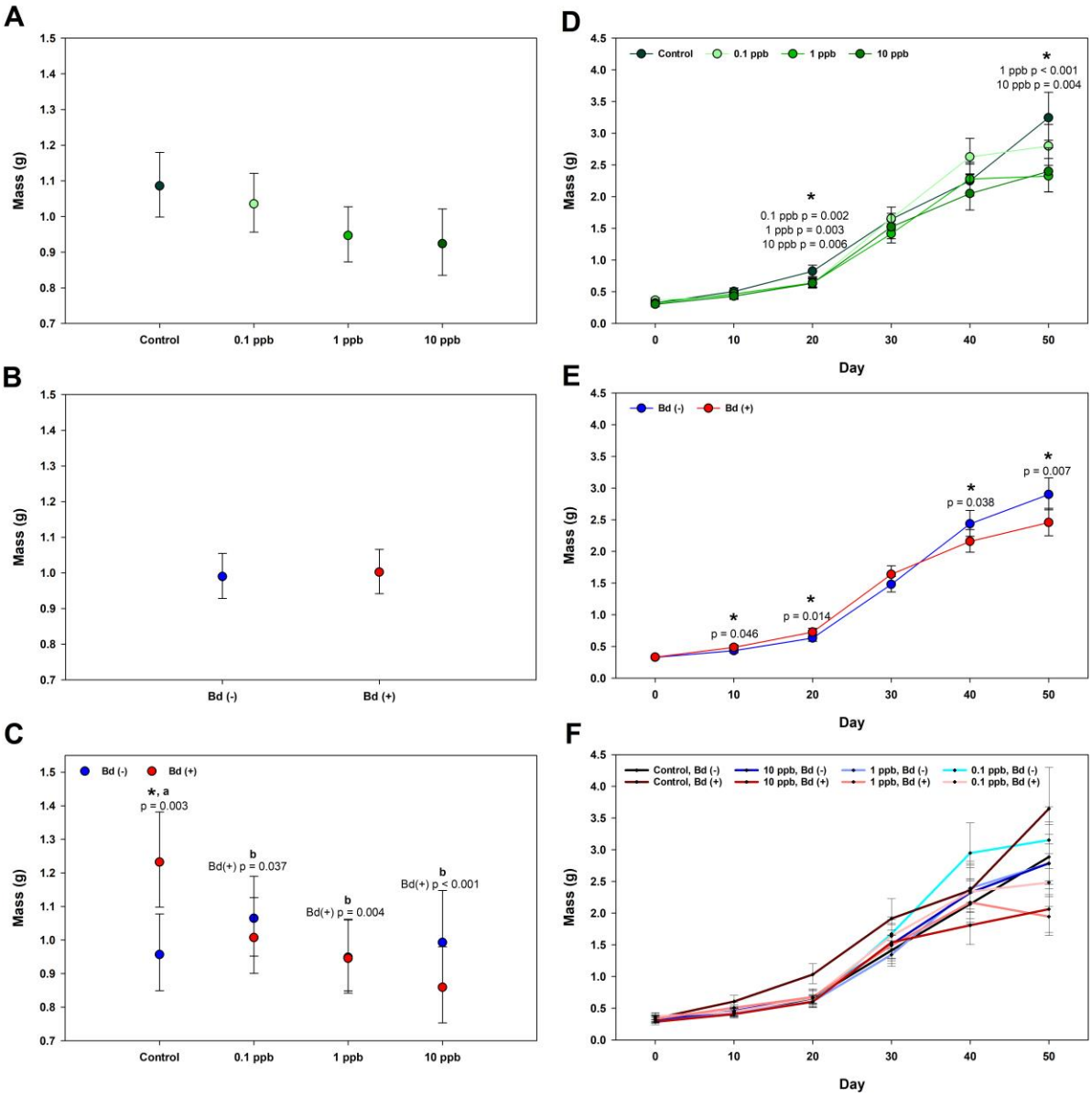


Figure 7: (A) Average mass in grey treefrog metamorphs following larval exposure to three concentrations of GenX. (B) Average mass of grey treefrogs after post metamorphic exposure to Bd. (C) Combined effects of larval exposure to three concentrations of GenX and post metamorphic exposure to Bd on average mass in grey treefrog metamorphs. (D) Average mass of grey treefrog metamorphs over time following larval exposure to three concentrations of GenX. (E) Average mass of grey treefrogs over time after post metamorphic exposure to Bd. (F) Combined effects of larval exposure to three concentrations of GenX and post metamorphic exposure to Bd over time on average mass in grey treefrog metamorphs. Across all panels, asterisks denote significant differences from controls ($p \leq 0.05$), in Panel C, letters denote differences within Bd treatment ($p \leq 0.05$).

