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Domestic Dogs and Horses as Sentinels of Per- and Polyfluoroalkyl Substance Exposure and Associated Health Biomarkers in Gray's Creek North Carolina

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The authors declare the following competing financial interest(s): The following authors disclose their associations that could be perceived as potential competing interests with the subject matter discussed in this manuscript: Dr. Kentley Dean is an employee of Southern Oaks Animal Hospital a full-service veterinary medical facility, located in Hope Mills, NC. Mike Watters and Debra Stevens-Stewart are residents of the study area and served as community representatives for this community engage participatory research study. The remaining authors declare no competing interests.

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.est.3c01146

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.3c01146.

Details on supplemental methods for PFAS analysis, mass spectrometry, quality controls, and SRM 1957 analysis (PDF)

Results of residential water testing for HFPO-DA (Table S1), pet demographics (Table S2), targeted PFAS analytes (Table S3), hematological parameters measured by VetScan blood chemistry rotors (Table S4), dog VetScan data (Table S5), horse VetScan data (Table S6), raw PFAS concentrations in dog serum (Table S7), raw PFAS concentrations in horse serum (Table S8), results of general linear model analysis for effect of water source (with household as a nested factor), sex, age, and body weight on serum PFAS concentrations in dogs (Table S9), results of general linear model analysis for effect of household, sex, age, and body weight on serum PFAS concentrations in horses (Table S10), and results of multiple linear regression analysis of log10(X+1) PFAS concentrations and health biomarkers in dogs (Table S11) and horses (Table S12) (XLSX)

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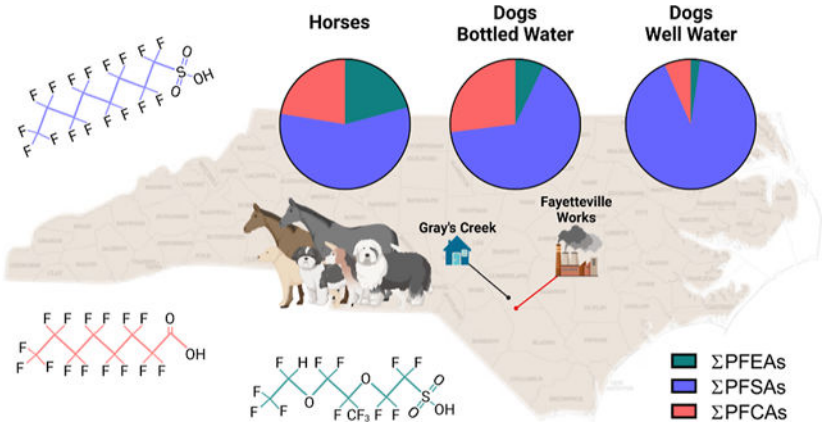
Abstract

Central North Carolina (NC) is highly contaminated with per- and polyfluoroalkyl substances (PFAS), in part due to local fluorochemical production. Little is known about the exposure profiles and long-term health impacts for humans and animals that live in nearby communities. In this study, serum PFAS concentrations were determined using liquid chromatography high-resolution mass spectrometry and diagnostic clinical chemistry endpoints were assessed for 31 dogs and 32 horses that reside in Gray's Creek NC at households with documented PFAS contamination in their drinking water. PFAS were detected in every sample, with 12 of the 20 PFAS detected in ≥50% of samples from each species. The average total PFAS concentrations in horses were lower compared to dogs who had higher concentrations of PFOS (dogs 2.9 ng/mL; horses 1.8 ng/mL), PFHxS (dogs 1.43 ng/mL, horses < LOD), and PFOA (dogs 0.37 ng/mL; horses 0.10 ng/mL). Regression analysis highlighted alkaline phosphatase, glucose, and globulin proteins in dogs and gamma glutamyl transferase in horses as potential biomarkers associated with PFAS exposure. Overall, the results of this study support the utility of companion animal and livestock species as sentinels of PFAS exposure differences inside and outside of the home. As in humans, renal and hepatic health in domestic animals may be sensitive to long-term PFAS exposures.

Graphical Abstract



Keywords

canine; drinking water; equine; fluoroether; One Health; nafion by-product 2; GenX chemicals; PFOA; PFOS

INTRODUCTION

Per- and polyfluoroalkyl substances (PFAS) are a diverse group of synthetic organofluorine chemicals that are characterized by the presence of one or more perfluoroalkyl or polyfluoroalkyl groups. Many are amphiphilic, heat resistant, and chemically stable, properties that make them useful for a wide variety of industrial and consumer applications.[1,2] High volume production and widespread use of PFAS-containing products, along with global transport of these chemicals through air and water, has led to ubiquitous contamination of indoor and outdoor environments.[3–10] Because many PFAS are non-degradable in the environment, or degrade to terminal perfluorinated end products, they persist in the environment and accumulate in biota.[11] Such ubiquity, persistence, high-mobility, and ability to bioaccumulate, combined with the robust evidence of toxicity, necessitates biomonitoring efforts to characterize the exposure and long-term health consequences of PFAS.

For decades, Fayetteville Works, a fluorochemical manufacturing facility located along the border of Cumberland County in central North Carolina (NC; Figure S1), has discharged PFAS into the surrounding environment through vent stack emissions and direct discharge of process wastewater into the Cape Fear River.[12–15] As a result, elevated levels of PFAS are present in residential drinking water and blood samples from fish, wildlife, and humans living near the Cape Fear River.[15–22] Owing to concerns regarding the persistence and toxicity of long-chain PFAS, US manufacturers participating in the 2010/2015 perfluorooctanoic acid (PFOA) stewardship program, put forth by the Environmental Protection Agency (EPA), report that they no longer produce PFOA and perfluorooctanesulfonic acid (PFOS).[23] Consequently, the production and use of shorter-chain alternatives, such as the perfluoroalkyl ether acid (PFEA) hexafluoropropylene oxide dimer acid (HFPO-DA), colloquially known as a GenX chemical, has increased.[11,24,25] Recent monitoring efforts have detected PFEAs in thousands of private wells in the eastern half of the state, including many in the Gray's Creek area of southern Cumberland County NC, with concentrations of HFPO-DA exceeding the EPA's drinking water health advisory limit of 10 parts per trillion (ppt; Table S1; Figure S2).[26–28] In conjunction with toxicity assessments for GenX chemicals finding evidence of adverse health effects in the liver, kidneys, thyroid, and immune system, there is considerable concern surrounding the potential long-term health impacts resulting from chronic consumption of PFAS-contaminated water.[29–31] However, the impacts of long-term exposure to the complex mixtures of PFAS found in the Gray's Creek area remain unknown.

Animal sentinels are animals that have ecological and physiological adaptations that make them sensitive to environmental hazards such as toxins, pollutants, or pathogens and can exhibit signs of adverse effects before humans would notice any effects. As a result, sentinel animals are indicators useful for detecting and providing advanced indication of environmental health hazards.[32] In a One Health context, there is increasing appreciation and use of domestic animals as sentinels of human exposure and health risks associated with household sources of chemical contamination. For example, studies of dogs and cats demonstrate that the prevalence and abundance of PFAS, pesticides, and flame retardants often parallel and, in some cases, match those of their owners.[33–37] Bolstering their

utility as sentinels for human health effects, domestic animals have substantial overlap in shared health risks, with 360 diseases in pet dogs that are analogous to human diseases.[38] Combined with their shorter lifespans, and therefore shorter disease latencies, companion animals can provide critical insights into human-relevant, exposure-associated adverse health outcomes. In addition to dogs and cats, horses represent a potentially useful, but underutilized, residential sentinel. Horses live in close proximity to their owner's homes but often spend their entire life outside, which gives them a unique exposure environment that is relevant to both humans and local wildlife. By studying sentinel animals that occupy both indoor and outdoor residential environments, we can ascertain information on situational environmental exposures and potential health risks.

