Title: Blood Concentrations of Per- and Polyfluoroalkyl Substances are Associated with Lupus-like Autoimmunity in the American Alligator

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Abbreviations: ACN: acetonitrile; CFR: Cape Fear River; CV: coefficient of variation; F: female; Infa interferon alpha type 1; IS: internal standard; LW: Lake Waccamaw; LOD: limit of detection; M: male; PCA: principal component analysis; RBC: red blood cell; SLE: systemic lupus erythematosus; SRM: standard reference material; SVL: snout to vent length; TBARS: thiobarbituric acid reactive substances

Highlights:

- Populations of American Alligator (*Alligator mississippiensis*) from the Cape Fear River in NC have elevated PFAS exposures compared to a nearby reference population
- Increased blood PFAS concentrations are associated with increased innate immune activities and hallmark phenotypes of autoimmune disease including increases in autoantibodies
- Compared the reference population, expression of interferon alpha responsive signature genes that are predictive of lupus erythematosus in humans was increased ~ 60- to 577-fold in the Cape Fear River Alligators with elevated blood PFAS

Abstract:

Surface and ground water of the Cape Fear River basin in central and coastal North Carolina is contaminated with high levels of per- and polyfluoroalkyl substances (PFAS). Elevated levels of PFAS have also been found in blood of fish and wildlife living in and around the Cape Fear River, and in the blood of human populations reliant on contaminated well or surface water from the Cape Fear River basin as a sources of drinking water. While the public and environmental health impacts of long-term PFAS exposures are poorly understood, elevated blood concentrations of some PFAS are linked with immunotoxicity and increased incidence of some chronic autoimmune diseases in human populations. The goal of this study was to evaluate PFAS exposure and biomarkers related to immune health in populations of American alligators (*Alligator mississippiensis*), a protected and predictive sentinel species of adverse effects caused by persistent toxic pollutants. We found that serum PFAS concentrations in a representative population of alligators from the Cape Fear River were increased compared to a reference population of alligators from the adjoining Lumber River basin. The elevated serum PFAS concentrations in the Cape Fear River alligators were associated with increased innate immune activities and hallmark phenotypes of the human autoimmune disease systemic lupus erythematosus. In addition to significant increases in double stranded-DNA binding autoantibodies in adult Cape Fear River alligators, our qRT-PCR analysis found remarkably high induction of Interferon- α signature genes implicated in the pathology of human autoimmune disease. We interpret the association of increased PFAS exposure with disrupted immune functions to indicate that PFAS broadly alters immune activities resulting in autoimmune disease in American Alligators. This work substantiates and extends evidence from experimental models and human epidemiology studies showing that some PFAS are immune toxicants.

Keywords: autoantibodies; autoimmune disease; crocodilian; immune toxicity; lupus; PFAS

Introduction:

Per- and polyfluoroalkyl substances (PFAS) are a class of synthetic organic chemicals that are global contaminants of both built and natural environments (OECD 2018). Owing to their extensively fluorinated aliphatic backbone, PFAS are chemically stable, resistant to thermal and enzymatic breakdown, and can persist in terrestrial and aquatic environments. Because of their widespread use over the past 70 years, many PFAS and their terminal breakdown products, have become ubiquitous contaminants of the land, water, and air to which humans and wildlife are exposed (De Silva et al. 2021). In the US alone, drinking water supplies for an estimated 200 million people are contaminated with PFAS, with recent analyses estimating the direct annual health-related cost due to PFAS exposure ranges from \$37-59 billion in the US and €52-84 billion per year in the European Union (Cordner et al. 2021; Andrews and Naidenko 2020)

Evidence from experimental animal and human epidemiologic studies has linked increased exposure to the most abundant PFAS with immunotoxicity and altered immune functions (DeWitt 2015). Those data led the United States National Toxicology Program to conclude that perfluorooctanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS) are hazards to the human immune system (DeWitt 2015; NTP 2016). Additional findings have also demonstrated associations between exposures to some PFAS with immunosuppression, with adverse health impacts linked to increased incidence in childhood infection, decreased antibody production in response to vaccination, and increases in severity of COVID-19 (Grandjean et al. 2020; Fenton et al. 2021). There is also evidence linking some PFAS exposures with an increased incidence of chronic autoimmune disorders, including thyroid disease and inflammatory bowel diseases

(Fenton et al. 2021). The mechanisms of PFAS-mediated immunotoxicity and their roles in autoimmunity are poorly understood.