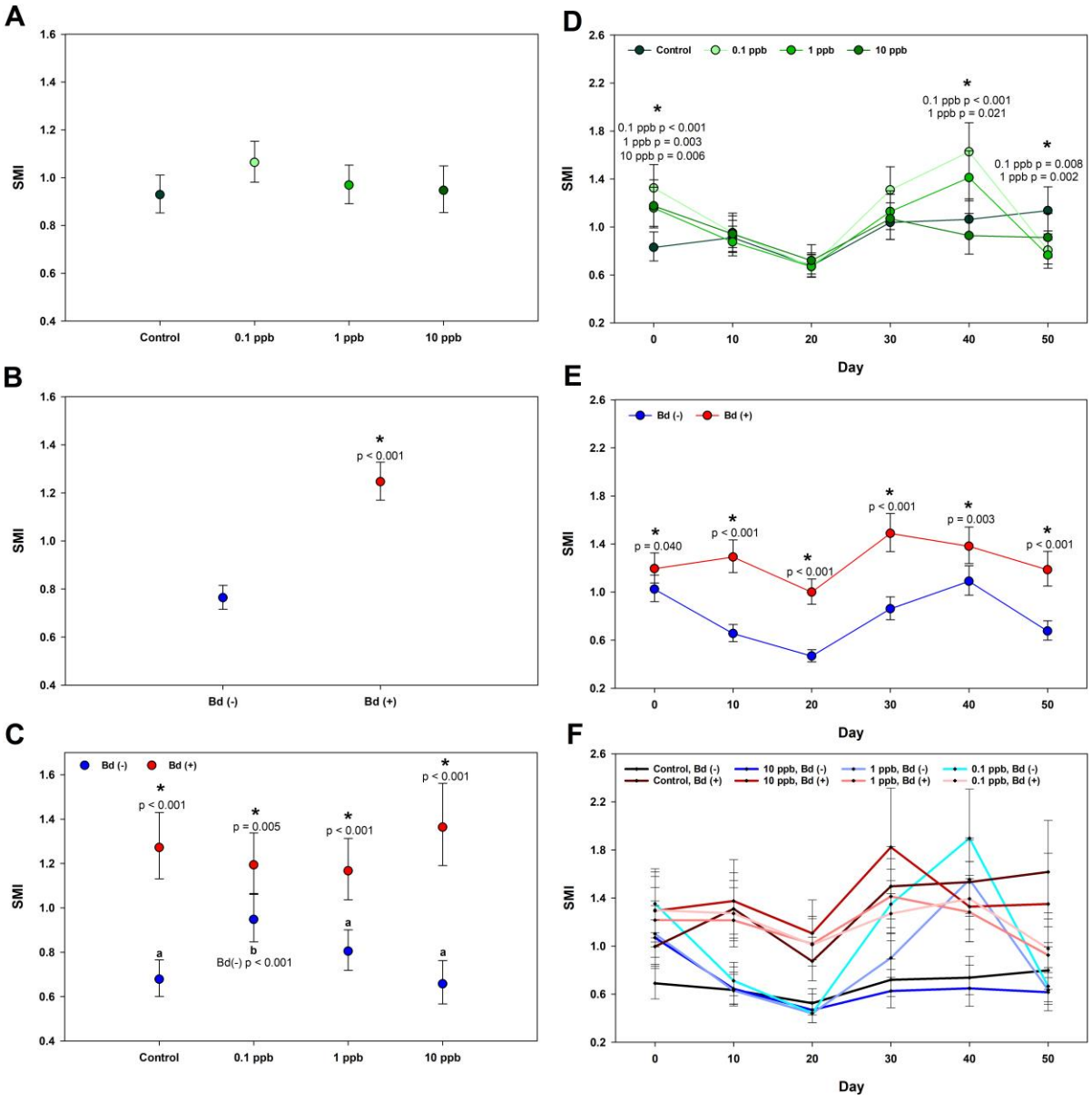
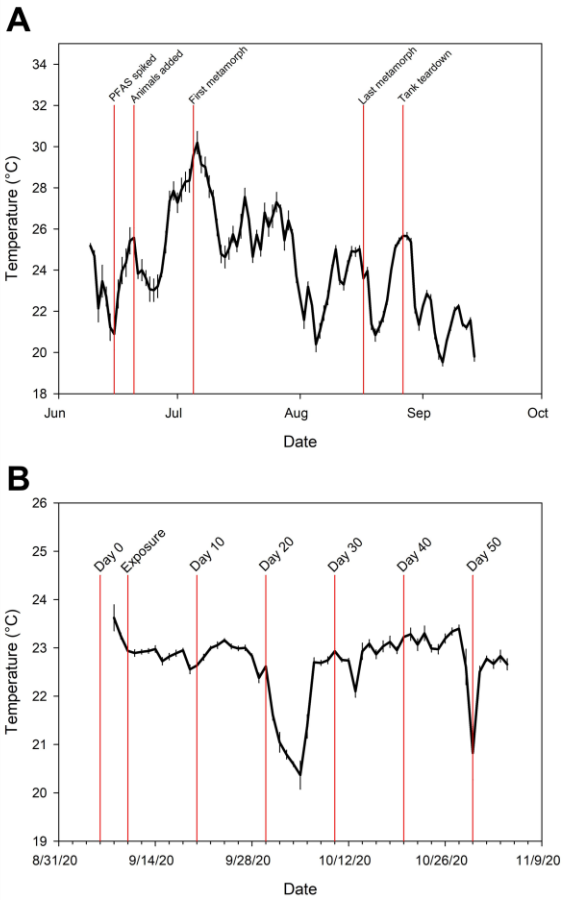