The "One Health" concept emphasizes the importance of understanding how shared environmental experiences contribute to the etiology of human and animal disease and ecosystem degradation. Although this approach has typically been applied to studies of zoonotic disease, there is growing interest in its application for identifying chemical-induced health risks to inform regulatory and public health response to chemical hazards like PFAS.[39] In the present study, we utilized a collaborative "One Health" approach, combining community-based participatory research with the expertise of veterinary, analytical, and toxicological scientists, to assess PFAS exposures and health biomarkers in pet dogs and horses living in the Gray's Creek area of Cumberland County NC. We tested multiple hypotheses in this study. First, because PFAS are ubiquitous contaminants of both the indoor and outdoor environment, we hypothesized that pets would have detectable levels of PFAS in their serum. Second, recent publications have shown that PFAS, including HFPO-DA, can have longer half-lives in male rodents;[31,40,41] therefore, we hypothesized that male study animals would have higher serum concentrations of PFAS. Third, because dogs and horses occupy different living environments, we hypothesized that differences in primary habitat contamination would influence serum PFAS composition and abundance. Specifically, we hypothesized that dogs would have higher overall PFAS exposure reflecting the indoor environment with PFAS exposure dominated by congeners found in drinking water and house dust contamination (i.e., PFOS, PFOA, and perfluorohexanesulfonic acid; PFHxS).[33,42,43] Fourth, due to concerns about drinking water contamination in this area, approximately half of the households that participated in this study gave their dogs bottled water, rather than well water, as their primary source of drinking water. We hypothesized that dogs provided with bottled water would have lower serum PFAS concentrations compared to those given well water. Finally, we hypothesized that the differences in serum PFAS would be associated with disparate health biomarker profiles across (i.e., horse vs dog) and within (i.e., dog bottled vs well) species.

MATERIALS AND METHODS

Study Population.

Owners provided informed consent prior to sample collection, completed a questionnaire about their companion and/or livestock animal's age, sex, weight, and source of drinking water, bottled or well, shared results confirming well water PFAS contamination, and allowed a veterinarian to take a blood sample for serum PFAS and health biomarker

analysis (Figure S1). Details regarding HFPO-DA concentrations detected in well water from participating households can be found in Table S1 and Figure S2. Pet demographic information is provided in Table S2. Owners and their animals (n = 31, dog; n = 32, horses) were recruited through social media and recruitment emails from August through September 2020. Dogs and horses were considered eligible if they lived in the Gray's Creek area and PFAS analysis had been completed on their drinking water source by the North Carolina Department of Environmental Quality (NCDEQ) between 2017 and 2021. The final study cohort comprised 22 households from an area that was within 18 kilometers of the fluorochemical production facility. A flow diagram detailing the stratification of samples across species, source of drinking water, household, and sex is shown in Figure S3. All study protocols and associated materials were approved by the NCSU Institutional Review Board (protocol number 21069) and Institutional Animal Care Use Committee (protocol number 20-336).

Sample Collection.

Blood samples (3–5 mL) were collected in September 2020 by a registered veterinary technician or licensed veterinarian using a 5 mL Luer lock syringe (BD, Franklin Lakes, NJ) with a 20-gauge 1 in. needle from the cephalic or jugular vein through standard venipuncture. Blood was transferred from the syringe into serum blood collection tubes (BD Vacutainer, Cat# 368045, plastic, Franklin Lakes, NJ) and allowed to clot at ambient temperature for at least 30 min. Serum was aliquoted into Teflon-free cryovials following centrifugation (1800g for 10 min at 4 °C) and transported on ice to NCSU. Serum fractions were stored at −80 °C until analysis.

Sample Preparation for PFAS Analysis.

Details on the materials used for sample preparation and PFAS analysis can be found in supplemental methods (Supporting Information Document 1). Serum (50 μL) was transferred into 2 mL polypropylene tubes, 10 μL of 0.1 M formic acid spiked with 24 isotopically labeled internal PFAS standards was added, and each sample was vortex mixed. Cold acetonitrile (300 μL; −20 °C) was added to denature and precipitate proteins. The sample was then vortex mixed and centrifuged at 10,000 rpm for 5 min, and the process was repeated. Following the second centrifugation, 100 μL of supernatant was transferred to a liquid chromatography vial containing 300 μL of 0.4 mM ammonium formate in diH_2O.

Mass Spectrometry.

The PFAS analyzed were chosen based on compounds that were previously detected in the NC Cape Fear River basin and the availability of appropriate authentic standards.[15,16,44,45] At the time of analysis, analytical standards were available for 33 PFAS of interest (Table S3). Methods for the preparation of standard curves, mass spectrometry conditions, and quality controls were identical to those used previously.[16,17] Additional details are also contained in supplemental methods (Supporting Information Document 1).

Analysis of Blood Chemistry Biomarkers.

Blood chemistry values for 14 parameters (Table S4) were obtained for all samples with sufficient volume and quality of serum necessary for analysis (dogs: $n = 29$, Table S5; horses: $n = 26$, Table S6) using a VetScan VS2 Whole Blood chemistry analyzer (Abaxis, Union City, CA) with commercially available diagnostic veterinary panels optimized for dogs (Abaxis, Comprehensive Diagnostic Profile Cat #500-1043) or horses (Abaxis, Equine Profile Plus Cat #500-1038). Two dogs, 1020 and 1036, and six horses, 1142–1147, were not analyzed due to insufficient serum volume for the dogs and missing metadata needed for statistical analysis for the horses (Supporting Information Document 1; Tables S7 and S8). In dogs, hemolysis hindered our ability to accurately measure total bilirubin ($n = 24$) and potassium ($n = 20$) in a subset of samples. Calcium ($n = 28$) concentration was also suppressed for a single dog in this study. All samples analyzed passed individual assay control QC assessments of the instrument and rotors. All procedures followed the manufacturer's protocols using 100 μL of serum.

Statistical Analysis.

Each blood sample was assigned a randomized number code by NCSU researchers at the time of collection. Samples were decoded for location, sex, age, weight, and drinking water source only after PFAS measurement and analysis was completed. Data were analyzed using Prism (version 9.3, GraphPad La Jolla, CA), JMP Pro (Version 16, SAS, Raleigh, NC, USA), SPSS (version 28.0, IBM, Armonk, NY, United States), and the R statistical environment (Version 4.1.1; R Core Team 2021). For all statistical analyses, a minimal level of statistical significance for differences in values among or between groups was considered ($p \leq 0.05$).

