AN ENVIRONMENTALLY RELEVANT BINARY MIXTURE OF PERFLUOROOCTANESULFONIC ACID AND PERFLUOROHEXANESULFONIC ACID RESULTS IN ANTAGONISM AND REDUCED BODY CONDITION IN NORTHERN LEOPARD FROGS.

by

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Dedicated to my daughter Azalea Pérez, my son Zyden Pérez, and my wife Deisy Pérez

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ABSTRACT

Perfluoroalkyl substances are synthetic organic chemicals of environmental concern because they have been associated with adverse effects in both human epidemiological studies and standard laboratory animals. In the environment, PFAS occur as mixtures, especially in areas with a history of PFAS application, such as aqueous film forming foam (AFFF) sites. Among the PFAS, perfluorooctanesulfonic acid (PFOS) and perfluorohexanesulfonic acid (PFHxS) are the most common, and occur at the highest concentrations. Thus, amphibian populations at or near AFFF sites are at risk of exposure to known bioaccumulative and persistent chemicals, likely compromising the physiology and body condition of the animals. Here, we exposed northern leopard frogs to environmentally relevant concentrations of 0.5 and 1 ppb PFOS and PFHxS, alone or as a mixture comprised of 0.5 ppb PFOS and 0.5 ppb PFHxS. Univariate analyses showed that in the larval stages, tadpoles exposed to PFAS had significantly reduced scaled mass indexes (SMI's) relative to the control, and only the organisms exposed to PFHxS 0.5 ppb were significantly larger. Sex did not significantly influence toxicity in the later stages (GS 42 & 46), indicating no sex-related effects. Altered body condition (i.e., fat stores) in the larval stages indicate potential effects to energy balance. There is a need to assess fitness-related effects as amphibians' transition into the terrestrial environment, and include endpoints such as: reproductive, developmental, immunological, mating, feeding, competition, and survival. Early developmental effects in the larval stages also suggests that earlier developmental endpoints may be of interest. Establishing ecological risk assessments for PFAS are necessary, as they are toxic, persistent, and bioaccumulative.

CHAPTER 1. PER- OR POLY-FLUOROALKYL SUBSTANCES (PFAS) IN THE ENVIRONMENT: BACKGROUND AND MIXTURE REVIEW

1.1 Background on PFAS

PFAS belong to the class of synthetic chemicals known as per- and polyfluoroalkyl substances. PFAS do not occur naturally in the environment, and as the name implies, are molecules of varying carbon chain lengths, where the hydrogen is completely or partially substituted by fluorine [1]. The PFAS family is estimated to include between 5,000 to 10,000 chemicals [2], of which only 4,700 are identified as being used or could have been used on the global market [3]. Needless to say, all the 4,700 PFAS have not been studied, and the uses may not be known [4]. Of the PFAS, perfluorosulfonic PFOS and PFHxS are strong acids with both hydrophobic and hydrophilic chemical properties [5]. In addition, because PFAS have stable carbon fluorine bonds that are only breakable at very high temperatures, these compounds are incredibly resistant to environmental degradation [6]. Ambient occurrence puts PFHxS as the third most common environmentally occurring PFAS, next to PFOS and perfluorooctanoic acid (PFOA) [6].

PFAS have been used since the 1940's, because of their amphiphilic properties (having both hydrophobic and hydrophilic characteristics). PFAS are markedly used in consumer products as a protective coating or a strong surfactant. Some consumer products include: non-stick cookware, furniture, carpets, water-repelling or stain-resistant textiles, paper or cardboard packaging, and most importantly, fire-fighting foams [7]. Having many industrial applications, PFAS are part of our daily lives. Since its initial use, PFHxS has been adopted as an alternative to PFOS, and is also ubiquitous in the environment, especially at aqueous film-forming foam (AFFF) sites [8, 9]. The low pKa values of PFOS and PFHxS [7], indicate that they dissociate in the aquatic environment, but their anion does not volatize because of its strong interaction with sediment and water [10]. Although PFAS volatility is low as an anion, volatilization of the neutral form does occur [11], permitting long-range atmospheric mobility of the compounds.

As a member of the same chemical category as PFOS, a persistent organic pollutant listed in Annex B in the Stockholm Convention, PFHxS, its salts, and related products, are in the process of being added to the Annexes A, B, and/or C of the Stockholm Convention on persistent organic pollutants [12]. PFHxS and all its related substances have been labelled as toxic, bioaccumulative,

and persistent by the Swedish Chemical Agency (KEMI) [13]. In the United States, long chain PFAS have been phased out through a cooperative effort made by the major manufacturers of fluoropolymer and telomer chemicals (3M, Arkema, Dow chemicals, etc.). Nevertheless, PFHxS and all its related compounds are still being used in the country due to the significant new use rule (SNUR) endorsed by the Toxic Substances Control Act (TSCA) [14]. In 2008, Australian reports recommended that PFOS and other PFASs like PFHxS, be restricted to essential use only, and any alternative chemical adopted as replacement, be less toxic and persistent in the environment than its counterparts [15]. PFAS chemicals are a global concern because their physicochemical properties make it a very stable molecule. In the environment, PFAS are persistent and remain resistant to photolysis, biodegradation, atmospheric photooxidation, and hydrolysis [16]. In areas with a history of application, like AFFF sites, PFAS can be a danger to aquatic ecosystems by altering trophic processes and populations of organisms, like amphibians.

1.2 Toxicity of PFAS mixtures and risk assessment challenges

To date, most of the PFAS mixtures performed are binary exposures of PFOS + PFHxS [17, 18] and PFOS + PFOA [19 – 24]. Although PFAS mixture toxicity data is limited, the results demonstrate inconsistent findings and complex toxicological interactions among binary mixtures and other more complex mixtures, including those artificially simulated [25 – 31]. For example, mixtures of PFAS report additive [17, 21, 26 (theoretically simulated)], antagonistic [18, 25, 32, 33], and synergistic [23, 26, 28] responses, depending on the test model, duration of exposure, dose, developmental stage, mixture components, and endpoints considered (survival, growth, reproduction, cellular marker, etc.) [34, 35]. Mixtures of PFOS and PFHxS specifically, report additive toxicity in Northern bobwhite quail (*Colinus virginianus*) [17], but antagonistic toxicity in the activation of mouse PPARα using transiently transfected COS-1 cells [25]. Furthermore, in reviewing an avian tissue and species-specific toxicity reference values (TRV), Dennis and colleagues [18], reported that PFOS absorbed and distributed differently in tissues of *C. virginianus* when coadministered with PFHxS, suggesting different modes of action (MOAs) for the chemicals. A similar trend in tissue accumulation was found in human samples collected from 20 autopsies [36].

Establishing a robust model that predicts the toxicity of PFAS mixtures is rather difficult, considering varied toxicity responses. PFAS toxicity is known to vary depending on chain length,

physicochemical properties (functional group, solubility, partition coefficients etc.), persistence, and bioaccumulative potential. In assessing the individual and joint effects of PFAS carboxylates and sulfonates on the viability of human liver cells (HL-7702), Hu and colleagues [28] found that perfluorosulfonic acids (PFSA) showed a higher stimulatory effect than perfluorocarboxylic acids (PFCA) with the same chain length. Complimentary findings also show that long-chained PFAS (≥ C8) and PFAS with sulfonate polar heads displayed the greatest toxic potential towards zebrafish (*Danio rerio*) embryos [33]. Short chain PFAS (C6 & C7) on the other hand, accumulated and produced significant alterations in behavior [33]. Using cultured hepatocytes from rare minnows (*Gobiocypris rarus*), Wei and his team [30], developed a toxicogenomic profile for six single PFAS and four formulations, demonstrating that all single PFAS and mixtures consistently regulated a particular set of genes, which indicated a regulatory hub, central to PFAS-mediated toxicity. Gene expression profiles also revealed a distinct gene set regulated by PFAS mixture exposures, and not by PFAS alone exposures, implicating several molecular processes and biological functions, including oxidative stress, xenobiotic metabolism, fatty acid metabolism and transport, and immune responses [30]. A review by Goodrum et al. [22], proposed multiple MOAs for PFAS, because short- and long-chained sulfonates, carboxylates, and fluorotelomers were able to interact with nearly two dozen nuclear receptors.

Adding to the equation is the ability of PFAS to absorb and distribute itself differently in tissues when co-administered as a mixture. The study by Dennis et al. [17] showed that PFOS distributed differently in the tissues of *C. virginianus* in the presence of PFHxS. Could the distribution of PFAS into different tissues explain the varied interaction responses observed for different endpoints? Some mixtures tend to reduce toxicity, while others tend to be additive, or synergistic. Ding et al. [20] exposed zebrafish embryos to mixtures of PFOS and PFOA and found additive, antagonistic, and synergistic interactions depending on the molar ratios. Synergistic effects were also seen on acute mortality and some developmental endpoints in the invertebrate *Daphnia magna*, upon exposure to a mixture of PFOS and PFOA [23]. In zebrafish embryo-larval stages, the two most toxic PFAS (PFHxS and PFOS) resulted in higher startle response, altered embryo-larval behavior, and made the fish more inclined to engage in burst swimming activities [33]. Differing interactions at different endpoints, suggests that PFAS mixture toxicity cannot be predicted by additive models that utilize data on single chemicals [33]. To say the least, the study of PFAS mixture toxicity is in its infancy and the available data are highly inconsistent [37].