The Cape Fear River basin, located in central and coastal North Carolina encompasses over 9,300 sq. miles of waterways that service ~5.2 million people in rural and urban communities, and is typical of many regions with high PFAS contamination of surface, ground, and drinking water (Fig. 1A). PFAS contamination in the Cape Fear originates primarily from fluorochemical production, manufacturing, wastewater treatment discharges, and the use of aqueous film forming foams (AFFF) as fire suppressants (McCord and Strynar 2019). In 2017, surface water sampling from the Cape Fear River revealed high levels of per- and polyfluoroether acids (PFEA) and fluoropolymer manufacturing byproducts with diverse chemical structures originating from a fluorotelomer and fluoropolymer PFAS production facility active since the mid-1980's (Hopkins et al. 2018; Sun et al. 2016). Both novel PFEAs such as HFPO-DA (GenX), Nafion byproducts, and a variety of other fluorochemicals from PFAS-based aqueous film forming foam used as a fire suppressant by military, municipalities, and regional airports have now been detected in the drinking water and blood samples of residents living near the Cape Fear River (McCord and Strynar 2019; Ruyle et al. 2021; Kotlarz et al. 2020). Recently, total organic fluorine analysis detected extremely high sum PFAS concentrations in excess of 100,000 ng/L in the Cape Fear River at drinking water treatment plant intakes suggesting that humans and aquatic ecosystems in the Cape Fear River basin have been experiencing very high levels of total PFAS exposure (Zhang et al. 2019). The potential for impacts on both ecosystems and human populations from this contamination has been confirmed by wildlife and human population based PFAS exposure studies finding increased levels of PFAS in fish, birds, and humans living near the CFR (Guillette et al 2020; Kotlarz et al. 2020; Robuck et al. 2021). The

public and environmental health impacts of long-term exposures resulting from these undocumented PFAS discharge are largely unknown.

The aim of this study was to evaluate the impacts of long-duration PFAS exposure on American alligators (*Alligator mississippiensis*), a top trophic carnivore at the human/wildlife interface of both rural and urban environments (Beal and Rosenblatt 2020; Somaweera et al. 2020). Alligators, renowned for their robust and highly protective immune systems, are an especially important aquatic biomonitoring and predictive sentinel species of adverse outcomes resulting from integrated effects of persistent toxic chemicals (Crain and Guillette 1998; Guillette et al. 2000; Finger and Gogal 201; Pérez and Wise 2018). Alligators living along the Cape Fear River are non-migratory apex predators with a life span exceeding 60 years that co-utilize human habitats contaminated with PFAS (IUCN 2018). Building from previous biomonitoring studies demonstrating utility of American alligators as effective indicators of environmental PFAS contamination (Bangma, Bowden, et al. 2017; Bangma, Reiner, et al. 2017). With a focus on immune health endpoints, we predicted that populations of American alligators would be sensitive sentinels of cumulative adverse health effects resulting from PFAS exposures common to humans living in the Cape Fear River basin.

Methods

Animal and sampling procedures

All animal procedures were performed with approval by the North Carolina State University Institutional Animal Care and Use Committee (protocol #18-085-O). Alligators were sampled using active capture methods that employed snatch hooks from locations with direct access to the Cape Fear River (CFR) and the adjacent Lumber River basin (Lake Waccamaw; 34.28726947673369, -78.50965117663293) in North Carolina. Cape Fear River sites included:

Greenfield Lake (34.2107388211135, -77.93707241283391) Wilmington, NC; Orton Pond in Brunswick County (34.045198995423966, -77.98399224982941); Oak Island, NC (33.91736426475394, -78.12476238375928), and Baldhead Island, NC (33.85295162890547, -77.97713359942439). Sampling occurred in 2018 (July 24 to October 25) and 2019 (April 18 to October 14).

Immediately following capture a 10-15 mL a whole blood sample was collected from the post-occipital spinal venous sinus using a sterile 20 g or 18 g needle and a 30 mL syringe (Myburgh et al. 2014). Whole blood samples were transferred to 8 mL serum and lithium heparin coated plasma tubes (Vacutainer, BD). Serum tubes were allowed to clot for 30 minutes at ambient temperature prior to storage on ice. Plasma tubes were inverted gently following collection, then stored on ice until centrifugation. After field collections, blood was centrifuged (1800 x g for 10 min at 4°C) and serum/plasma fractions were immediately aliquoted into Teflon-free cryovials and stored at -80°C until analysis. Following the blood sample collections, alligators were visually examined for general health, and external injuries were noted and photographed. Sex was determined by cloacal examination with total length, snout to vent length, tail girth, head/snout, and rear foot measurements recorded. Following sample/data collection, alligators were released at the site of capture. Typical time from first contact to release of animal was dependent on animal size with the average time to release being < 15 minutes.