Where necessary, PFAS detected at concentrations less than the LOD were assigned an interpolated concentration value of $\mathrm{LOQ}/\sqrt{2}$ prior to statistical analyses (Tables S7 and S8). Shapiro–Wilk's test was used to assess normality of data; however, no PFAS, except for perfluoro(3,5-dioxahexanoic) acid (PFO2HxA) in horses, passed normality. Individual PFAS concentrations were transformed using $\mathrm{Log}_{10}(X + 1)$, where the addition of 1 allows for the inclusion of non-detects (0) and quantifiable values < 1 following data transform.[46]

Principal components analysis (PCA) was performed to dimensionally reduce PFAS concentrations using a restricted maximum likelihood estimation. Ward's general agglomerative hierarchical clustering was performed on $\mathrm{Log}_{10}(X + 1)$ transformed PFAS concentrations. Differences between PFAS concentration means were analyzed by two-way ANOVA with Sidak's multiple comparisons post-hoc test. Fisher's exact test was used to compare individual PFAS detection frequencies across and within species. General linear modeling (GLM) was used to examine the impact of water source (dogs only), household, sex, age, and weight, on serum concentrations of individual PFAS with detection frequencies ≥50% for individual compounds. Water source and sex were considered fixed factors, household was considered a fixed factor within water source, and age and weight were treated as covariates (Tables S9 and S10). Adjusted correlation coefficients were considered negligible (±0.0–0.1), weak (±0.1–0.39), moderate (±0.4–0.69), strong (±0.7–0.89), and very strong (±0.9–1) based on previously established criteria.[47]

To evaluate the magnitude of effect for differences between means and GLM analyses, effect sizes were calculated. ANOVA effect size was determined by calculating Eta squared (η^2), effects of which are defined as small at 0.01, medium at 0.06, and large at 0.14.[48] Effect sizes for post-hoc Sidak's corrections for multiple comparisons were calculated by Cohen's d, effects of which are defined as small at 0.2, medium at 0.5, and large at 0.81.[48]

To identify the associations between serum PFAS concentrations and health parameters, multiple linear regression analysis was performed using PFAS concentrations as independent variables and biochemical parameters as dependent variables, with household and water source as covariates (Tables S11 and S12). Shapiro–Wilk's test was used to assess normality of data. Health parameters that did not pass normality were natural log transformed for regression analysis. In dogs, because serum PFAS concentrations were significantly impacted by the source of drinking water, separate regression analyses were run for dogs given bottled water and dogs given well water.

RESULTS

Unique PFAS Exposure Profiles of Dogs and Horses.

From the targeted list of the 33 PFAS analyzed, we identified 20 different PFAS including PFEAs, perfluorosulfonic acids (PFSAs), and perfluorocarboxylic acids (PFCAs, Table S3). Of those 20, 16 had a ≥50% detection in one species. The PFSAs, specifically PFOS and PFHxS, had the highest concentrations in all serum samples, with the highest levels observed for PFHxS in dogs and PFOS in horses. Two congeners were detected only in dogs, PFHxS and perfluoroethocysulfonic acid (NVHOS), while three congeners, perfluoropentanoic acid (PFPeA), HFPO-DA, and perfluoro(3,5,7,9,11-pentaoxadodecanoic) acid (PFO5DoDA), were detected only in horses (Table 1). Species-specific differences in detection frequency were observed for perfluorohexanoic acid (PFHxA; $p < 0.0001$), perfluoropentanesulfonic acid (PFPeS; $p = 0.05$), PFHxS ($p < 0.0001$), PFOS ($p = 0.003$), and perfluorododecanoic acid (PFDoDA; $p = 0.0006$), with PFHxA, PFPeS, PFHxS, and PFOS more frequently detected in dogs and PFDoDA more frequently detected in horses.

Unsupervised hierarchical clustering analysis and PCA of $\mathrm{Log}_{10}(X + 1)$ transformed concentration data demonstrated unique exposure profiles between dogs and horses, with principal components (PC) 1 and PC2 accounting for 38% of the variance (Figure 1A,B). Analysis of the difference between mean $\mathrm{Log}_{10}(X + 1)$ PFAS concentrations revealed a significant effect of species [$F(1, 1220) = 47.92$; $p < 0.0001$; $\eta^2 = 0.02$] and congener [$F(29, 1220) = 60.77$; $p < 0.0001$; $\eta^2 = 0.42$] and a significant interaction between species and congener [$F(19, 1220) = 16.54$; $p < 0.0001$; $\eta^2 = 0.12$]. Šidák's multiple comparisons test indicated that mean ΣPFAS, PFOS, PFHxS, and PFOA were significantly higher in dogs ($p < 0.0001$; $d = 0.97$; $p < 0.0001$; $d = 0.90$; $p < 0.0001$; $d = 1.26$; $p = 0.006$; $d = 1.19$), and Nafion Byproduct 2 (NBP2) was higher in horses ($p = 0.001$; $d = 0.79$; Figure 1C).

Unique PFAS Exposure Profiles in Dogs given Drinking Well or Bottled Water.

No significant differences in detection frequencies for any individual PFAS were observed between dogs given bottled water or well water. However, the detection frequency of all

PFAS congeners except PFHxA was higher for dogs consuming primarily well water. Notably, ΣPFSA concentrations were higher in dogs given well water, and ΣPFCA concentrations, particularly PFOA, were moderately higher in dogs drinking bottled water (Table 1). Unsupervised hierarchical clustering analysis and PCA of $Log_{10}(X + 1)$ transformed data demonstrated unique exposure profiles between dogs given bottled water and dogs given well water, with 46% of the variance explained by PC1 and PC2 (Figure 2A,B). A significant effect of water source [$F(1, 580) = 24.59$; $p < 0.0001$; $\eta^2 = 0.01$], congener [$F(19, 580) = 56.91$; $p < 0.0001$; $\eta^2 = 0.6$], and interaction [$F(19, 580) = 9.42$; $p < 0.0001$; $\eta^2 = 0.10$] on the difference between mean $Log_{10}(X + 1)$ PFAS concentrations was observed. Sidák's multiple comparisons test indicated that mean ΣPFAS, PFOS, and PFHxS are significantly higher in dogs that consumed well water compared to those in dogs consuming bottled water ($p < 0.0001$; $d = 1.26$; $p < 0.0001$; $d = 1.32$; $p < 0.0001$; $d = 1.18$; Figure 2C).

Correlations between Clinical Biomarkers and PFAS Concentrations.

For dogs, we identified a significant overall effect of water source, with household as a nested factor, on PFAS concentrations ($F = 8818.22$; $p < 0.001$; $\eta^2 = 1.0$). No significant overall effect of sex, age, or weight were observed (Table S9). A significant effect of sex was identified for NBP2, with males having higher levels than females [difference between means (ΔM) = 0.04; 95% CI: 0.02, 0.06; $p = 0.003$].

Multiple linear regression analysis for dogs was adjusted for household and water source to evaluate the associations between health biomarkers and PFAS concentrations (Tables 2; S11). Significant positive correlations between PFHpS and alkaline phosphatase (ALP), perfluorobutanesulfonic acid (PFBS) and alanine aminotransferase (ALT), PFO2HxA and blood urea nitrogen (BUN), ΣPFAS and BUN, perfluoroethoxypropyl carboxylic acid (PEPA) and glucose, PFO2HxA and total protein, and PFO2HxA and globulin were observed. Significant negative correlations were observed for PFHxS and ALP, NBP2 and ALP, PMPA and BUN, and PFO2HxA and albumin/globulin ratio.