Based on the diverse interactions observed in PFAS mixture toxicity studies, Ankley and colleagues [38], concluded that it is currently not feasible to predict how different PFAS will interact with each other in the environment. In laboratory studies, even single PFAS are potent enough to induce toxicity relative to a mixture. In the study by Hue et al. [28], the authors concluded that stimulatory responses of HL-7702 cells to single PFAS may likely induce adverse effects at relevant mixture concentrations. Thus, it may be relevant to continue to understand the toxicity of single PFAS along with a combination of mixtures. Currently, there is a need for mixture ecological risk assessments to manage the hazards mixtures may pose to aquatic communities and populations in the environment, especially at or near contaminated AFFF sites. However, more PFAS mixture data is needed, as inconsistent results in the literature make it difficult to develop reliable predictive models. A recent review by McCarthy et al. [37], discusses the approaches that exist for mixture risk assessment as it relates to PFAS mixtures. Approaches to address the risk of PFAS will likely include novel methods that rely on in-vitro assays, quantitative structure activity relationships (QSAR), read-across, or whole-organisms toxicity studies [37].

1.3 Thesis intent

At PFOS-based AFFF sites, amphibians are exposed to a mixture of PFAS primarily composed of PFOS and PFHxS. Accordingly, these PFAS should be prioritized for testing and evaluation based on their toxicity, frequency of occurrence, and environmental concentrations in an effort to simplify risk assessment [8, 38]. Multiple lines of evidence suggest that PFOS and PFHxS are the most abundant at AFFF sites [8, 9], more bioaccumulative, and putative endocrine disruptors. Currently, ecological risk assessment models for PFAS mixtures are being developed, and the impacts of PFAS on aquatic populations and communities, at or near AFFF sites, are being studied. Using environmentally relevant concentrations that mirror occurrence at AFFF sites, we asked two questions: (1) Would a mixture of the two most abundant PFAS (PFOS and PFHxS) affect the growth (length, mass, developmental stage) and body condition (SMI) of northern leopard frogs? (2) Would the interaction between PFOS and PFHxS, a long-chain and short-chain sulfonate, conform to an additive response? Our endpoints included: snout-vent length (SVL), body mass, developmental stage, scaled mass index (SMI), time to stage, and sex.

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CHAPTER 2. EFFECTS OF AN ENVIRONEMNTALLY RELEVANT MIXTURE OF PFOS AND PFHXS ON NORTHERN LEOPARD FROGS

2.1 Abstract

Northern leopard frog tadpoles were exposed to perfluorooctanesulfonic acid (PFOS) and perfluorohexanesulphonic acid (PFHxS); two common PFAS at AFFF impacted sites. Using a randomized block design, Gosner stage 25 (GS 25) northern leopard frog larvae were exposed through tail absorption (GS 46) to 0.5 and 1 ppb PFOS and PFHxS alone, plus a mixture comprised of 0.5 ppb PFOS and 0.5 ppb PFHxS. Our results demonstrated that after 31 days of exposure, the tadpoles exposed to PFHxS were significantly larger than those exposed to 0.5 ppb PFHxS. Significant differences in the scaled mass index (SMI) were also detected between the control and all other treatments. A single sample t-test was used to evaluate response additivity at day 31. Our results indicated an antagonistic response when tadpoles were co-exposed to PFOS and PFHxS. On the other hand, organisms sampled at GS 42 and 46 showed no significant differences in snout-vent length, body mass, SMI, or time to stage. In addition, there were no differences in responses between males and females. Different effects at different developmental periods are of concern, and indicate that PFAS studies need to incorporate more developmental endpoints in an effort to capture transient toxicity. Ecologically, effects on SMI by PFAS in the larval stages suggests potential alterations to amphibian life history traits, and a risk to amphibian populations at or near AFFF sites.

2.2 Introduction

Poly and perfluoroalkyl substances (PFAS) are found as mixtures near the surface waters of aqueous film forming foam (AFFF) sites such as airports, petrochemical facilities, and military bases. Amphibian populations near sites with a history of PFAS application are at risk of exposure, due to the persistent, bioaccumulative, and toxic nature of some PFAS. Perfluorooctane sulfonate (PFOS) and perfluorohexane sulfonate (PFHxS) dominate occurrence in surface waters at AFFF-impacted sites [1]. Both are ubiquitous and co-occur at high concentrations [2]. For example, in surface waters of assumed medium-volume release sites (hangers and buildings), PFHxS ranged between 0.36 to 2700 µg/L, while PFOS ranged between 0.39 and 190,000 µg/L [2]. On the other

hand, in assumed high-volume release sites (testing and maintenance), PFHxS ranged between 4.4 and 6.7 µg/L, while PFOS ranged between 34 and 42 µg/L [2].

PFOS and PFHxS are amphiphilic synthetic organic compounds with polar heads and are characterized as long and short chained PFAS, respectively. Perfluorinated sulfonates are of environmental concern because they are more bioaccumulative [3 – 5] and more toxic [6 – 7] than similarly chained perfluorinated carboxylates. Effects attributed to PFOS exposure are well documented in many *in vivo* and *in vitro* studies with laboratory animals and human systems [8]. Adverse effects include: thyroid disruption, hepatotoxicity, immunotoxicity, neurotoxicity, and cardiovascular, reproductive, pulmonary, and renal toxicity [9 – 14]. Similar adverse effects have also been reported for PFHxS in both *in vivo* and *in vitro* studies with animals and human systems [15].

Amphibians at AFFF sites are likely exposed to a mixture of PFAS that is primarily composed of PFOS and PFHxS [1]. Acute and chronic PFAS toxicity studies on amphibians show effects to body mass, snout-vent length (SVL), and time to developmental stage [16 – 18]. Sublethal effects at environmentally relevant concentrations have been reported [18] and suggest further studies on the impact of PFAS mixtures. Amphibians are an ideal model system to evaluate PFAS mixture toxicity because of their biphasic lifestyle (i.e., an aquatic and terrestrial life-history) and permeable skin. Amphibians that inherently spend a longer time in the larval stages, like that of the northern leopard frog, are of particular interest, because prolonged exposure to PFAS in the sensitive stages of development, could alter later developmental stages as the organism transitions into the terrestrial environment. To our knowledge, no study has attempted to characterize mixture toxicity for amphibians from the larval stages through post-metamorphic development, using a mixture that mirrors environmentally relevant concentrations of the two most common PFAS at AFFF sites, PFOS and PFHxS [1].

In an effort to establish a toxicity reference value (TRV) for PFOS and PFHxS on amphibians, our objective was to evaluate the sublethal mixture effect of PFOS and PFHxS on the growth and body condition of the northern leopard frog. We estimated 0.5 ppb concentrations for each of the chemicals as the lowest concentration to be tested based on literature [2].

We hypothesized that the toxicity of single PFAS would differ from a mixture, and that the total chemical load would be driving the response. In our prediction, higher PFAS loads would result in higher toxicity that conforms to response additivity in a mixture. For risk assessment

purposes, understanding the partial-life cycle toxicity of amphibians at AFFF was important, as metamorphosis is critical for the survival of a species.

2.3 Methods

2.3.1 Test chemicals and stock preparation

Stocks were prepared on April 4, 2019 using technical grade PFOS and PFHxS. Stocks were prepared by adding 500 and 1000 mg PFOS and PFHxS, respectively, to 1 L Ultrapure water (MilliQ) in polypropylene bottles and were stirred for 24 hours prior to use. A second stock was prepared for each chemical by diluting each stock above 100 times with MilliQ water. The final concentration was estimated to be 5 mg/L PFOS and 10 mg/L PFHxS.

2.3.2 Test organisms

We collected four northern leopard frog egg masses from an ephemeral pond at the Purdue Wildlife Area. We transferred egg masses to separate 200-L outdoor tanks containing 150 L aged well water and covered with 70% shade cloth until larvae reached GS 25 [19]. Hatchlings were fed 5 pellets of rabbit chow daily, and water changes were performed periodically.

2.3.3 Test setup

Once reared to the free-swimming stage, hatchlings were randomly collected with mesh nets from their holding tanks and transferred to buckets with ~ 20 to 25cm aged well water. Hatchlings from the four egg masses were combined to ensure a diverse genetic pool. Upon collection, hatchlings were transported to the main laboratory for sorting. Our inclusion criteria at sorting were defined as those tadpoles that had no visible irregularities in morphology, coloration, or behavior, and were between Gosner 25 to 28.

After pooling egg masses, hatchlings were gently poured into sorting trays and twenty tadpoles were haphazardly assigned to each experimental unit. Tadpoles were allowed 30 minutes to acclimate to room temperature before adding to the experimental units. The stocking number per aquaria was confirmed immediately, and dead or injured individuals were replaced. Ten individuals were euthanized as initial samples in buffered tricaine methanesulfonate (MS-222) for initial phenotypic measurements: SVL, mass, developmental stage, and body burden analysis

(Table S.1). Pooled samples (5 per tube) were stored in 1.5 mL polypropylene tubes at -20°C. Twenty-four hours later, 10 mL water samples were taken from each experimental unit and stored in 15 mL polypropylene tubes at 4°C. Samples were extracted from two separate areas within the aquaria in 5 mL portions.