Figure 8: (A) Average SMI in grey treefrog metamorphs following larval exposure to three concentrations of GenX. (B) Average SMI of grey treefrogs after post metamorphic exposure to Bd. (C) Combined effects of larval exposure to three concentrations of GenX and post metamorphic exposure to Bd on average SMI in grey treefrog metamorphs. (D) Average SMI of grey treefrog metamorphs over time following larval exposure to three concentrations of GenX. (E) Average SMI of grey treefrogs over time after post metamorphic exposure to Bd. (F) Combined effects of larval exposure to three concentrations of GenX and post metamorphic exposure to Bd over time on average SMI in grey treefrog metamorphs. Across all panels, asterisks denote significant differences from controls ($p \leq 0.05$), in Panel C, letters denote differences within Bd treatment ($p \leq 0.05$).

APPENDIX: SUPPLEMENTARY INFORMATION



Supplemental Figure 1: Daily temperature variation (mean ± SEM) throughout grey treefrog exposures to **(A)** PFAS in larval stages and **(B)** Bd as post-metamorphic froglets.

Supplemental Table 1: **(A)**All chemical measurements for water samples analyzed from mesocosms. The "Volume (mL) or Mass (g)" column lists the volume of water sampled (mL). Columns with NA indicate chemical not quantified. LLOQs are provided for each compound with units; columns labeled "(chemical) < LLOQ?" show whether each chemical-specific measurement was < LLOQ (listed as "1") or > LLOQ "listed as "0". **(B)** Water quality parameters (mean ± SEM) measured in control mesocosms throughout larval exposures.

Supplemental Table 2: Mean ± SEM, minimum, and maximum values for all survival and phenotypic endpoints measured across the larval stage exposures to PFAS. Mean ± SEM values in bold are significantly different than controls, mean ± SEM values in bold italics indicate significant differences between PFOA and GenX at 10 ppb ($p \leq 0.05$).

Supplemental Table 3: Statistical summaries for all phenotypic analyses of grey treefrog tadpoles at (A) day 14 post exposure, (B) at metamorphic climax, and (C) across metamorphosis. Statistically significant p-values ($p \leq 0.05$) are in bold.

Supplemental Table 4: Statistical summary tables for two-way ANOVAs of (A, D) SVL, (B, E) Mass, and (C, F) SMI of grey treefrogs exposed to 10 ppb PFOS, PFOA, or GenX (A, B, C) or one of three concentrations of GenX (D, E, F). Statistically significant values ($p \leq 0.05$) are in bold.