Because 51.4% of the horses in this study resided at the same location, a significant overall effect of household ($F = 58.77$; $p < 0.001$; $\eta^2 = 1.0$) was evident in our GLM results. However, no overall effects of sex, age, or weight were observed (Table S10). Weak to moderate effects of body weight were observed for PFDoDA ($F = 5.52$; $r = 0.42$; $p = 0.03$; $\eta^2 = 0.25$), PFO2HxA ($F = 6.92$; $r = 0.18$; $p = 0.02$; $\eta^2 = 0.29$), and PFPeS ($F = 5.99$; $r = 0.20$; $p = 0.03$; $\eta^2 = 0.26$). Sensitivity analysis conducted by removing samples with non-detectable concentrations of PFPeS ($n = 5$), did not identify a significant relationship between PFPeS and animal body weight.

For biomarker analysis, our multiple linear regression analysis for horses was adjusted for household and identified significant positive correlations between PFBS and creatine kinase, and PFUnDA and calcium. Negative correlations for PFHpA and total carbon dioxide, PFOA and creatine kinase, PFDoDA and gamma glutamyl transferase (GGT), and PFHpA and albumin/globulin ratio were observed (Tables 3 and S12).

DISCUSSION

Analysis of serum PFAS concentrations in domestic dogs and horses from Gray's Creek NC revealed that these companion animals and livestock are ubiquitously exposed to PFAS. Of the 20 PFAS detected, 12 were detected in more than half the samples from the two species. The PFSAs were measured at the highest concentrations, with PFOS accounting for 43% of PFSAs and 41% of ΣPFAS in dogs. Those findings are consistent with previous studies of domestic cats in NC where PFOS (G.M = 8.89 ng/mL) and PFHxS (G.M = 6.91 ng/mL) were about twofold higher than the concentrations we observed in pet dogs drinking well water (PFOS G.M = 4.93 ng/mL; PFHxS G.M = 3.26 ng/mL).[33] Further, the median concentrations of PFOS and PFHxS in dogs consuming well water (PFOS 4.65 ng/mL; PFHxS 3.25 ng/mL) were similar to the concentrations found in children residing in Wilmington NC (PFOS 5.1 ng/mL; PFHxS 1.9 ng/mL).[44] This finding suggests that dogs may serve as an important sentinel of household PFAS exposure that is particularly relevant to humans during sensitive developmental windows.[49,50] PFAS concentrations in horse serum was also dominated by PFSAs, with PFOS accounting for 85% of PFSAs and 48% of ΣPFAS, but the total concentrations were threefold lower than the concentrations found in dogs. Compared to dogs, horse serum was enriched for PFEAs; this difference was the result of increased concentrations of NBP2, which is a production intermediate of Nafion fluoropolymer manufacture. Collectively, these findings highlight the utility of companion animal and livestock species as sentinels of PFAS exposure differences inside and outside of the home.

The relative overall composition of PFAS detected in pet serum appears reflective of their primary living environment and source of drinking water. Exposure to an indoor environment was associated with higher ΣPFAS concentrations in dogs, which is consistent with previous reports showing lower concentrations of PFAS in feral cats compared to indoor domestic cats.[33] Notably, dog serum was dominated by PFOS, PFHxS, and PFOA, an exposure profile reflective of the dominant perfluorinated compounds previously reported in human serum samples.[44,51] These similarities likely relate to shared exposure routes within indoor environments including water, house dust, and PFAS treated textiles.[42,43,52,53] While we did not specifically look at PFAS concentrations in dog food, others have reported concentrations in the parts per billion (i.e., ng/g) range for PFOS, PFOA, and PFHxS. Dog food often contains food industry by-products and fish meal, which in part may explain the higher concentrations of these PFAS in dog serum compared to horses.[54–56] Follow-up studies looking at the total daily intake of PFAS from food and water sources in dogs may clarify the contribution of the ambient environment to circulating PFAS concentrations as opposed to dietary sources.[54]

Source of drinking water was also a major contributing factor to serum PFAS concentrations in dogs, with about half of the dogs in this study given well water and the other half provided bottled water for drinking. Dogs whose primary drinking water source was from the well had higher concentrations of PFSAs, mainly PFOS and PFHxS, as compared to dogs given bottled water. Those two PFSAs are consistently detected as the primary contaminants found in biota living near the Cape Fear River watershed, a drinking water source that services up to 1.5 million North Carolinians. Further, PFCAs,

including PFOA, PFHxA, and PFHpA, have also been frequently detected in surface water samples from the Cape Fear River often at concentrations higher than PFSAs.[20] Nevertheless, recent findings from our lab and others have reported PFOS as the PFAS found at the highest concentrations in wildlife and humans that co-utilize the Cape Fear River, potentially demonstrating high degrees of bioaccumulation for this perfluoroalkyl acid and/or decomposition of polyfluoroalkyl compounds into PFOS as a terminal breakdown product.[16,17,21,22,44] Additional studies are needed to determine if these findings are driven by (1) unidentified sources of PFOS exposure, including dietary sources that may retain PFOS (e.g., fish and vegetation), (2) differences in biological half-lives (PFOS > PFOA) in these species, and/or (3) terminal transformation of longer-chain precursors into PFOS that result in the high concentrations detected in serum.[57–59]

In contrast to dogs, horses living in the same area had higher concentrations of NBP2 in their serum and unique, albeit limited, detection of HFPO-DA and PFO5DoDA. Some of these PFEAs, like HFPO-DA, have been shown to have substantially shorter half-lives than their longer-chain predecessors (e.g., PFOA), on the order of hours rather than years. Therefore, it is possible that we only see detectable levels of HFPO-DA and PFO5DoDA in horses due to contamination of the outdoor environment, more time spent outdoors, and consumption of grasses, hay, or other forage, which may contain PFAS that are taken up from contaminated water and soil or deposited from contaminated air and rainwater.[60–64] However, it is also important to consider that differences in the latency between water consumption and the time of blood collection, in conjunction with reduced half-lives of shorter-chain PFAS, may have contributed to the limited detection of HFPO-DA in horses and NVHOS in dogs. This seems particularly relevant in the case of HFPO-DA, where we only see detectable levels in horses that reside at the same household, which also had the highest concentrations of HFPO-DA in well water.

Since NBP2 was first identified in the Cape Fear River in 2017 it has been consistently detected in humans and animals that reside in and around this river basin.[14,16,17,44] To our knowledge, this is the first study to report PFAS exposure in horses. The relatively high frequency of detection of NBP2 (64.2%) and the higher geometric mean concentration of NBP2 in horses (0.57 ng/mL) compared to those in dogs (0.12 ng/mL) emphasize the utility of these animals as sentinels of contaminant exposure in the natural environment from a point source. It is notable that PFHxS, one of the most commonly detected PFAS, was not detected in any of the horses in this study. While more work is needed to understand why PFHxS was not detected in horses, this finding highlights the importance of looking at sentinel species that occupy different environments and may suggests that behavioral or physiological differences of species may contribute to differences in the ability to detect even common PFAS congeners.