Chemicals

Ethanol (Optima®, Lot 183859), ammonium formate (99%, AC401152500), acetonitrile (ACN; Optima®, Lot 184819), and formic acid (99.5%, A117-50) from Fisher Scientific Waltham, MA) was used for extractions and instrument solvent gradient. All water used for extraction, in aqueous buffers, and solutions were prepared in sterile Milli-Q A10 water (18Ω; 3

ppb total oxidizable organics), with the purified water analyzed for contamination prior to use. All laboratory glassware was rinsed with sterile Milli-Q water and methanol prior to use. The control material for the instrument methods was National Institute of Standards and Technology (NIST) Standard Reference Material (SRM) 1957 organic contaminants in non-fortified human serum. Chemicals for buffers and other standard laboratory chemicals were also purchased from Fisher Scientific and as indicated below.

LC-MS analysis of Serum PFAS Concentrations

Matrix matched (serum) calibration solutions (n = 23; ranging from 0.1 ng/mL to 100 ng/mL) were prepared from neat standards in charcoal stripped fetal bovine serum (Life Technologies, Grand Island, NY; cat #10437, Lot #1754113; total protein 3.7 g/dL). An internal standard (IS) solution was prepared by diluting a solution containing 10 mass-labeled PFAS in water with 0.1 M formic acid (99.5%, A117-50, Fisher Scientific, Waltham, MA): $^{18}\text{O}_2$ -PFBS, $^{13}\text{C}_4$ -PFBA, $^{18}\text{O}_2$ -PFHxS, $^{13}\text{C}_4$ -PFOA, $^{13}\text{C}_4$ -PFOS, $^{13}\text{C}_5$ -PFNA, $^{13}\text{C}_9$ -PFDA, $^{13}\text{C}_4$ -PFBA, $^{13}\text{C}_2$ -PFHxA, $^{13}\text{C}_2$ -6:2FTS (Wellington Labs, Guelph, ON). An additional matrix matched quality control (QC) sample was made by combining randomly selected aliquots of sampled American alligator serum (n = 12). The resulting alligator QC sample and NIST SRM 1957 (n = 6) was used to test the method for reproducibility and stability (Table S2). Charcoal stripped fetal bovine serum was also spiked with 5 ng/mL of PFAS standards and used as an internal control for accurate measurement (n = 4). Additional control samples included fetal bovine method serum blanks (n = 7), and purified water field blanks (n = 5) made by injecting 4 mL of Milli-Q water into vacutainer tubes during field sampling on 5 different days during the sampling period.

All control and experimental samples were extracted and analyzed using methods identical to those detailed in our previous studies (Guillette et al. 2020). Briefly, 50 μL of each sample was

aliquoted into a 2 mL polypropylene tube to which 100 μ L of 0.1 M formic acid and internal standards (12.5 ng) were added. Ice-cold ACN (450 μ L) was added to the tube and then vortexed for 3 seconds. Samples were then centrifuged at 12,500 x g for 5 min at room temperature. The resulting supernatant (100 μ L) was collected and added to 300 μ L of aqueous 0.4 mmol of ammonium formate and then transferred to polypropylene autosampler vials. PFAS were analyzed using a Vanquish UPLC system (Thermo Fisher Scientific, Waltham, MA) equipped with an Accucore C18+ column (2.1 mm x 100 mm x 1.5 μ) at a flow rate of 300 μ L/min, injection volumes of 100 μ L, and a binary mobile phase gradient composed of 95:5 H₂O:ACN, 0.4 mM ammonium acetate and 95:5 ACN:H₂O, 0.4 mM ammonium acetate. A Thermo Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific, Waltham, MA) with a heated electrospray ionization (HESI) source operated in negative mode was used to detect PFAS. For compound validation, data was collected in data dependent mode with a preferred ion list consisting of the quantitated PFAS standards (Table S1). PFAS quantitation was based on an eight-point calibration curve with three injections per concentration randomly assigned in the sample run of the internal standard normalized integrated peak area of the extracted ion chromatogram of the [M-H]⁻ ion with a 5-ppm mass tolerance. The r^2 of all calibration curves used for analysis were above .97; limits of detection (LOD) were defined as the estimated mean concentration of method blanks plus three standard deviations (Table S2). Multiple replicates of SRM 1957 (n = 6) were compared to the values on the Certificate of Analysis for the NIST SRM 1957 standards and were within 15.89 % of expected values (Table S4).