The tadpoles were subject to an acclimation period of three days under laboratory conditions. The average room temperature and humidity were 21.8 ± 0.6 °C and 50.9 ± 9.7 % relative humidity, respectively. Animals were fed on the first day and no water changes occurred during the acclimation period.

2.3.4 Experimental design and approach

The study was conducted from May 10, 2019 to September 2, 2019 at the Purdue Wildlife Area facility. Our exposure treatments consisted of a control, 0.5 and 1 ppb PFOS, 0.5 and 1 ppb PFHxS, and a mixture containing 0.5 ppb PFOS + 0.5 ppb PFHxS. Using a randomized block design, exposures were conducted on a rack system, whereby each shelf on the rack contained one replicate from each treatment for a total of 24 experimental units, plus 4 feeding controls, which were used to calibrate feeding rates throughout the study. Experimental units or testing chambers consisted of 15 L precleaned polypropylene plastic aquaria, filled with 7.5 L aged filtered well water.

2.3.4.1 Experimental procedure

Using the diluted stocks, each experimental unit was dosed with the appropriate chemical volume after acclimation (PFOS 0.5 ppb = 750 µL, PFOS 1 ppb = 1500 µL, PFHxS 0.5 ppb = 375 µL, and PFHxS 1 ppb = 750 µL). The mixture was spiked with 750 µL PFOS and 375 µL PFHxS. The units were then stirred and allowed a minimum of 2 hours to attain equilibrium. The day of first dosing was defined as experimental day 0, and on this day, 10 mL water samples were extracted and stored as described above. Mortality and organism health was monitored daily. Dead organisms were removed from the units and stored separately in 1.5- or 5 mL polypropylene tubes at -20°C. Room temperature and humidity were also monitored daily and water quality parameters (temperature, DO, pH, conductivity, ammonia, and total ammonia nitrogen) were measured periodically throughout the study. Average measurements are provided in Table S.2.

The food controls were handled once weekly and used to establish the feeding regimes. All tadpoles in each food control aquarium were captured with a mesh net, blotted dry on paper towels, and weighed on an analytical balance with a tared cup of aquarium water. The daily food ration was calculated and defined as 10% of the average individual animal mass from all 4 aquaria. Considering that the feeding schedule was once every other day, the daily ration was multiplied by the number of surviving animals in each experimental unit to obtain the appropriate amount of ground Tetramin flakes that was to be added to each unit on feeding days. The feeding regime and schedule was adjusted as needed to reduce biological loading and associated effects on water quality.

Water changes were conducted once every four days initially, but on experimental day 25, it was adjusted to once every other day due to DO issues. A third set of water samples (one 10 mL and two 50 mL volumes in polypropylene tubes) was collected just prior to the first water change using the same methods aforementioned. Old and new water samples were collected once every two weeks thereon. To perform water changes, all tadpoles in each experimental unit were gently captured with a mesh net and temporarily transferred into a 2 L container with aquarium water. Separate nets were used for each treatment to eliminate cross-contamination. For each unit, the old exposure media was dumped, wiped with paper towels to remove excess debris, rinsed, filled with 7.5 L aged well water, spiked with its respective PFAS, and then mixed well before the organisms were moved back to their respective units. Tanks holding the aged well water were replenished to facilitate equilibrium of dissolved gases and temperature.

2.3.4.2 Animal sampling

Test organisms were sampled at three periods during the study: day 31, metamorphosis (GS 42) and tail resorption (GS 46). Our first sampling event was triggered when at least 50% of the animals in 50% of the food controls had developed beyond stage 29. The food controls were monitored weekly until criteria were met. On day 31, five tadpoles were haphazardly selected from each experimental unit and consolidated in a treatment-specific 1 L plastic container. The tadpoles were euthanized in buffered MS-222, rinsed in well water, and gently blotted on paper towels to remove excess water. Immediately following this procedure, the organisms were weighed, measured, and staged to examine phenotypic endpoints. Of the five, two organisms were

designated for body burden analysis and three were designated for potential RNA isolation and molecular work.

The animals designated for potential RNA isolation were processed first and immediately after euthanasia. Each tadpole was dissected with a dissecting scissor and placed in a 15 mL falcon tube containing RNA stabilization solution at a tissue to volume ratio of 1 gram tissue:10 mL RNALater. Separate dissecting equipment were used for each treatment to eliminate cross-contamination. The RNALater was allowed to perfuse for at least 24 hours at room temperature and then stored at -20°C for future analysis. On the other hand, animals designated for body burden were stored in 5 mL polypropylene tubes and stored at -20°C until transfer to the analytical lab. Due to density effects, all experimental units were subject to density reduction on day 36. The extracted individuals were randomly removed from each unit, euthanized, pooled, and stored in a 15 mL falcon tube at -20°C. Each unit housed 12 organisms after density reduction.

Animals were sampled daily upon reaching target stages 42 and 46. Euthanasia, rinsing, sample storage and processing rotations were the same as the first sampling event. The processing rotations for our phenotypic data (SVL, mass, and stage) were also the same for each experimental unit. The first two metamorphs that emerged from each experimental unit were processed for potential RNA isolation as described previously. At this time, we also excised the tails from these individuals, flash froze them in liquid nitrogen, and stored at -80°C for tentative thyroid hormone extraction. Only samples for molecular work were collected at GS 42, but samples for body burden and molecular work were collected at GS 46.

Once sample sizes for each experimental unit were met at Gosner 42, subsequent metamorphs were reared through tail absorption. Metamorphs that were destined for tail absorption were sampled at Gosner 42 first, assigned a temporary ID, and then transferred to an individual deli container with ~ 1cm treatment water. The deli containers were made from polypropylene material and measured 4.5 inches in diameter and 3 inches in height. The containers were placed at an angle with the use of a plastic screw cap, providing a dry area for emergence and a wet area for rehydration. No water changes occurred during this time, which resulted in ammonia buildup within the first 48 hrs. On experimental day 88, the exposure water from two containers with dead individuals were measured with 0.35 mg/L unionized NH_3. Two other containers, each from the previous days (experimental day 86 and 87) also measured 0.35 and 0.18 mg/L unionized NH_3. To

prevent the ammonia from reaching a toxic level of 1.5 mg/L [20] over several days, we reverted to daily water changes thereafter.

The study concluded after we observed that most tadpoles remaining were not going to metamorphosize because of their size. The study concluded on experimental day 116. Euthanasia, rinsing, sample storage, and processing procedures were the same as with the previous sampling events. Each animal was preserved in a 15 mL falcon tube for potential body burden analysis at -20°C.

2.3.5 Chemical analyses

PFAS loads in water and tissues were measured using previous validated methods. A detailed description of the methods can be found in the studies by Flynn et al. [16, 21 – 22] and Hoover et al. [18].

2.3.6 Statistical analyses

All statistical analyses were performed in R version 3.6.1 [23]. To assess differences in water concentrations, body accumulation, phenotypic endpoints, and body condition, we performed one-way parametric analyses (ANOVA). Interactions, covariates (developmental stage and time to stage), block, dependent (SVL, mass, stage, SMI) and independent (chemical treatment) variables were included in all univariate models, but the block, interactions and covariates were dropped from the analysis when they were not significant. A complete list of the reduced statistical models performed are presented in Table S.3 to S.8. Assumptions of normality and equal variance were verified through diagnostic tests such as Shapiro-Wilk and Bartlett's test, respectively. A Box-Cox test was performed on the continuous variables to determine the appropriate transformation that would improve model fit. Outliers were examined by visualizing Cook's distance and influence plots. Significant differences among treatments were resolved by the use of a post-hoc Tukey test.

PFOS and PFHxS concentrations in water were analyzed separately by sampling time. Sample sizes were sufficient for days 1 (acclimation), 3 (initiation), and 115 (termination), but not for day 15. A significant portion of our water chemistry data violated ANOVA assumptions of normality and homomgenous variance. However, model results are presented with those violations

since ANOVA are robust to deviations from normality. Body burden data was only evaluated for organisms sampled at day 31.

To perform our analyses on the phenotypic endpoints, we subsetted the data according to the three sampling points (day 31, GS 42 and 46). Given that some animals were sexed, the GS 42 data was further divided into all individuals vs. sexed individuals to determine sex-specific effects. For these analyses, when the results with and without a transformation were quantitatively similar via a Tukey test, the raw data was visualized.

The scaled mass index (SMI) was calculated using the methods published by Peig and Green [26]. The SMI is a reliable indicator of condition in amphibians and is ideal because it reduces bias in estimates, compared to other body condition indices [27, 28]. After the removal of an outlier from each data set (Day 31, GS 42 and 46), a standardized major axis (SMA) regression was performed on a bivariate plot of ln mass versus ln SVL to obtain the scaling factor (b_{sma}). The control organisms from each data set were used to calculate the scaling factor to ensure that scaling relationships were not influenced by treatment. However, to make sure that the scaling factor was not biased, a second scaling factor was obtained, which included all individuals in each data set (i.e., including the controls). The results from both scaling factors were quantitatively similar in all cases, confirming that there was no bias in our rationale.