In addition to living environment and source of drinking water, we identified sex and body weight as factors influencing serum concentrations for some PFAS. In dogs, we observed a significant effect of sex on NBP2 concentration, with males having higher serum NBP2 concentrations than females, a trend that has been previously shown for other PFEAs in rodent models.[31,40,41] In horses, negative correlations between body weight and serum concentrations of PFDoDA and PFO2HxA were observed. Previously, we reported

similar findings in Striped Bass, with negative correlations for PFOS, PFDA, and NBP2 with increasing body weight and size.[16] While a more robustly powered population study is required to evaluate the biological significance of these relationships in each species, our findings do suggest and support previous studies showing that factors including sex and body weight contribute to differences in absorption, distribution, and/or excretion of PFAS.[25,65]

Our multiple linear regression analysis found few changes across blood chemistry parameters that were correlated with individual PFAS and no significant correlations, except for BUN in dogs, with total PFAS. Within dogs, two key diagnostic biomarkers used to assess liver function, ALP and ALT, were correlated with serum PFAS concentrations. Specifically, positive correlations between ALP and PFHpS and ALT and PFBS were observed. Although ALT was within the normal range for the majority of animals, ALP was found to be above the normal range in 38% of dogs. Previous studies, including studies in domestic dogs, have shown that long-term exposure to PFAS can increase ALP, highlighting the liver as a critical target of PFAS toxicity.[54,66–70] In addition to elevations in ALP, elevated glucose levels may also be a sign of liver toxicity as chronic liver damage can lead to glucose intolerance and even diabetes.[71,72] Glucose levels were above the expected normal range in 21% of animals; however, animals in this study were not fasted prior to blood collection, which may confound the positive relationship observed between PEPA and glucose. Levels of BUN, total protein, and globulin, diagnostic indicators of liver and kidney diseases, were all positively correlated with PFO2HxA, but concentrations of these biomarkers primarily fell within the normal range, except for globulin, which was lower in 31% of dogs. This finding is consistent with numerous human and animals studies that found suppression of adaptive immune activities associated with PFAS; however, future studies looking at markers of both innate and adaptive immune system in these animals are warranted to better characterize the physiological basis for the observed changes in globulins.[73–76] Few studies have evaluated the impact of environmentally relevant mixtures and replacement PFEAs on the kidneys or liver.[16,70,77–79] Our findings provide further suggestive evidence of renal and hepatic sensitivity to PFAS exposure, and have identified ALP, glucose, and globulins as potential biomarkers of environmentally relevant PFAS exposures in dogs.

In horses, biomarkers of liver and kidney function were also associated with serum concentrations of PFAS. Significant correlations between serum PFAS concentrations and total carbon dioxide, creatine kinase, calcium, GGT, and albumin/globulin ratio were observed. Strikingly, albumin concentration was elevated in 62% of horses. Albumin serves as the primary transport protein for a number of PFAS, including PFOS, PFOA, perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), and PFHxS, which may have important ramifications for the half-lives of PFAS and the function and half-life of serum albumin itself.[80–87] Additional samples are needed to establish if there are any interactions between albumin concentrations and PFAS exposure in horses living in the Gray's Creek area. While GGT was elevated for 23% of animals, the other biomarkers, total carbon dioxide, creatine kinase, and calcium, generally fell within a clinically normal range for the majority of horses in this study. Increases in GGT are a general clinical indicator of liver disease; however, the importance of the negative correlation between GGT and

PFDoDA observed in these animals remains unclear but may suggest a subclinical vitamin B6 and magnesium deficiency in the study animals, which we consider unrelated to PFAS exposures.

Companion animals and livestock, like dogs and horses, can provide insight into environmentally relevant human and wildlife exposures due to their shared indoor and outdoor environments. While this sentinel niche has previously been established for dogs, horses have been underutilized in this domain. It is important to reiterate that this study was conducted to explore the potential associations between serum PFAS concentrations and health biomarkers that can be used as clinical indicators of underlying conditions. Therefore, reported associations are unable to define causal relationships. Nonetheless, our findings build upon previous studies of PFAS exposure in sentinel animals and help to establish horses as a useful biomonitoring tool of residential PFAS contamination from a nearby point source. A more robustly powered study, with a greater diversity of participating households, is needed to ascertain the strength of associations between the observed changes in diagnostic biomarkers and PFAS exposure in dogs and horses living in Gray's Creek NC. The ubiquity of domestic animals, availability of diagnostic tools for health assessments, and overlap with human diseases emphasizes the importance of utilizing a "One Health" approach to study shared environmental exposures and adverse health outcomes for humans and animals in the industrialized world.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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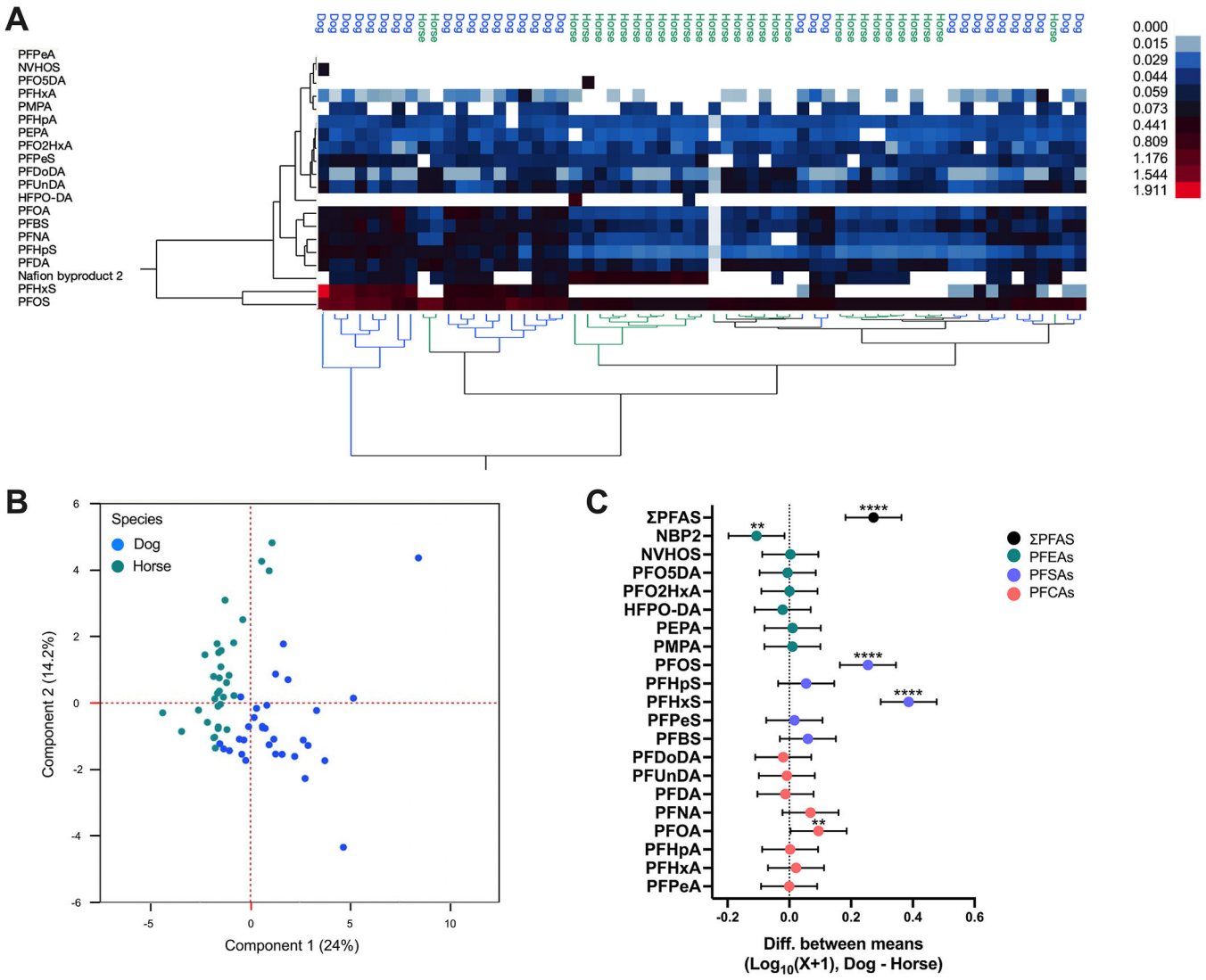

Figure 1.