Lysozyme assay

The EnzChek® Lysozyme Assay (Thermo Fisher Scientific, Waltham, MA, Cat. E-22013) was used according to manufacturer's protocols to determine lysozyme activity in serum of a subset of American alligator serum samples (Lake Waccamaw: n = 22, Wilmington CFR: n =

21). Samples were diluted 1:5 in sample buffer and analyzed in triplicate, the average relative standard deviation (RSD) for QC and experimental samples were 7% and 9%, respectively.

Alligator serum/plasma complement assay

Packed Sheep red blood cells (RBCs, Innovative Research Novi, MI) were aliquoted into 15 ml polypropylene conical tubes and centrifuged for 5 minutes at 800 x g. Cell pellets were washed with phosphate buffered saline (pH 7.4; PBS) and then gently suspend in PBS and recentrifuged. Washed cells were resuspended at a final concentration of 2% RBCs in PBS or PBS supplemented with a final concentration of 50 mM EDTA. Cells were rocked gently and 150 µl of the cell suspension was rapidly aliquoted into 1.7 ml microcentrifuge tubes containing 150 µl of each alligator serum sample (plasma samples were analyzed in parallel and yielded comparable results) and mixed by inversion. All sample aliquoting for each analysis was completed in ≤ 2 minutes. Samples were incubated at room temperature for 20 minutes and then rapidly placed onto ice/water bath for 10 minutes, samples were centrifuged at 3000 x g for 5 minutes and then rapidly returned to ice. Supernatant (100 µl) was aliquoted in duplicate into clear 96-well microtiter plates. Absorbance at 540 nm was determined using a Tecan microplate reader. Controls on each analysis plate included a blank control containing 1x PBS only, 1% RBC in PBS, 1% RBC in 1x PBS supplemented with 50 mM EDTA, and a positive control of maximum RBC lysis containing 2% RBCs to which an equal volume of deionized water was added. Percent maximal complement activity for each sample was calculated by subtracting the mean absorbance from the EDTA containing samples from the mean absorbance of the sample lacking EDTA. That difference was divided by mean maximal absorbance of the positive control samples, and the product was multiplied by 100. Each sample was analyzed a minimum of two times. The mean % CV for maximal RBC lysis positive controls was 1.75 ± 1.42 % and for all experimental samples was 3.41 ± 4.43 %.

dsDNA ELISA Analysis

Individual wells of 96 well microtiter plates were coated overnight at 4°C with 500 ng of double stranded calf thymus DNA suspended in DNA coating solution (Pierce, Rockford IL, cat # 17250). Plates were washed extensively with PBS containing 0.05 % Tween 20 (Fisher Scientific), incubated at room temperature for 2 hours in blocking buffer consisting of PBS supplemented with 10% bovine serum albumin (BSA; fraction V, Fisher Scientific), and then rewashed with PBS containing 0.05% Tween 20. Alligator plasma samples were diluted 1:500 and 1:1000 into PBS containing 10% BSA. Diluted samples (100 µl) were added to wells in duplicate or triplicate and incubated overnight at 4° C. Wells were then washed extensively with PBS/0.05% Tween 20, blotted dry by inversion on to paper towels, and then incubated for 1 hour at room temperature with affinity purified polyclonal horse radish peroxidase (HRP) conjugated goat anti-alligator IgG antiserum (Bethyl Labs, Montgomery, TX; Cat # A140-119P; Lot# A140-119P-6) that was diluted 1:10,000 (0.1 µg/ml) in PBS 10% BSA. Samples were extensively washed with PBS 0.05 % Tween 20, blotted dry, and 50 µl of 1x TMB Solution (Invitrogen, Cat # 00-4201-56) was added to each well. The peroxidase reaction was terminated following incubation for 30 minutes at room temperature by the addition of an equal 50 µl volume of 2M sulfuric acid, and the absorbance of each well measured at 450 nm. Control samples on each analyzed plate included calf serum diluted 1:500 (Gibco), blocking buffer only, 1:500 dilution of alligator plasma or calf serum sample lacking HRP-conjugated anti-alligator antiserum, and coated wells lacking DNA. Following optimization of alligator plasma concentrations and alligator antiserum dilutions, specificity of DNA binding was confirmed by observing significant decreases in immunoreactivity in DNA-coated wells that were treated with DNase1 (10 units; RQ1 Dnase, Promega Madison, WI), and in samples of alligator plasma pretreated with 100 µg of calf thymus DNA. Experimental plasma samples were analyzed in duplicate or triplicate on a