2.4 Results

2.4.1 Water chemistry

2.4.1.1 Measured PFAS in water

During acclimation (24 hrs. after) the concentrations of PFOS and PFHxS in the experimental units were negligible. A spiking error occurred at initiation, where all PFAS treatments were spiked with either 0.5 ppb PFOS or PFHxS (Fig. 2.1-B, both PFAS), respectively. This technical error was corrected at the second water change four days later. At termination, PFAS loads were near nominal, and significant differences between the 0.5 and 1 ppb treatments were detected (Fig. 2.1-C, both PFAS). In the mixture, the PFOS load was not significantly different relative to the 0.5 ppb PFOS treatment, but the PFHxS load was significantly higher in comparison to its 0.5 ppb PFHxS pair (Fig. 2.1-C, both PFAS).

2.4.2 Body burden at day 31

In general, tissue burdens were higher when the load of the PFAS was higher, and in the mixture, tissue burdens were intermediate to the 1 ppb pairs (Fig 2.2 – A &B). Also, PFOS was the only one that bioaccumulated. For example, tadpoles exposed to the mixture accumulated similar burdens of PFOS, like the tadpoles exposed to 0.5 ppb PFOS (Fig 2.2 – B) (P = 0.21). An antagonistic response between PFOS and PFHxS is reported below.

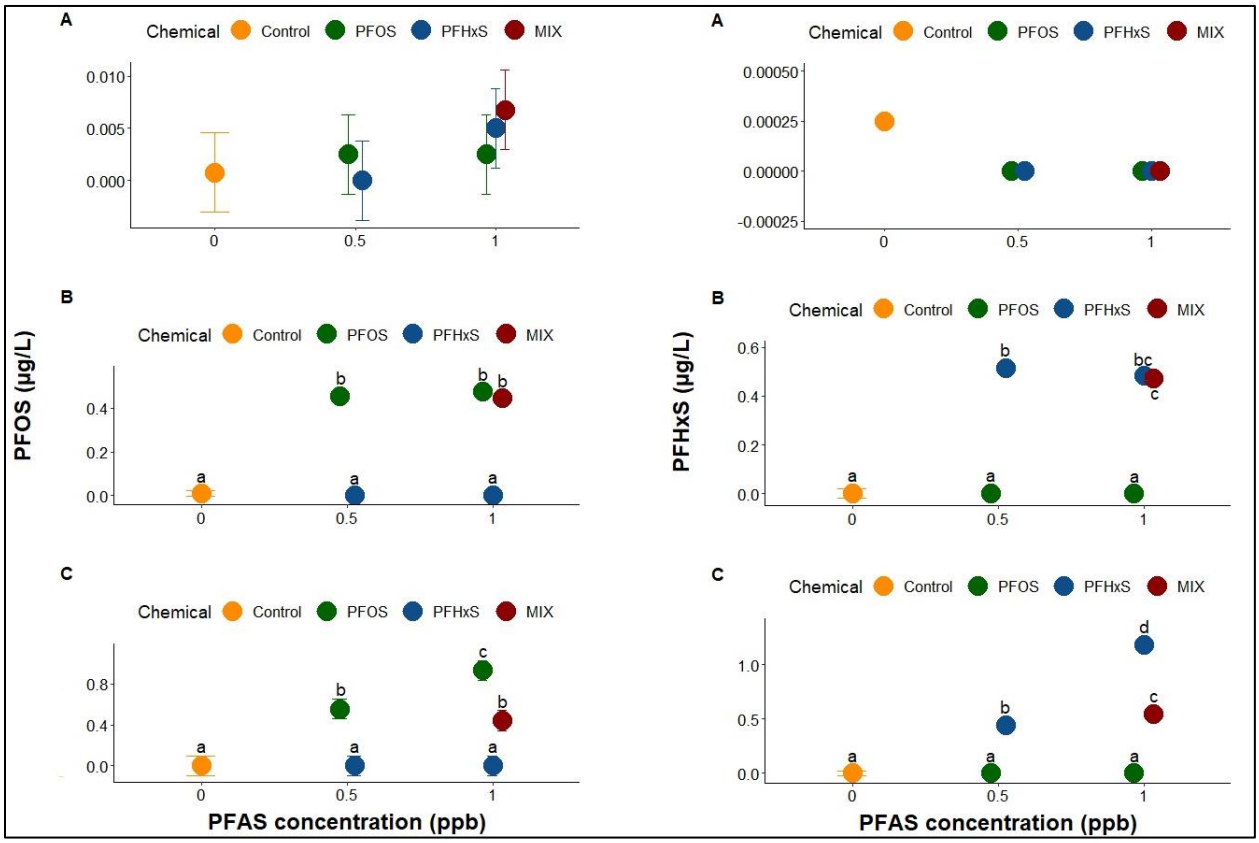

Figure 2.1. Measured PFOS and PFHxS in water throughout the study. (A) PFOS and PFHxS measured during acclimation (24 hrs. later). (B) PFOS and PFHxS measured at initiation. (C) PFOS and PFHxS measured at termination. Error bars represent 95% confidence intervals. No confidence intervals are provided for figure A because only one control replicate measured 0.001 ppb, while the remaining 23 bins measured 0.000 ppb. Treatments with different letters are statistically significant (P < 0.05).

No significant differences in PFHxS accumulation between the control and the mixture (Fig. 2.2 – A), or PFOS 1 ppb and the mixture (Fig. 2.2 – A), suggests a dietary source of PFAS exposure, which is discussed below.

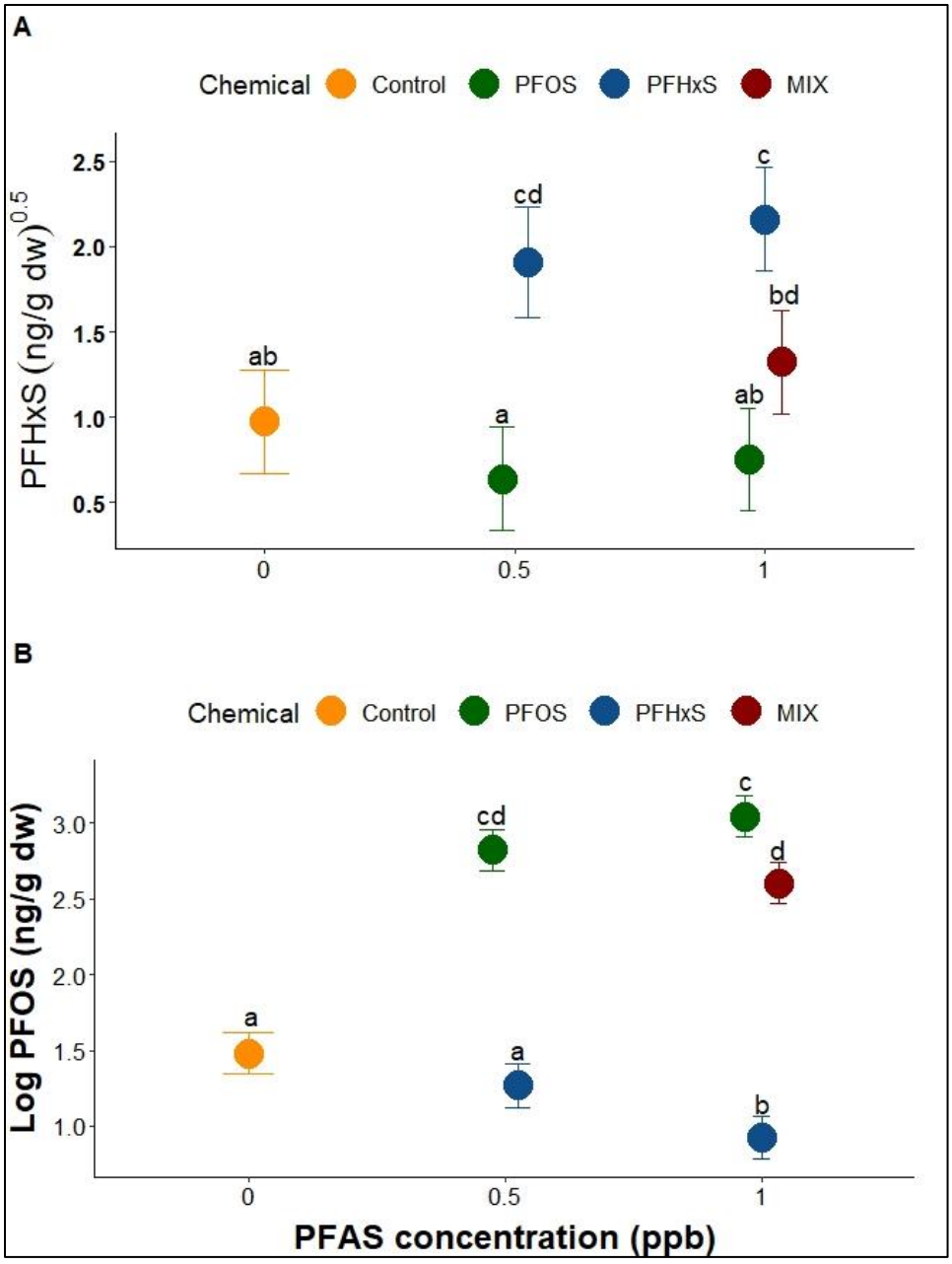

Figure 2.2. Measured PFAS body burdens in *Lithobates pipiens* at 31 days of exposure. (A) PFHxS body burdens. (B) PFOS body burdens. Error bars represent 95% confidence intervals. Treatments with different letters are statistically significant ($P < 0.05$).