Comparison of PFAS in dogs and horses. Results of (A) unsupervised hierarchical clustering and (B) principal component analysis of $\mathrm{Log}_{10}(X+1)$ transformed serum PFAS concentrations from dogs and horses. (C) Differences between mean $\mathrm{Log}_{10}(X+1)$ transformed serum PFAS concentrations with 95% confidence intervals, dog–horse (fluoroethers, green; sulfonics, purple; carboxylics, orange; $^{**}p \leq 0.01$; $^{****}p \leq 0.0001$); dog $n = 31$ and horse $n = 32$.

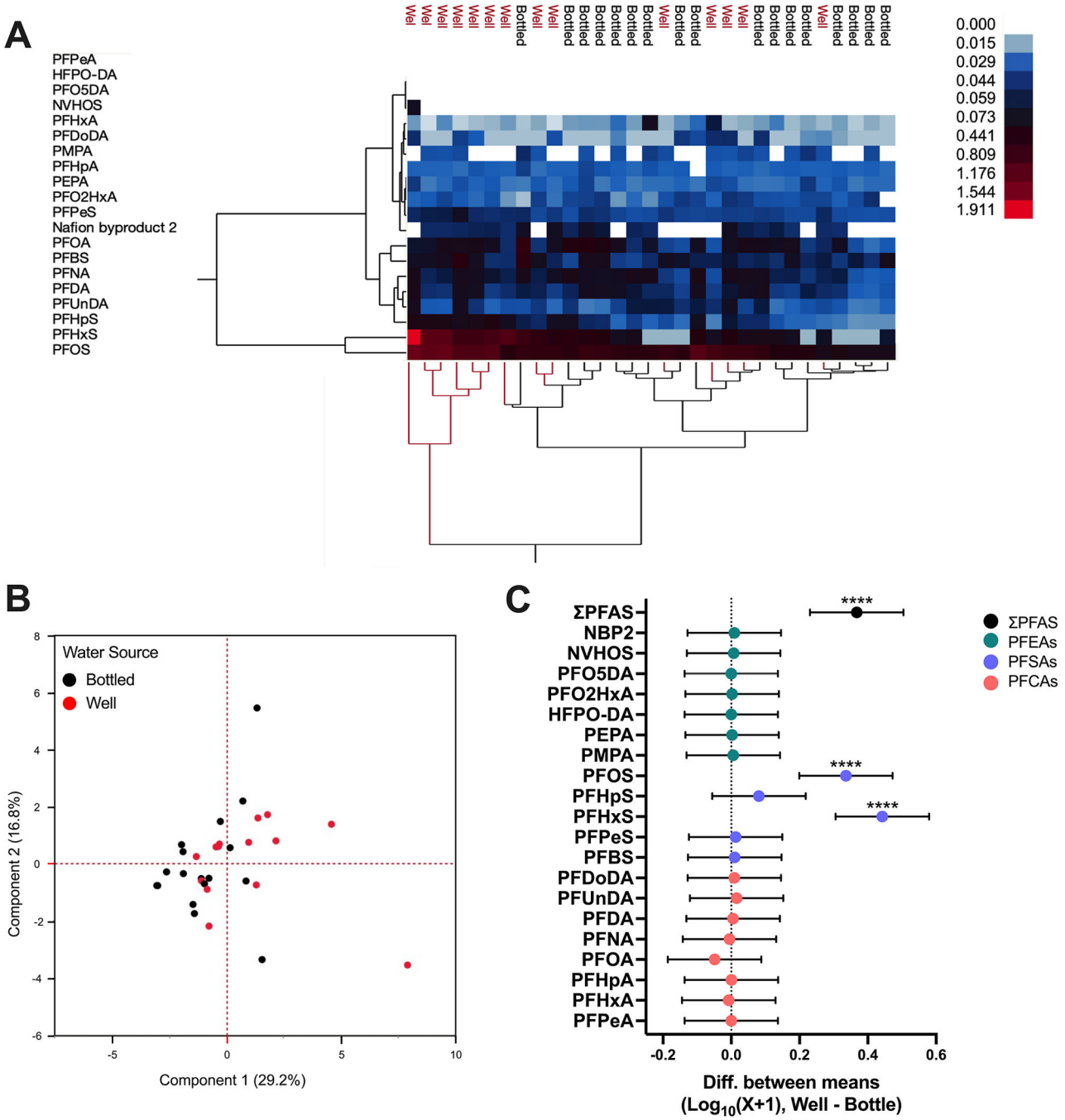

Figure 2.

Comparison of PFAS in dogs' drinking well or bottled water. Results of (A) unsupervised hierarchical clustering and (B) principal component analysis of $\mathrm{Log}_{10}(X+1)$ transformed serum PFAS concentrations from dogs given bottled water and well water. (C) Difference between mean $\mathrm{Log}_{10}(X+1)$ transformed serum PFAS concentrations with 95% confidence intervals, well–bottled (fluoroethers, green; sulfonics, purple; carboxylics, orange; ****$p \leq 0.0001$). Dogs given well water ($n = 14$) compared to bottled water ($n = 17$).

Table 1.

Detection Limits, Detection Frequency, and Concentrations of PFAS in Dog and Horse Serum[a]