single plate at 1:500 dilution and on the same plate using an independent dilution of 1:1000, with each analysis independently replicated. The average intra-assay coefficient of variation for all experimental samples was $5.9 \pm 1.4\%$ (1:500) and $4.5\% \pm 1.1\%$ (1:1000). The average between plate coefficient of variation was $7.0 \pm 1.7\%$ (1:500) and $6.0 \pm 1.5\%$ (1:1000). The average Absorbance 450 nM observed for each sample diluted at 1:500 was 2.05 ± 0.02 times greater than samples diluted at 1:1000.

Whole blood cell counts and leukocyte differential

Blood smears were prepared from ~10 µl of peripheral whole blood taken from a subset of alligators. Blood smears were prepared in the field and allowed to air dry at room temperature on Superfrost plus slides (Fisher Scientific, Pittsburgh, PA). Slides were stained with Hemacolor® according to the manufacturer's protocols (Sigma-Aldrich, Cat: 1.11661). Peripheral whole blood cell counts were made by evaluation of stained blood smears using 40X and 100X objectives on a Nikon Eclipse 80i microscope (Nikon; Melville, NY). Each slide was examined independently by three different investigators blinded to sampling site, size, and sex. Leukocyte identity was determined by cellular morphology and staining characteristics (Sykes and Klaphake 2015; Stacy, Alleman, and Sayler 2011). A minimum of 400 leukocytes and thrombocytes were counted to calculate percentage of each cell type. Leukocytes were categorized into lymphocytes, basophils, heterophils, eosinophils, azurophils, and basophils with percent of each cell type determined by dividing the number of each cell type by the total cells counted minus the number of thrombocytes and then multiplying by 100. Variation between individual researcher counts was determined to be less than 7.9% for each cell type.

TBARS Assay

Plasma lipid peroxidation was analyzed for 100 µl alligator plasma samples ran in duplicate using TBARS (TCA method) Assay kit (# 700870, Cayman Chemical, Ann Arbor, MI)

according to manufacturer's supplied protocols. Malondialdehyde concentrations were calculated by interpolation from a standard curve generated by serial dilution of manufacturer supplied standards.

Quantitative RT-PCR analysis

Total RNA from archived (stored at -80°C) American alligator whole blood was isolated using the Quick-RNA Whole Blood Kit (Zymo Research, Irvine, CA). One µg of RNA was reverse transcribed using the high-capacity cDNA reverse transcription kit following manufacturer's recommendations (Applied Biosystems; Grand Island, NY). Standard Fast TaqMan PCR amplification was performed in triplicate on a Step One Plus Real-Time PCR System (Applied Biosystems; Grand Island, NY) in a final volume of 20 µL containing ~10 ng of cDNA (1.5 µL of RT product), 1x Universal Master Mix and custom TaqMan expression assay primers specific for each target alligator mRNA (**Table S5, S6**) (Applied Biosystems; Grand Island, NY). Relative expression was quantified using the $2^{-\Delta\Delta C_t}$ method, in which $\Delta\Delta C_t$ is the normalized value. Alligator Gapdh expression was used as an independent reference gene for normalization.

Data and Statistical Analysis

Each animal/blood sample was assigned a randomized numeric code by NC State University researchers at the time of collection. Samples were decoded for site and length/size/sex only after PFAS measurement/analysis and biological markers analysis were completed. Blood slides were further deidentified to mask sample identity for whole blood cell counts. Samples used for each analysis were randomly selected based only on availability of sufficient material for analysis, to balance analysis for comparable numbers of males and females, life stage (adult SVL > 90 cm/juvenile SVL < 90 cm), and to control for possible effects

on seasonality samples were date matched. When necessary, SVL and sex were included in statistical models as covariates.