2.4.3 Survival at termination

Models to assess differences among the treatments included survival as the response variable and chemical concentration as the independent variable. Average treatment survival ranged between 79.4 and 86.8 % and was not affected by treatment.

2.4.4 Phenotypic effects at day 31, GS 42, and 46

2.4.4.1 Snout-vent length

To evaluate differences in growth in all sampling points, we designed models that included SVL as a response variable, chemical concentrations as the independent factor, and developmental stage (day 31), or time to stage (GS 42) as covariates. There was a significant increase in SVL between the 0.5 ppb PFHxS treatment and the control at day 31 (Fig. 2.3) (Tukey test, $P = 0.00029$). The effect plot also demonstrates that the organisms exposed to 0.5 ppb PFOS were significantly different than all treatments containing PFHxS, including the mixture (Fig. 2.3) ($P < 0.05$). Time to stage also significantly lowered SVL at metamorphosis ($P < 0.001$), but there were no significant differences in SVL among the treatments for GS 42 or 46 (Table S.5). Sex was added as a covariate to some models at GS 42 and 46, but were eliminated because they were not significant (data not shown).

2.4.4.2 Body mass

Mass was assessed with models that included developmental stage (day 31), or time to stage (GS 42) as covariates, mass as a dependent variable, and chemical concentration as the independent variable (Table S.6). At day 31, developmental stage significantly affected mass ($P < 0.001$), but the only differences were between the tadpoles exposed to 0.5 ppb PFOS and those exposed to PFHxS (0.5 and 1 ppb) and the mixture (Tukey test, $P = 0.0001$, $P = 0.007$, and $P = 0.024$, respectively) (Fig. 2.4). At metamorphosis (GS 42), time to stage was negatively associated with mass ($P < 0.001$), but no significant differences were detected among the groups (Tukey test, $P > 0.05$, all comparisons) (data not shown). A similar trend was obtained at tail resorption, but the effect was not significant. With regards to sex-related effects, no significant differences among the PFAS groups or between the sexes were seen.

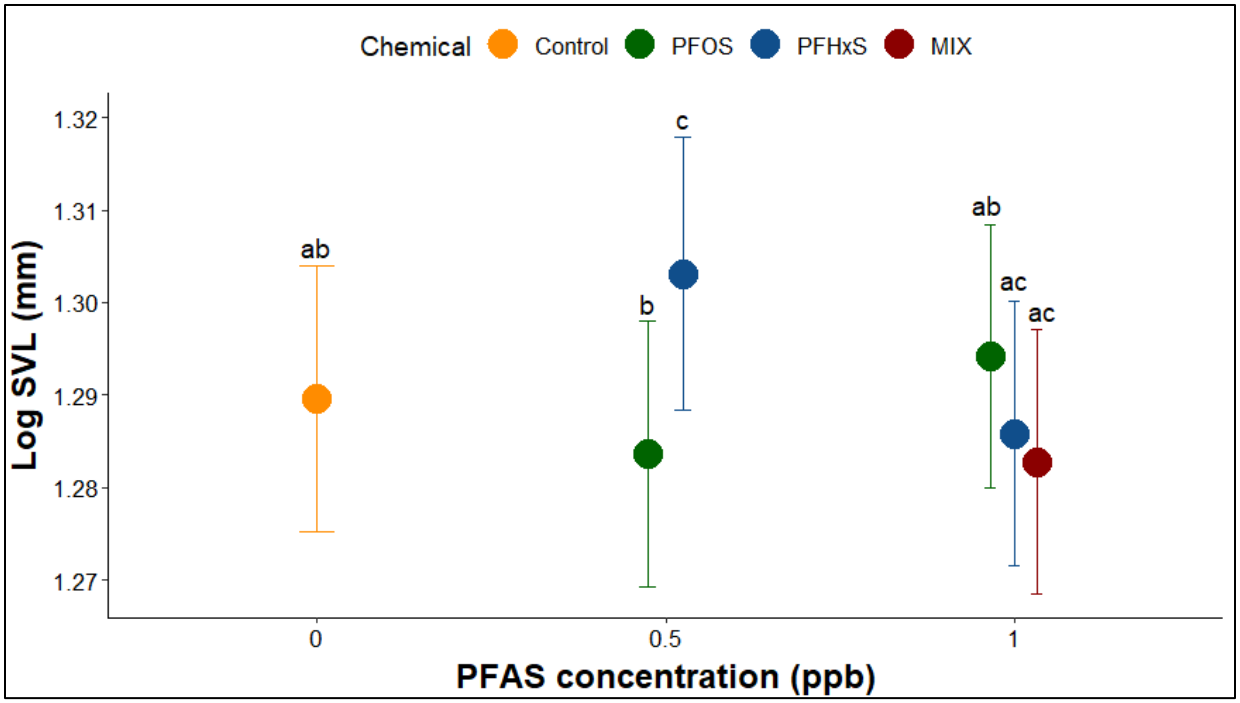

Figure 2.3. Chronic effects of PFAS on the snout-vent length of *Lithobates pipiens* at 31 days of exposure. Error bars represent 95% confidence intervals. Treatments with a different letter are statistically significant ($P < 0.05$).

2.4.4.3 Developmental stage and time to stage

To assess treatment differences in developmental stage (day 31) and time to stage (GS 42 and 46), all GLMs included that variable as the response, while chemical concentration was considered the independent variable. No treatment effects were found (Table S.7).

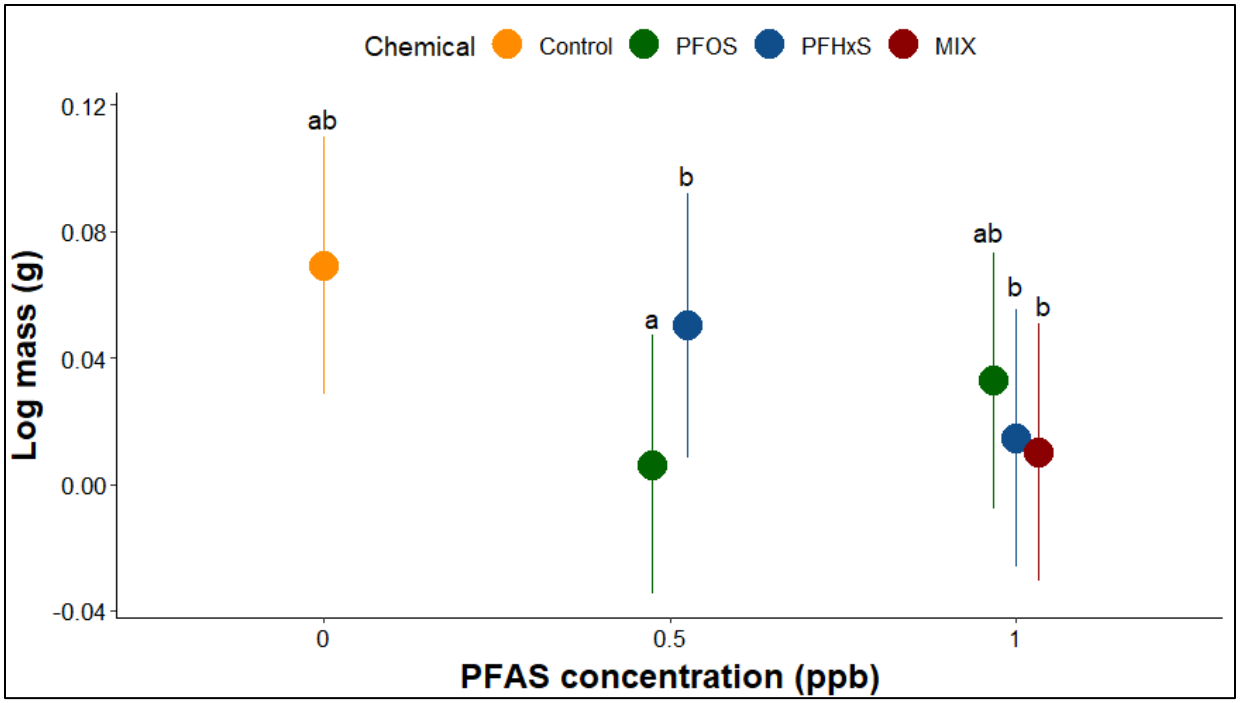

Figure 2.4. Chronic effects of PFAS on the body mass of *Lithobates pipiens* at 31 days of exposure. Error bars represent 95% confidence intervals. Treatments with a different letter are statistically significant ($P < 0.05$).