PFAS	LOD (ng/mL)	detection frequency (% >LOD)		concentration (ng/mL, GM and range)		detection frequency (% >LOD)		concentration (ng/mL, GM and range)	
		dog (*n* = 31)	horse (*n* = 32)	dog (*n* = 31)	horse (*n* = 32)	dog bottle (*n* = 17)	dog well (*n* = 14)	dog bottle (*n* = 17)	dogwell (*n* = 14)
PFCA									
PFPeA	0.03	0	3	<LOD	<LOD (<LOD-0.05)	0	0	<LOD	<LOD
PFHxA	0.03	94[c]	31	0.064 (0.02–0.34)	0.02 (<LOD-0.05)	100	86	0.07 (0.03–0.34)	0.05 (0.02–0.21)
PFHpA	0.03	97	97	0.097 (<LOD-0.13)	0.09 (0.02–0.11)	94	100	0.10 (<LOD-0.13)	0.10 (0.09–0.11)
PFOA	0.01	100	97	0.37 (0.09–2.63)	0.10(0.01–0.13)	100	100	0.43 (0.09–2.63)	0.28 (0.11–0.55)
PFNA	0.01	100	91	0.28 (0.10–0.64)	0.09 (<LOD-0.16)	100	100	0.29 (0.10–0.40)	0.27 (0.12–0.56)
PFDA	0.01	100	97	0.21 (0.10–0.57)	0.25 (0.01–0.88)	100	100	0.20 (0.10–0.40)	0.22 (0.13–0.57)
PFUnDA	0.04	100	97	0.15 (0.08–0.42)	0.17 (0.03–0.36)	100	100	0.13 (0.08–0.21)	0.17 (0.09–0.42)
PFDoDA	0.05	42	84[c]	0.07 (0.04–0.16)	0.12 (0.04–0.23)	35	50	0.06 (0.04–0.15)	0.08 (0.04–0.16)
PFSA									
PFBS	0.01	100	97	0.30 (0.12–2.08)	0.13 (0.01–0.24)	100	100	0.28 (0.12–2.08)	0.31 (0.15–1.88)
PFPeS	0.02	100[a]	84	0.14 (0.11–0.25)	0.10 (<LOD-0.18)	100	100	0.13 (0.11–0.17)	0.16 (0.12–0.25)
PFHxS	0.06	77[c]	0	1.43 (0.04–80.55)	<LOD	65	93	0.54 (0.04–4.01)	3.26 (0.04–80.55)
PFHpS	0.03	100	97	0.23 (0.06–0.83)	0.09 (0.02–0.27)	100	100	0.13 (0.06–0.33)	0.36 (0.08–0.83)
PFOS	0.77	87[b]	50	2.88 (0.54–17.60)	1.82 (0.54–11.77)	82	93	1.74 (0.54–8.78)	4.93 (0.54–17.60)
PFEA									
PMPA	0.01	55	38	0.06 (<LOD-0.16)	0.04 (<LOD-0.11)	47	64	0.06 (<LOD-0.16)	0.07 (<LOD-0.13
PEPA	0.02	100	89	0.10 (0.08–0.14)	0.07 (<LOD-0.11)	100	100	0.09 (0.08–0.14)	0.10 (0.08–0.14)
HFPO-DA	0.01	0	6	<LOD	0.11 (<LOD-3.25)	0	0	<LOD	<LOD
PFO2HxA	0.05	94	97	0.10 (<LOD-0.15)	0.10 (0.04–0.16)	88	100	0.10 (<LOD-0.15)	0.11 (0.06–0.15)
PFO5DoDA	0.1	0	3	<LOD	0.02 (<LOD-0.49)	0	0	<LOD	<LOD
NVHOS	0.1	3	0	0.007 (<LOD-0.25)	<LOD	0	7	<LOD	0.02 (<LOD-0.25)
NBP2	0.01	65	63	0.12 (ND-0.26)	0.57 (ND-2.38)	59	71	0.11 (<LOD-0.24)	0.13 (<LOD-0.26)
Totals									
ΣPFAS				6.93 (1.74–101.90)	3.75 (1.52–14.02)			4.41 (1.74–13.50)	11.61 (2.58–101.90)

PFAS	LOD (ng/mL)	detection frequency (% >LOD)		concentration (ng/mL, GM and range)		detection frequency (% >LOD)		concentration (ng/mL, GM and range)	
		dog (*n* = 31)	horse (*n* = 32)	dog (*n* = 31)	horse (*n* = 32)	dog bottle (*n* = 17)	dog well (*n* = 14)	dog bottle (*n* = 17)	dogwell (*n* = 14)
ΣPFEAs				0.39 (0.09–0.61)	0.78 (0.14–2.63)			0.36 (0.09–0.61)	0.43 (0.29–0.61)
ΣPFSAs				5.03 (0.91–99.33)	2.13 (0.75–12.19)			2.79 (0.91–10.49)	9.59 (1.25–99.3)
ΣPFCAs				1.23 (0.54–3.62)	0.84 (0.11–1.60)			1.28 (0.54–3.62)	1.16 (0.80–2.08)

[a]Letters indicate statistical differences in detection frequencies across species based on Fisher's exact test. a = $p \leq 0.05$. b = $p \leq 0.01$. c = $p \leq 0.001$.

Abbreviations: GM, geometric mean; LOD, limit of detection; PFCA, perfluorocarboxylic acid; PFSA, perfluorosulfonic acid; PFEA, perfluoroalkyl ether acid.

Table 2.

Correlations between PFAS Concentration [$\text{Log}_{10}(X+1)$] in Dogs and each Biomarker[a]

biomarker	N	PFAS β (95% CI)							
		PFBS	**PFHxS**	**PFHpS**	**PMPA**	**PEPA**	**PFO2HxA**	**nbp_2**	**ΣPFAS**
albumin (g/dL)	29	1.03	−0.22	−1.03	−2.30	−3.26	−1.43	2.74	−2.30
		(−3.13, 5.37)	(−1.10, 0.67)	(−8.52, 6.45)	(−10.64, 6.05)	(−33.03, 26.51)	(−15.78, 12.92)	(−4.79, 10.27)	(−10.64, 6.05)
alkaline phosphatase (U/L)	29	3.07	**−3.79** [b]	**21.12**[a]	−17.58	15.39	−10.49	**−21.46**[a]	−17.58
		(−9.34, 15.47)	**(−6.31, −1.27)**	**(−0.26, 42.50)**	(−41.43, 6.27)	(−69.65, 100.43)	(−51.48, 30.51)	(−42.96, 0.04)	(−41.43, 6.27)
alanine aminotransferase (U/L)	29	**10.93**[a]	−1.33	14.31	0.84	19.78	6.59	−6.21	0.84
		(0.41, 21.45)	(−3.47, 0.81)	(−3.81, 32.44)	(−10.39, 21.06)	(−52.31, 91.88)	(−28.16, 41.35)	(−24.44, 12.02)	(−19.39, 21.06)
amylase (U/L)	29	1678.36	−377.37	245.28	−2453.74	−5551.19	−4001.65	−1669.17	−2453.74
		(−2219.66, 5576.38)	(−1169.45, 414.71)	(−6470.10, 6960.65)	(−9947.19, 5039.71)	(−32,266.75, 21,164.37)	(−16,881.31, 8878.01)	(−8423.44, 5085.10)	(−9947.19, 5039.71)
total bilirubin (mg/dL)	24	3.95	−2.27	3.09	−18.19	25.63	−14.86	−1.16	−18.19
		(−7.43, 15.33)	(−5.82, 1.28)	(−21.47, 27.64)	(−43.90, 7.53)	(−40.76, 92.02)	(−61.31, 31.59)	(−19.83, 17.50)	(−43.90, 7.53)
blood urea nitrogen (mg/dL)	29	1.39	0.42	−2.89	**−9.65**[b]	−12.88	**14.19**[b]	0.46	**−9.65**
		(−2.22, 5.00)	(−0.32, 1.15)	(−9.11, 3.33)	**(−16.59, −2.71)**	(−37.63, 11.87)	**(2.26, 26.13)**	(−5.80, 6.71)	**(−16.59, −2.71)**
calcium (mg/dL)	28	5.11	0.22	3.57	−1.90	22.43	9.46	10.44	−1.90
		(−10.89, 21.10)	(−2.95, 3.38)	(−25.09, 32.22)	(−33.07, 29.27)	(−84.59, 129.45)	(−41.98, 60.91)	(−16.55, 37.44)	(−33.07, 29.27)
phosphorus (mg/dL)	29	−0.81	−0.23	1.80	−0.66	−7.23	−4.93	−3.63	−0.66
		(−3.94, 2.31)	(−0.86, 0.41)	(−3.59, 7.18)	(−6.67, 5.35)	(−28.65, 14.20)	(−15.26, 5.40)	(−9.05, 1.79)	(−6.67, 5.35)
creatinine (mg/dL)	29	−1.38	0.72	−4.23	−1.75	−7.52	12.10	4.79	−1.75
		(−5.59, 2.84)	(−0.13, 1.58)	(−11.49, 3.03)	(−9.85, 6.35)	(−36.40, 21.35)	(−0.183, 26.02)	(−2.52, 12.08)	(−9.85, 6.35)
glucose (mg/dL)	29	1.66	0.35	−0.49	3.18	49.99[a]	−1.29	9.49	3.18
		(−5.19, 8.52)	(−1.05, 1.74)	(−12.30, 11.31)	(−9.99, 16.35)	(3.03, 96.96)	(−23.94, 21.37)	(−2.38, 21.35)	(−9.99, 16.35)
sodium (mmol/L)	29	−0.04	0.00	0.39	0.29	−1.89	0.27	−0.14	0.29
		(−0.34, 0.27)	(−0.06, 0.06)	(−0.14, 0.93)	(−0.31, 0.88)	(−4.01, 0.22)	(−0.75, 1.29)	(−0.67, 0.39)	(−0.31, 0.88)
potassium (mmol/L)	20	10.29	−0.27	7.51	−5.69	−64.57	30.57	−17.47	−5.69
		(−21.32, 41.90)	(−4.37, 3.82)	(−20.53, 35.56)	(−37.59, 26.21)	(−147.09, 17.95)	(−26.34, 87.48)	(−37.70, 2.77)	(−37.59, 26.21)
total Protein (g/dL)	29	8.65	0.69	−1.45	1.02	−44.83	**42.09**[b]	−3.16	1.02