Data was analyzed and visualized using Prism (version 9.3, GraphPad La Jolla, CA); R statistical programming environment version 3.5.2; SPSS (Version 27.0, IBM, Armonk, NY); JMP Genomics 9 (SAS, Cary, NC). For PFAS concentration data, means and concentration ranges were determined from values above the LOD and interpolated ($[PFAS] = LOD/\sqrt{2}$) values were used when detected PFAS congeners were detectable but below the LOD ($LOD = mean_{blanks} + 3(SD_{blanks})$), non-detects were replaced with a value of zero. A Shapiro-Wilk's test was used to assess normality of data; for data requiring normalization a natural log transformation was used, [PFAS] data was $\log_{10}$ transformed, and percentage/ratiometric data were arcsine transformed. Non-parametric statistics were used for data not meeting analysis model assumptions following data transformation. Linear regression and Pearson's correlation coefficient or Spearman's rank correlation coefficient were used to calculate relationships between total PFAS serum concentrations and serum biomarkers. A 2-way analysis of variance (ANOVA) was used to evaluate peripheral blood counts data (cell type, location), and $\sum \log_{10}$ [PFAS] (size class, location) data and biomarker activities were initially evaluated at each site using multivariate ANOVA (biomarker activity, $\sum \log_{10}$ [PFAS], and/or location). Principal component analysis (PCA) was performed to dimensionally reduce PFAS concentrations using a restricted maximum likelihood estimation (REML) and components with a minimum eigen value of 1. Ward's general agglomerative hierarchical clustering procedure was performed on serum PFAS congener concentrations ($\log_{10}$ transformed) to demonstrate large scale differences in PFAS clustering using JMP Genomics 9. A minimal level of statistical significance for differences in values among or between groups was considered $p \leq .05$.

Results and Discussion

We used liquid chromatography and high-resolution mass spectrometry to determine concentrations of 23 different PFAS present in serum samples of 75 adult and juvenile alligators from North Carolina (Guillette et al. 2020). Alligators were sampled beginning in July 2018 and continued through October of 2019 at sites experiencing both point and non-point source PFAS exposures along the Cape Fear River (CFR; n = 49), and from Lake Waccamaw (LW; n = 26), a site < 50 km away and in the adjoining Lumber River watershed with no known fluorochemical production (Fig. 1A). From the targeted list of 23 PFAS analyzed (Table S1), we found 14 different PFAS, including long and short chain perfluoroalkyl acids (PFAA), perfluoroether acids (PFEA) and the fluorotelomer 6:2 FTS in these alligator blood samples (Table S2, S3).

As anticipated from previous wildlife exposure studies along the Cape Fear River, PFOS was the dominant PFAS detected in alligator serum (Guillette et al. 2020; Robuck et al. 2021). We detected PFOS in 100% of samples analyzed and it accounted for 79.7% and 75.8% of the total PFAS present in alligator serum samples from the CFR and LW respectively. The median number of PFAS congeners detected in serum samples from CFR alligators was 10 (range 4-12), whereas a median of 5 (range 2-9) were detected in LW samples (Table S3). The relative composition of long and short chain PFAAs and PFEAs detected in blood of CFR American alligators were in general agreement with those found in the blood of adults and children exposed to PFAS from drinking water in Wilmington, NC (Kotlarz et al. 2020).

Results of our principal component, multiple linear regression, unsupervised hierarchical clustering, and statistical analysis of total serum concentrations demonstrated both increases in PFAA concentrations and an exposure profile enriched for Nafion byproduct 2, PFO₅DoDA, NVHOS, HFPO-DA and 6:2 fluorotelomer sulfonate (6:2 FTS) was characteristic and predictive of samples from the CFR (Fig. 1 B-D). Concentrations and detection frequencies of PFEAs

(HFPO-DA, Nafion byproduct-2), PFAS congeners related to upstream production and 6:2 FTS discharge from the Chemours Fayetteville Works facility, were exclusive to, or greatly enriched in the CFR serum samples (Fig. 2C, D; Table S2). By contrast the PFAS exposure profiles observed in blood of LW alligators were characterized by the presence of bioaccumulative six-carbon and longer PFAAs (Fig.1D). However, the PFEAs PMPA (15%), PDO4DA (23%), and

Fig 1. Location of study sites and serum PFAS exposure profiles. (A) Location of samples collected at Lake Waccamaw (LW; blue) and from the Cape Fear River basin (CFR; orange). Results of (B) principal component and (C, D) unsupervised hierarchical clustering of log₁₀ transformed serum PFAS congener concentrations from the CFR and LW. Females and are indicated with a circle in panel (B) or a capital F (C, D) and males are indicated with a triangle or a capital M. (E) Mean of log₁₀ transformed PFAS concentrations (ng/ml) found in serum of adult (SVL ≥ 90 cm) and juvenile/sub-adult American alligators from LW (adult n = 15; juvenile n = 11) and the CFR (n = 26; juvenile = 23).