2.4.4.5 Scaled mass index

Body condition was assessed by GLMs that considered SMI as the response variable and chemical concentration as the independent variable. All PFAS treatments (PFOS 0.5 ppb, PFOS 1 ppb, PFHxS 0.5 ppb, PFHxS 1 ppb, and the MIX) affected the scaled mass index relative to the control at day 31 (Fig. 2.5). A Tukey comparison showed the following significant reductions ($P = 0.014$, 0.0033, 0.00015, 0.0041, and 0.013, respectively). No differences in body condition were detected at GS 42 or 46 (Table S.8).

2.4.5 Testing response additivity at day 31

To evaluate the assumption of response additivity for PFAS alone and their mixture, a single sample t-test was used on the SMI data. The method was adopted from Billet et al. [29]. Relative to the control, reductions in SMI were calculated for each PFAS alone treatment (PFOS 0.5 ppb and PFHxS 0.5 ppb), and those differences were summed to derive the expected response difference under additivity. The observed response difference in SMI was the reduction observed in the mixture, relative to the control. In calculating the t-statistic, the expected response difference

was the population mean ($\mu = 0.2923$) and the observed response difference was the sample mean ($\bar{x} = 0.1225$). With a standard deviation (s) of 0.0958 and sample size (n) of 4, our results indicate significant departure from additivity ($p < 0.05$). Our SVL data (Fig. 2.3) indicate that there is an antagonistic response.

2.5 Discussion

2.5.1 Phenotypic effects

Recently, East and colleagues [1] reported that PFHxS occurs as frequently as PFOS and often approaches high concentrations, similar to PFOS. Average concentrations of PFOS and PFHxS at 256 AFFF sites were 0.25 and 0.23 µg/L, respectively [1]. This data supports that our estimates for PFOS and PFHxS are slightly higher, but representative of an AFFF site.

In this study, we exposed northern leopard frogs to 0.5 and 1 ppb PFOS or PFHxS, alone or in a mixture (0.5 ppb PFOS + 0.5 ppb PFHxS), from the larval stages (GS 25) through tail absorption (GS 46). We hypothesized that the toxicity between mixture and single PFAS would produce different responses in amphibians, and that the load would be driving the response. In our prediction, higher PFAS loads would result in higher toxicity that conforms to response additivity in a mixture. Our results do not support our hypothesis, because the load was not driving the response, and the toxicity in the mixture did not conform to response additivity. For example, tadpole effects on SMI in all PFAS groups at day 31 (Fig. 2.5) show that the load was not driving this response. Univariate analyses also showed that the lower load of PFHxS (0.5 ppb) significantly increased tadpoles' growth (measured as SVL) at day 31, as opposed to the control animals (Fig. 2.3).

After exposing *X. laevis* tadpoles to PFOS from Nieuwkoop and Faber (NF) stages 46/47 to 62 [30] (i.e., similar from GS 25 to GS 42 on the Gosner scale in this study), Cheng and colleagues [31], reported no effects on the time to metamorphosis, even though PFOS exposure upregulated thyroid related genes: thyroid receptor beta A (TRβA), type II deiodinase, and basic transcription element-binding protein (BTEB). The authors only described an increasing trend in the time to metamorphosis as PFOS concentrations increased [31]. The results of this study compliment that trend, and also report no effects on sex, time to metamorphosis, SVL, mass, time to tail resorption, or SMI in the later stages (GS 42 and 46) by PFOS or PFHxS, and their mixture.

Like the study by Cheng et al. [31], Hoover and colleagues [18] also found an increasing trend in time to metamorphosis of *L. pipiens* exposed to either PFOS or PFHxS, with a lowest observed effect concentration (LOEC) of 10 µg/L for PFHxS and of 100 µg/L for PFOS. Previously, developmental delays had only been reported at higher doses for PFOS (3000 µg/L) in *L. pipiens* [17]. In univariate space, PFHxS had a LOEC of 0.5 ppb in SVL and SMI, while PFOS had a LOEC in SMI at day 31.

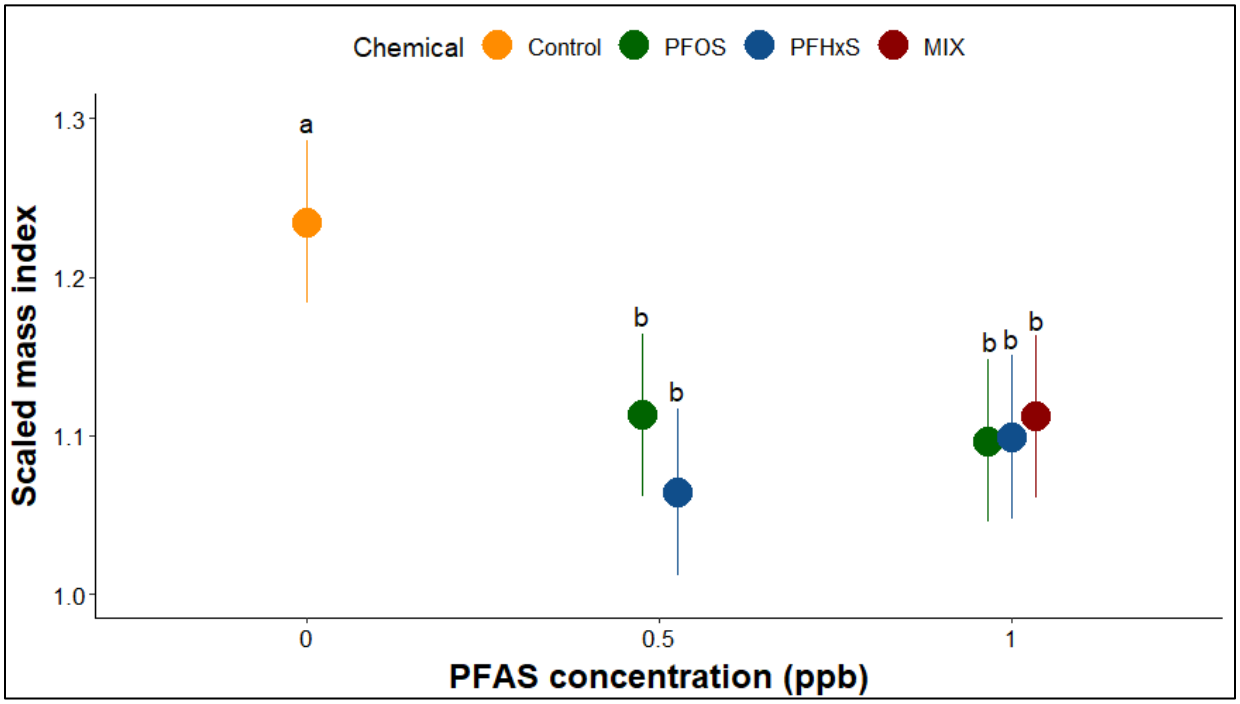

Figure 2.5. Chronic effects of PFAS on the scaled mass index of Lithobates pipiens at 31 days of exposure. Error bars represent 95% confidence intervals. Treatments with a different letter are statistically significant ($P < 0.05$).

Relative to the control, PFOS alone did not induce adverse phenotypic effects at any timepoint throughout this study, although it is known to be bioaccumulative and toxic. Consistent with a partial life-cycle study, *L. pipiens* appeared insensitive to PFOS with regards to bioconcentration potential and direct toxicity [17]. Likewise, *X. laevis* also appeared phenotypically insensitive to PFOS with regards to survival and time to stage [31]. Importantly, the effects of PFOS on amphibians are not consistent, as effects on time to stage have been reported for *L. pipiens* [18]. Thus, it appears that the differing effects on amphibians could be due to differences in dose, length and route of exposure, and response measured. PFHxS on the other

hand, was toxic at environmentally relevant concentrations in this study and in Hoover's [18], making it a PFAS of concern.

Although PFOS bioaccumulates, it has a lower serum half-life than PFHxS [32]. As a result, tissues exposed to PFHxS for prolonged periods of time may display more pathological effects. In our study, PFHxS proved to be more toxic by inducing increased SVL in the 0.5 ppb treatment at day 31 when developmental stage was considered as a covariate (Fig. 2.3). Our body burden data shows that organisms exposed to PFHxS alone had higher accumulation of PFHxS relative to those exposed to PFOS alone or the controls (Fig. 2.2). The mixture however, only contained higher accumulation of PFHxS relative to 0.5 ppb PFOS and not the control or 0.5 ppb PFHxS (Fig. 2.2). These results suggest that PFHxS accumulation was coming from another source and not the water or the organisms, because the control was contaminated. Analyses on the food showed that both Tetramin fish flakes and rabbit chow had higher levels of PFHxS compared to PFOS. The influence of this exposure on the results of this study are unknown at this time.

Effects to single PFAS exposures at environmentally relevant concentrations are concerning, and support the need for further inquiry into environmentally relevant PFAS mixture exposures. Currently, several studies have attempted to model PFAS mixture toxicity, but the results are highly inconsistent and are affected by dose, developmental stage, test duration, test model, and endpoints considered [33, 34]. Since PFHxS is more toxic to the liver, and binds with a higher affinity to transthyretin [35], it appears that both chemicals have distinct MOAs, and may bind with different affinities to substrates (proteins, enzymes, cofactors, receptors) and ligands, altering different molecular initiating events.