biomarker	N	PFAS β (95% CI)							
		PFBS	**PFHxS**	**PFHpS**	**PMPA**	**PEPA**	**PFO2HxA**	**nbp$_2$**	**ΣPFAS**
		(−0.41, 17.71)	(−1.15, 2.54)	(−17.06, 14.16)	(−16.40, 18.44)	(−106.93, 17.27)	**(12.16, 72.03)**	(−18.86, 12.54)	(−16.40, 18.44)
globulin (g/dL)	29	7.31	0.81	−0.42	3.21	−43.75	**43.03**^{b}	−6.26	3.21
		(−1.07, 15.68)	(−0.89, 2.52)	(−14.85, 14.01)	(−12.89, 19.32)	(−101.16, 13.66)	**(15.36, 70.71)**	(−20.77, 8.26)	(−12.89, 19.32)
		Albumin/Globulin							
ratio	29	−3.37	−0.23	−1.46	−2.38	16.89	**−22.28**^{b}	3.90	−2.38
		(−8.28, 1.53)	(−1.23, 0.76)	(−9.91, 6.99)	(−11.81, 7.05)	(−16.72, 50.51)	**(−38.49, −6.08)**	(−4.59, 12.40)	(−11.81, 7.05)

[a]Data in bold indicate a significant association. Letters indicate p-values with a = $p \leq 0.05$ and b = $p \leq 0.01$. β represents the beta coefficients and CI represents the 95% confidence intervals.

Table 3.

Correlations between PFAS Concentration [$\text{Log}_{10}(X+1)$] in Horses and each Biomarker[a]

biomarker	N	PFAS β (95% CI)				
		PFHpA	**PFOA**	**PFUnDA**	**PFDoDA**	**PFBS**
sodium (mmol/L)	26	−3.30	−1.98	2.39	0.49	1.46
		(−11.88, 5.28)	(−8.45, 4.49)	(−0.88, 5.66)	(−1.78, 2.77)	(−1.02, 3.95)
potassium (mmol/L)	26	−4.96	−66.67	53.16	−4.79	19.33
		(−198.13, 188.22)	(−212.46, 79.12)	(−20.43, 79.12)	(−56.00, 46.43)	(−36.59, 75.24)
total carbon dioxide (mmol/L)	26	**−378.26** [a]	−16.80	110.83	44.28	5.52
		(−758.30, 1.77)	(−303.61, 270.02)	(−33.95, 255.61)	(−56.47, 145.00)	(−104.48, 115.53)
creatine kinase (U/L)	26	13.48	**−53.48** [b]	18.59	−4.38	**19.78** [a]
		(−38.59, 65.55)	**(−92.78, −14.18)**	(−1.25, 38.43)	(−18.18, 9.43)	**(4.71, 34.85)**
glucose (mg/dL)	26	−2869.64	2743.38	−335.55	−192.46	−95.69
		(6596.67, 857.38)	(−69.46, 5556.21)	(−1755.44, 1084.34)	(−1180.54, 795.62)	(−1174.50, 983.13)
calcium (mg/dL)	26	−2.27	−3.49	**5.39** [a]	−0.32	1.93
		(−16.52, 11.98)	(−14,24, 7.27)	**(−0.04, 10.82)**	(−4.10, 3.45)	(−2.20, 6.06)
bBlood urea nitrogen (mg/dL)	26	−187.74	−68.39	37.08	−12.75	61.91
		(−617.09, 241.62)	(−392.43, 255.65)	(−126.49, 200.66)	(−126.58, 101.08)	(−62.37, 186.19)
creatinine (mg/dL)	26	−18.39	11.91	5.23	0.75	6.11
		(−59.30, 22.52)	(−18.97, 42.78)	(−10.36, 20.82)	(−10.10, 11.59)	(−5.74, 17.95)
aspartate Aminotransferase (U/L)	26	−3581.06	−3507.56	3039.93	−1599.30	1411.47
		(−13,041.88, 5879.77)	(−3507.56, 3632.64)	(−564.37, 6644.24)	(−4107.49, 908.89)	(−1327.03, 4149.97)
total bilirubin (mg/dL)	26	3.29	−2.12	1.51	1.09	−0.45
		(−53.03, 59.60)	(−44.62, 40.39)	(−19.95, 23.00)	(−13.84, 16.02)	(−16.76, 15.85)
GGT (U/L)	26	30.38	−15.39	23.92	**−17.46** [a]	2.04
		(−35.65, 96.41)	(−65.22, 34.45)	(−1.24, 49.08)	**(−34.96, 0.05)**	(−17.07, 21.15)
albumin (g/dL)	26	−2.82	−5.74	1.66	2.11	3.26
		(−14.68, 9.04)	(−14.69, 3.21)	(−2.86, 6.18)	(−1.03, 5.26)	(−0.18, 6.69)
total protein (g/dL)	26	52.81	−49.95	3.62	2.33	10.69
		(−69.44, 175.06)	(−142.22, 42.31)	(−42.96, 50.19)	(−30.08, 34.74)	(−24.69, 46.08)
globulin (g/dL)	26	61.39	−29.40	−0.11	−4.76	−0.87

biomarker	N	PFAS β (95% CI)				
		PFHpA	**PFOA**	**PFUnDA**	**PFDoDA**	**PFBS**
		(−34.92, 157.71)	(−102.08, 43.29)	(−36.80, 36.59)	(−30.29, 20.78)	(−28.75, 27.01)
				Albumin/Globulin		
ratio	26	**−37.41** [a]	11.41	2.92	4.76	2.04
		(−72.81, −2.00)	(−15.31, 38.13)	(−10.57, 16.41)	(−4.63, 14.15)	(−8.21, 12.29)

[a]Data in bold indicate a significant association. letters indicate p-values with a = $p < 0.05$ and b = $p < 0.01$. β represents the beta coefficients and CI represents the 95% confidence intervals.