PFO5DoDa (19%) were also detectable in some alligator serum samples from LW suggesting that there are low levels of PFEA contamination within the Lumber river basin ecosystem (Fig. 1D, Table S2).

Concentrations of sum total PFAS detected in serum of CFR alligator was increased compared to alligators sampled from LW (Fig. 1E), two-way ANOVA found a significant effect of location on total serum PFAS concentration ($F(1, 71) = 27.3, p < .0001$). We did not detect differences in total PFAS concentrations between juveniles/sub-adults (SVL < 90 cm) and adults ($F(1, 71) = 0.0018, p = .965$) at either site, and there was not an interaction between groups ($F(1, 71) = 0.989, p = .323$). Post hoc analysis indicated that serum concentrations of PFAS were significantly increased in both the juveniles/sub-adult ($p = .006$) and adult alligators ($p < .0001$) from the CFR (Fig. 1E).

The American alligator is renowned for having a particularly robust and protective antibacterial and antiviral innate immune system (Merchant and Britton 2006; Finger and Gogal 2013). Initially focusing on biomarkers of innate immune function, we found that anti-microbial lysozyme activity was significantly increased in adult CFR alligators compared to adult alligators from LW (Fig. 2A). Those findings were similar to our previous findings that high concentrations of PFAS were associated with elevated lysozyme activity in adult striped bass (*Morone saxatilis*) from the CFR (Guillette et al. 2020). By contrast, we did not observe a significant difference between sites for lysozyme activities of juvenile/subadult alligators, although at LW the lysozyme activity of sexually immature animals compared to adults from the same location was significantly increased ($p < 0.0001$; Fig. 2A). The apparent age-related difference in lysozyme activity at LW was unrelated to differences in PFAS exposure, a difference that is likely related to ecological, population density, or other undefined factors at this location. Because of these site-specific differences in lysozyme activity, associations

Figure 2. Impacts and relationship of PFAS exposure on innate immune functions. (A) Violin plots comparing median lysozyme activity in juvenile (SVL < 90 cm) and adult American alligators (SVL > 90 cm) from Lake Waccamaw (LW; thick lines indicate median; thin lines indicate quartiles) and from the Cape Fear River (CFR). (B) Relationship of total PFAS concentrations with lysozyme activity in adult alligators. Solid line is the best fit linear regression, with shaded area represents 95% CI. (C) Violin plots of complement activity in plasma samples of alligator from LW and CFR. Shown is the percent of maximal EDTA sensitive sheep red blood cell lysis activity (thick lines indicate median; thin lines indicate quartiles; LW: n = 22, CFR n = 44).

between total PFAS concentrations and lysozyme activity were evaluated only in sexually mature adult alligators. Using Spearman's ranked correlation to assess the relationship between serum total PFAS concentrations and lysozyme activity in adult alligators from both sites, we observed a positive correlation between serum PFAS and lysozyme activity, $r(29) = .45, p = .006$ (Fig. 2B). Serum complement activity, a key component of the humoral innate response that has been linked to autoimmunity (Zipfel 2009), was increased in the entire population of sampled CFR alligators (Fig. 2C). As observed for lysozyme, log10 transformed total PFAS concentrations and serum complement activity were found also to be moderately positively correlated, $r(71) = .23, p = .05$.

Despite living in environments with near-constant exposure to pathogenic microorganisms, and having especially high burdens of fecal coliform and other pathogenic bacteria (Johnston et al. 2010), crocodilians, including American alligators, rarely suffer from systemic or skin infections from these microorganisms (Barksdale, Hrifko, and van Hoek 2017; van Hoek 2014). However, during our sampling we observed a notably uncharacteristic increase in incidence of