2.5.2 Antagonistic interaction in the mixture

Our results indicate that the mixture toxicity between PFOS and PFHxS was antagonistic. The SVL of tadpoles exposed to 0.5 ppb PFHxS was larger compared to the controls and animals exposed to 0.5 ppb PFOS (Fig. 2.3). A significant difference was also detected between the mixture and 0.5 ppb PFOS (Fig. 2.3). This type of interaction between PFHxS and PFOS can best be described as antagonistic, because the effects observed in PFHxS 0.5 ppb were reduced when PFHxS was co-administered with an equal amount of PFOS. This pattern is consistent with an interactive toxicity between PFOS and PFHxS at the site of uptake, or inside the organism, or both. Consistent with the literature, the study by Carr et al. [36] found antagonistic responses between

several binary mixtures, including PFOS + PFHxS. However, the authors considered a conservative approach and adopted an assumption of additivity with a potential for antagonistic interactions. With regards to mass, the tadpoles exposed to 0.5 ppb PFOS were significantly smaller than the organisms exposed to PFHxS (0.5 and 1 ppb) and the mixture. It appears that there is potential for an antagonistic interaction in mass, but it was inconclusive because no treatment was significantly affected relative to the control (Fig 2.4). No effects were observed in the later stages either.

The mixture was not toxic to northern leopard frog larvae at day 31, GS 42, or GS 46, but PFHxS alone was, at the earliest timepoint. Like the studies by Menger and colleagues [37] and Kar et al. [38], single PFAS have showed higher toxicity than a mixture. When both PFOS and PFHxS were combined, a reduction in toxicity was observed at day 31. An antagonistic interaction suggests that PFOS and PFHxS induce toxicity through different modes of action (MOAs), and may likely interact with similar ligands during development. In *C. virginianus*, PFOS and PFHxS showed interactive effects with avian receptors, that likely was a result of different MOAs depending on dose and co-occurrence [39]. On the contrary, Goodrum et al. [40], indicated a similar MOA for PFOS and PFHxS because of their ability to activate the peroxisome proliferator-activated receptor alpha (PPARα). The activation of PPARα is consistent with PFAS toxicity, and is of interest, because PFHxS has been recognized as being more toxic to the liver than PFOS [41], which may explain our results as discussed below.

The PPARα signaling pathway is a complex cellular response that requires activation by a ligand (a fat-soluble molecule), which regulates lipid transport and synthesis, fatty acid β-oxidation, and the transcription level of liver-lipid metabolizing genes [42]. Target organs for PPARα signaling are heart, kidney and liver adipose tissue, because it is expressed in tissues that metabolize fatty acids rapidly [42]. Thus, activation of PPARα generally results in a reduction of triglyceride concentrations, which alters the homeostatic control of energy balance [43]. In evaluating the toxicity of PFHxS on mice, Das et al. [44] and Bijland et al. [45], found effects to cholesterol metabolism, which resulted in increased lipase activity, followed by reductions in fatty acids, glycerol, lipoproteins and triglycerides. Significant reductions in male serum cholesterol and triglycerides were also reported, along with male-specific pathologically enlarged centrilobular hepatocytes. With regards to the PPARα-receptor, Wolf and colleagues [46], found that PFHxS increased the activity of the mouse PPARα-receptor in vitro. PFHxS, as

a short-chain PFSA, could potentially be more toxic depending on several factors, which include: test model, developmental stage, dose, endpoint and time of exposure (33, 34 & 40).

Using quantitative structure-toxicity relationship (QSTR) models, Kar et al. [38] showed that PFAS mixtures interacted in a manner consistent with concentration addition, suggesting a similar MOA. On the other hand, Good rum et al. [40], proposed multiple MOAs, because short- and long-chained PFAS sulfonates, carboxylates, and fluorotelomers were able to interact with nearly two dozen nuclear receptors. Our results were consistent with the latter, indicating that PFOS and PFHxS may interact with similar proteins, enzymes, or co-factors, with different affinities. For example, PFHxS's stronger affinity for transthyretin, competitively inhibits PFOS from binding to it [35], potentially increasing its toxicity. Ecologically, altered molecular signaling events induced by PFAS, because of interactions with nuclear receptors and transport molecules like transthyretin, suggest that these chemicals may pose a risk to wild populations of amphibians. Other studies need to evaluate transgenerational exposures, as well as fitness-related endpoints as animals' transition into the terrestrial environment.

2.5.3 Scale mass index as a measure of body condition

Body condition has been widely accepted as an important determinant of fitness [27], as it is closely related to an animal's health. The SMI is a preferred method for determining body condition, because it standardizes body mass at a fixed length, by using a scaling relationship between length and mass [27], which reduces bias in estimates. The authors reported that structural size was related to energy stores, because scaling relationships varied depending on variables of interest, or between species (potentially populations as well). Therefore, SMI also measures the variability in lipid stores as it relates to size.

At day 31, all tadpoles exposed to PFAS had significantly reduced SMI compared to controls (Fig. 2.5), but not at GS 42 or 46. Consistent with PPARα activation and lipid dysregulation, PFAS exposure may have affected the pathways related to lipid degradation in lipid rich tissues like the liver and fat bodies of amphibians. Dale et al. [47] found increased liver lipid degradation enzymes in Atlantic cod (*Gadus morhua*) after exposure to a PFAS mixture. PFAS exposure disrupted lipid homeostasis by upregulating enzymes involved in fatty acid degradation pathways, like fatty acid β-oxidation [47]. In a study with young adults, Chen et al. [48] concluded that increased fatty acid oxidation and lipolysis by PFAS, likely contributed to the impaired

responses observed in glucose metabolism. Thus, glucose may be another major energy molecule that PFAS may be altering as well. Hence, in northern leopard frogs, a reduction in SMI in the larval stages (day 31) could mean a reduction in fat stores, as lipid dysregulation is a MOA for PFAS.

No effects in SMI were observed at the later stages between control and PFAS exposed animals. An analysis on the food (n of 2) showed that Tetramin flakes contained 12.48 and 8.43 ppb PFHxS and 1.77 and 2.21 ppb PFOS. On 5/28/2019, eighteen days after initiation, Tetramin flakes were dropped as a food source; and rabbit chow was adopted for the remainder of the study. An analysis on rabbit chow (n of 2) showed that it contained 3.95 and 2.57 ppb PFHxS, and 0 ppb PFOS. Since PFAS alters lipid homeostasis, it is likely that PFAS in the food altered the fat stores of control frogs. Since PFHxS is more toxic to the liver and has a longer half-life, it is likely that the control frogs experienced prolonged exposure to PFHxS at the later stages, which may have reduced their fat store, rendering unhealthy individuals at the end of a lengthy exposure.

Effects to body condition at day 31 can affect tadpole fitness. Fitness describes an organism's ability to survive, find a mate, and reproduce, in an effort to pass on its genes to the next generation. All PFAS exposed tadpoles had significantly reduced SMI's in the larval stages, but not at the later stages (GS 42 and 46). In fact, no other endpoint (SVL, mass, time to stage, sex) was significantly different to the controls in the later stages. Prolonged exposure to PFHxS by diet may have influenced the results, however, because the controls did not perform consistently, it is unclear as to whether the effects were induced by PFAS, or the organism's health. Nonetheless, effects to SMI in the larval stages suggests PFAS acted early in development and merits further inquiry into effects at earlier developmental periods.

Reduced SMI in the larval stages also means reduced fitness, potentially compromising the ability for organisms to survive and reproduce in the adult stages. As an amphibian transitions into the terrestrial environment, PPAR activation is important to consider because it is involved in major diseases and cellular and biochemical processes, such as fertility, reproduction, neurodegenerative disorders, diabetes, cancer, lung disease, pain and obesity [43]. In a molecular sense, these include effects to peroxisome proliferation, fatty acid metabolism, lipid homeostasis, glucose homeostasis, and macrophage function. Likely impaired by a host of cellular processes, young developing amphibians with dysregulated lipid stores might be at risk of developing neurodevelopmental and metabolic disorders that may impair their fitness to reproduce, feed,

assimilate, fight off pathogens and disease, develop tolerance, locomote, escape predators, and ultimately survive. The fat bodies in females are especially important as well, because those rich lipid tissues are necessary for the production of offspring. With lower energy stores, a female impacted by PFAS may likely produce less offspring, since clutch size and body mass are highly correlated [49].

2.6 Limitation

PFAS containing food

The food sources we used in this study (Tetramin fish flakes and rabbit chow), contained both PFHxS and PFOS. Given that PFHxS has a longer half-life and is more toxic to the liver, it may have affected the condition of northern leopard frogs in the later stages. The constant supply of food with higher levels of PFHxS likely induced toxic effects.

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2.8 **Supplemental Information**

Table S.1. Phenotypic measurements for ten organisms at initiation.

Snout-vent length (mm)	Mass (g)	Stage
5.06	0.023	25
5.43	0.029	25
4.47	0.019	25
5.31	0.021	25
5.25	0.024	25
5.35	0.033	25
5.38	0.026	25
5.18	0.028	25
4.53	0.015	25
4.38	0.016	25

Table S.2. Average water quality parameters measured periodically throughout the study.

Statistic	Temp (°C)	DO (mg/L)	pH	Conductivity (µs/cm)	NO_3 (ppm)	NH_3 (mg/L)	NH_3-NH_4^+ (ppm)
Average	21.82	4.40	7.96	568.13	1.30	0.02	0.57
Std. Dev	0.65	1.68	0.14	44.46	0.02	0.02	0.39

Note: (i) NO_3 was only measured once, and (ii) ammonia concentrations did not get to toxic levels.

Table S.3. Statistical models performed for PFAS in water.

Mo. #	**Response variable (s)**	**Independent variable (s)**	**Sampling time**	**Covariate (s)**	**F-value**	**DF**	**P-value**
1	PFHxS in water	Chemical concentration	Acclimation (day 1)	-	1	5	0.45
2	PFHxS in water	Chemical concentration	Initiation (day 3)	-	778.7	5	< 0.001
3	PFHxS in water	Chemical concentration	Termination (day 115)	-	1911.3	5	< 0.001
4	PFOS in water	Chemical concentration	Acclimation (day 1)	-	2.0	5	0.13
5	PFOS in water	Chemical concentration	Initiation (day 3)	-	1119.9	5	< 0.001
6	PFOS in water	Chemical concentration	Termination (day 115)	-	69.1	5	<0.001

Note: Reduced models are presented, nonsignificant interactions, block, and covariates have been removed.

Table S.4. Statistical models performed for PFAS accumulation in tadpole body at day 31.

Mo. #	**Response variable (s)**	**Independent variable (s)**	**Sampling time**	**Covariate (s)**	**F-value**	**DF**	**P-value**
7	PFHxS accumulation	Chemical concentration	Day 31	-	16.9	5	< 0.001
8	PFOS accumulation	Chemical concentration	Day 31	-	177.3	5	< 0.001

Note: Reduced models are presented, nonsignificant interactions, block, and covariates have been removed.

Table S.5. Statistical models performed for PFAS effects on SVL in northern leopard frogs.

Mo. #	Response variable (s)	Independent variable (s)	Sampling time	Covariate (s)	F-value	DF	P-value
9	Snout-vent length	Chemical concentration	Day 31	Developmental stage	I = 1.1 C = 419	I = 5 C = 1	I = 0.35 C = < 0.001
10	Snout-vent length	Chemical concentration	GS 42	Time to stage	I = 1.0 C = 13	I = 5 C = 1	I = 0.44 C = < 0.001
11	Snout-vent length (Sexed individuals)	Chemical concentration	GS 42	-	1.2	5	0.34
12	Snout-vent length	Chemical concentration	GS 46	-	2.0	5	0.09

Note: Reduced models are presented, nonsignificant interactions, block, and covariates have been removed. *Two F-values, DFs, and P-values are reported for models that included a covariate (I = independent variable and C = covariate).*

Table S.6. Statistical models performed for PFAS effects on body mass in northern leopard frogs.

Mo. #	Response variable (s)	Independent variable (s)	Sampling time	Covariate (s)	F-value	DF	P-value
13	Body mass	Chemical concentration	Day 31	Developmental stage	I = 1.5 C = 381.6	I = 5 C = 1	I = 0.20 C = < 0.001
14	Body mass	Chemical concentration	GS 42	Time to stage	I = 0.7 C = 13.4	I = 5 C = 1	I = 0.61 C = < 0.001
15	Body mass (sexed individuals)	Chemical concentration	GS 42	-	1.2	5	0.32
16	Body mass	Chemical concentration	GS 46	-	1.5	5	0.21

Note: Reduced models are presented, nonsignificant interactions, block, and covariates have been removed. *Two F-values, DFs, and P-values are reported for models that included a covariate (I = independent variable and C = covariate).*

Table S.7. Statistical models performed for PFAS effects on Gosner stage and time to stage in northern leopard frogs.

Mo. #	Response variable (s)	Independent variable (s)	Sampling time	Covariate (s)	F-value	DF	P-value
17	Developmental stage	Chemical concentration	Day 31	-	1.4	5	0.21
18	Time to metamorphosis	Chemical concentration	GS 42	-	1.1	5	0.36
19	Time to metamorphosis (sexed individuals)	Chemical concentration	GS 42	-	0.9	5	0.48

20	Time to tail resorption	Chemical concentration	GS 46	-	0.9	5	0.47

Note: Reduced models are presented, nonsignificant interactions, block, and covariates have been removed. Two F-values, DFs, and P-values are reported for models that included a covariate (I = independent variable and C = covariate).

Table S.8. Statistical models performed for PFAS effects on survival and SMI in northern leopard frogs.

Mo. #	**Response variable (s)**	**Independent variable (s)**	**Sampling time**	**Covariate (s)**	**F-value**	**DF**	**P-value**
21	Survival	Chemical concentration	Termination	-	0.4	5	0.85
22	Scaled mass index	Chemical concentration	Day 31	-	5.1	5	< 0.001
23	Scaled mass index	Chemical concentration	GS 42	-	0.6	5	0.71
24	Scaled mass index	Chemical concentration	GS 46	-	0.7	5	0.62

Note: Reduced models are presented, nonsignificant interactions, block, and covariates have been removed. Two F-values, DFs, and P-values are reported for models that included a covariate (I = independent variable and C = covariate).

CHAPTER 3. FUTURE DIRECTIONS AND IMPLICATIONS

3.1 Future Directions

PFAS induced toxicity early in amphibian development makes it a potential toxicant to amphibian embryogenesis. Early developmental endpoints to consider in future PFAS exposures include: gamete viability, fertilization rates, and effects to earlier GS stages (GS 2 to 24). A suite of embryonic stages should be evaluated, and incorporate timepoints such cleavage, blastula formation, gastrulation, and organogenesis. Mixtures of different PFAS could prove more toxic to sensitive developmental periods. Furthermore, understanding organ toxicity at earlier developmental timepoints might be an approach to consider in other mixture toxicity studies, as PFAS tend to distribute differently among dissimilar tissues, likely due to differences in affinity to proteins, enzymes, cofactors, receptors, and ligands. This approach might be useful in the development of more complex predictive models needed for PFAS mixtures.

As an activator of PPAR, PFAS may pose a hazard to wild populations of amphibians. Effects to SMI by PFAS are pronounced in the larval stages, which implies effects to fat stores and energy balance. Other important life-cycle endpoints that merit scientific inquiry are those which consider fitness-related performance as amphibians' transition to the terrestrial environment, as well as long-term life-history traits. For example, maternal transfer experiments coupled with transgenerational epigenetic and transcriptomic probes might be useful in determining potential developmental or metabolic abnormalities associated with PPAR activation. More studies evaluating the effects associated with larval and post-metamorphic development are needed to develop a TRV that can be included in ecological risk assessments.

The non-monotonic responses observed with single PFAS exposures appear to be dependent on factors such as test model, dose, endpoint, developmental stage, and time of exposure. Simple PFAS mixtures also tend to interact differently depending on the same factors. Therefore, it may be impossible to establish a predictive model with so much noise to consider. In an effort to establish an improved model, focusing on a single endpoint in multiple species, might be more rewarding. For example, focusing on a single molecular endpoint, like PPAR activation, a known MOA for PFAS, might allow us to better understand the molecular mechanisms that drive PFAS-induced toxicity.

3.2 Implications

Data interpretations may be confounded by the limitation in the study. Nevertheless, mixture toxicity studies with PFAS are highly inconsistent, but the activation of PPAR is not. Since PPAR is involved in a host of cellular and biochemical processes (lipid and glucose homeostasis, fatty acid metabolism, and peroxisome proliferation) it is important to perform toxicity studies that probe for molecular endpoints that are associated with biological functions (i.e., reproduction, fertility, growth). The goal would be to link the biological responses at the molecular and cellular level, to those occurring at the organ and organism level. By extension, those responses can also be associated to responses at the population, community, and ecosystem levels. Incorporating biological hierarchy into the equation may help us to improve our understanding of PFAS toxicity in the environment. Understanding molecular mechanisms of PFAS toxicity is also important, because it will give us insight into latent diseases associated with PFAS, and in the process, increase our odds of developing therapeutic drugs to ameliorate potentially damaging diseases.

PFAS are known endocrine disruptors to thyroid hormones and glucocorticoids. While both are involved in the metabolism of proteins, fats, and carbohydrates, it is likely that PFAS is altering the biochemical signaling associated with both glands (thyroid and adrenals). In a broader sense, PFAS not only affects energy molecules such as triglycerides and glucose, but also the hormones that regulate them. For example, many enzymes involved in glycolysis and the citric acid cycle are upregulated or downregulated by glucocorticoid and thyroid hormone expression. In particular, glucocorticoids affect the activity of pyruvate, hexokinases, phosphofructokinase, and glycogen phosphorylase during glycolysis. Overall, PFAS appears to parasitize and alter several mechanisms associated with oxidative metabolism (glycolysis, fatty acid β-oxidation, and oxidative phosphorylation), which are important for optimal performance. This characteristic of PFAS makes it dangerous to both wildlife and human populations.