Fig. 3. Autoimmune disease like phenotypes associated with PFAS exposure. (A) Examples of cutaneous lesions and slow to heal wounds associated with PFAS exposure in 3 representative animals sampled from the CFR in 2019. Lower case letters indicate body location of lesion shown in each panel. (B) Violin plots comparing median thrombocyte numbers quantified from whole blood smears in alligators from Lake Waccamaw (n = 16) and the Cape Fear River (n = 39). (C) Representative photomicrograph of a schistocyte (arrowhead) observed in a whole blood sample isolated from an alligator from the CFR. Bar = 10 μ m (D) Violin plots comparing median anti-dsDNA immunoreactivity in male (n = 4) and female (n = 4) alligator serum samples from each site. (E) Quantitative RT-PCR analysis of Inf α signature gene expression analysis. Note the X-axis scale is Log₁₀ relative fold-change. All increases in expression are significant (P < .0001). For violin plots median is indicated by a thick line and thin lines indicate quartiles.

skin lesions, slow healing, and infected wounds in ~20% of sampled CFR alligators (Fig. 3A). Those lesions were considered reminiscent of autoimmune disease related vasculitis in humans (Shanmugam et al. 2017). Although fresh and healed wounds, including loss of limbs that were likely related to territorial and mating interactions were observed, similarly infected and slow to heal wounds were not observed in alligators sampled from LW. We also observed decreased clotting in some CFR alligators following blood collection; that phenotype was consistent with our findings of modestly decreased numbers of thrombocytes in whole blood cell counts from CFR alligators (Fig. 3B). Additionally, we found an increased incidence of abnormal red blood

cell phenotypes including schistocytes (helmet cells) associated with PFAS exposure in the CFR samples (Fig. 3C, Table S2). Our analysis of whole blood cell counts found no evidence for a general increase in chronic stress; no relationships were detected between sample site or PFAS concentrations and leukocyte differentials, or heterophile/lymphocyte ratios. We also measured relative levels of lipid peroxidation products in plasma which also found no evidence of increased impacts related to general systemic mechanisms of cellular injury and stress in the CFR alligators (Fig. S1).

Based on the observed increases in helmet cells and modest thrombocytopenia associated with increased PFAS exposure in CFR alligators, we evaluate other hallmark phenotypes of autoimmune diseases (Crow, Olferiev, and Kirou 2019; Sirobhushanam, Lazar, and Kahlenberg 2021). Using an alligator specific anti-double stranded DNA (dsDNA) immunoglobulin (IgG) binding enzyme-linked immunosorbent assay, we evaluated a subset of adult male and female alligators from the CFR and LW and found that dsDNA binding IgGs were significantly increased in CFR alligator plasma samples (Fig. 3D). Differences between males and females were not detected. Finally, our RT-PCR analysis of type 1 interferon (Ifn- α) responsive gene expression in alligator whole blood samples found extremely high expression of four different Ifn- α signature genes implicated in pathology of human autoimmune diseases including systemic lupus erythematosus (SLE) (Crow, Olferiev, and Kirou 2019). Relative to samples from LW, Ifn- α responsive gene expression was induced > 60-fold for *Rsad2* (viperin), and >400-fold for *Cxcl10*, *Mx1*, and *Oas1* in CFR alligator blood samples (Fig. 3E). The finding of extremely high induction of Inf- α signature gene expression in the CFR alligators supports a central role for excessive production of type I interferons in increasing the innate immune functions associated with elevated PFAS exposures. We interpret the greatly increased Inf- α signature gene expression, elevated autoantibodies, vasculitis-like skin lesions, elevated incidence of slow

healing wounds, and increases in innate immune activity observed in the CFR population as indicative of an SLE autoimmune-like disease process in American alligators.

Conclusions

Overall, our work found strong associations across multiple hallmark phenotypes of SLE-like autoimmune disease and elevated PFAS exposures in American alligators exposed to elevated levels of PFAS from the CFR. The PFAS-associated changes in both the innate immune and adaptive immune system may represent a significant health concern for wildlife and human health that may leave exposed populations at greater risk for infections and autoimmune disease. Uniquely, for this work we implemented an integrated One Environmental Health approach using American alligators as sentinels to more fully understand the consequences of long-term exposure to complex and poorly understood mixtures of PFAS with the aim of improving health of impacted human, animals, and ecosystems (Gibbs 2014; Pérez and Pierce 2018). In addition to being long lived, and sharing their environment with humans, American alligators and other crocodilians, by virtue of having innate immune functions optimized to eliminate microbial pathogens, may serve as an ideal predictive model for detecting autoimmune hazards caused by chemical pollutants. Our findings of adverse immune-health effects in American alligators that have exposure profiles similar to the surrounding human population illuminates and reaffirms the need to reduce exposure and cease production and use of a chemical class that, through its ubiquity and persistence, is a global environmental health concern.

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