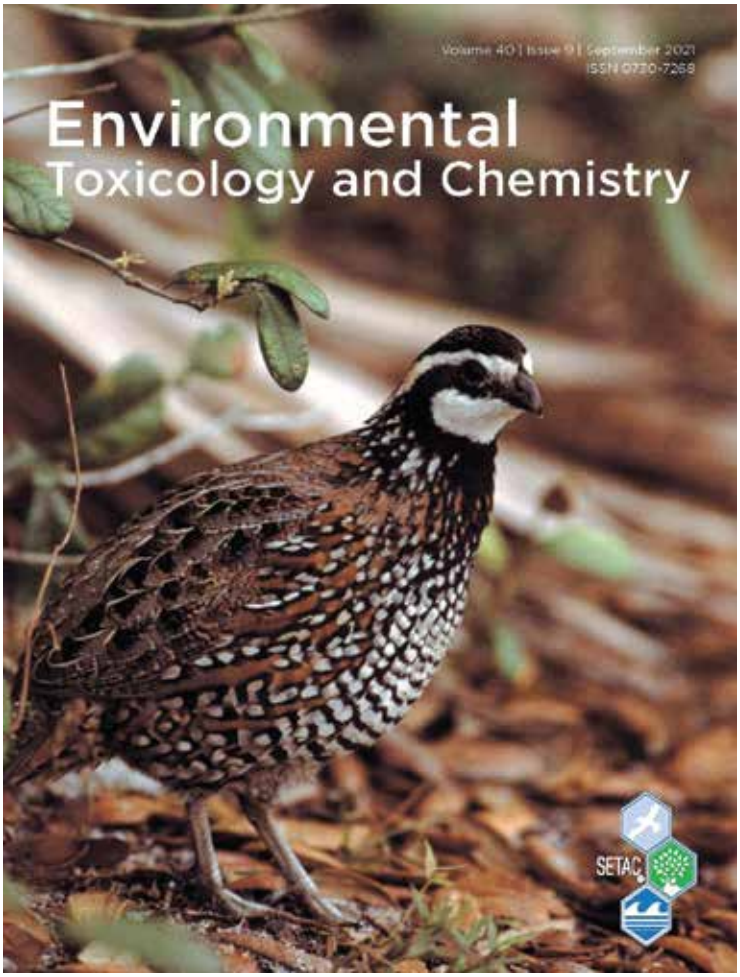
Environmental Toxicology and Chemistry

Volume 40, Issue 9 / p. 2601-2614



Chronic Reproductive Toxicity Thresholds for Northern Bobwhite Quail (Colinus virginianus) Exposed to Per fluorohexanoic Acid (PFHxA) and a Mixture of Per fluorooctane SulfonicAcid (PFOS) and PFHxA

Nicole M. Dennis ✉,Farzana Hossain,
Seenivasan Subbiah,
Adcharee Karnjanapiboonwong,Michael L. Dennis,
Chris McCarthy,Christopher G. Heron,
W. Andrew Jackson,Jordan P. Crago,Jennifer A. Field,
Christopher J. Salice,Todd A. Anderson

First published: 08 June 2021
https://doi.org/10.1002/etc.5135

Abstract

Terrestrial toxicology data are limited for comprehensive ecotoxicological risk assessment of ecosystems contaminated by per- and polyfluoroalkyl substances (PFAS) partly because of their existence as mixtures in the environment. This complicates logistical dose–response modeling and establishment of a

threshold value characterizing the chronic toxicity of PFAS to ecological receptors. We examined reproduction, growth, and survival endpoints using a combination of hypothesis testing and logistical dose–response modeling of northern bobwhite quail (*Colinus virginianus*) exposed to perfluorohexanoic acid (PFHxA) alone and to PFHxA in a binary mixture with perfluorooctane sulfonic acid (PFOS) via the drinking water. The exposure concentration chronic toxicity value (CTV) representative of the lowest-observable–adverse effect level (LOAEL) threshold for chronic oral PFAS toxicity (based on reduced offspring weight and growth rate) was 0.10 ng/mL for PFHxA and 0.06 ng/mL for a PFOS:PFHxA (2.7:1) mixture. These estimates corresponded to an adult

LOAEL average daily intake CTV of 0.0149 and 0.0082 μg × kg body weight^{-1} × d^{-1}, respectively. Neither no-observable–adverse effect level threshold and representative CTVs nor dose–response and predicted effective concentration values could be established for these 2 response variables. The findings indicate that a reaction(s) occurs among the individual PFAS components present in the mixture to alter the potential toxicity, demonstrating that mixture affects avian PFAS toxicity. Thus, chronic oral PFAS toxicity to avian receptors represented as the sum of the individual compound toxicities may not necessarily be the best method for assessing chronic mixture exposure risk at PFAS-contaminated sites.

Environ Toxicol Chem 2021;40:2601–2614.

INTRODUCTION

The ongoing persistence of per- and polyfluoroalkyl substances (PFAS) in nearly every environmental compartment is of major concern because these compounds evoke toxicity in both humans and laboratory animals at environmentally relevant concentrations (see Giesy and Kannan 2001; Moody et al. 2003; Van de Vijver et al. 2005; Haukås et al. 2007; Greaves et al. 2012; Anderson et al. 2016; Agency for Toxic Substances and Disease Registry 2018; Salice et al. 2018; US Environmental Protection Agency 2019; East et al. 2021). Toxicology data for even the most frequently occurring

or abundant PFAS (e.g., perfluorooctane sulfonic acid [PFOS], perfluorohexane sulfonic acid [PFHxS], perfluorooctanoic acid, and perfluorohexanoic acid [PFHxA]) are limited for comprehensive ecotoxicological risk assessment (ERA) of PFAS-contaminated ecosystems (see McCarthy et al. 2017; Agency for Toxic Substances and Disease Registry 2018; Divine et al. 2019; US Environmental Protection Agency 2019; Conder et al. 2020). This general dearth of PFAS toxicity data prompted the US Department of Defense to fund PFAS toxicology research for terrestrial species, generating numerous studies that have since improved our understanding of chronic PFAS toxicology (Strategic Environmental Research and Development Program 2016; Salice et al. 2018; Flynn et al. 2019, 2020, 2021; Dennis

et al. 2020, 2021; East et al. 2021; Rewerts et al. 2021; Suski et al. 2021; Tornabene et al. 2021; Weir and Salice 2021; McCarthy et al. 2021b). A synopsis of these works was provided by Leeson et al. (2021).

Many factors currently exist that complicate the characterization of chronic oral PFAS toxicity to avian ecological receptors at environmentally relevant concentrations and mixture ratios. For example, chemicals like PFAS that are chronically ingested at low experimental doses are probable endocrine-disrupting and carcinogenic compounds and occur in mixtures that often produce nonmonotonic, biphasic, and/or hormetic dose–response relationships that confound traditional dose–response modeling and threshold derivation (Organisation for

Economic Co-operation and Development 2006; Jensen and Leffers 2008; Calabrese 2009; Buck et al. 2011; Anderson et al. 2016; Dennis et al. 2020; Goodrum et al. 2020; Temkin et al. 2020; East et al. 2021; McCarthy et al. 2021a). In addition, findings are inconsistent for the same health endpoint among and between avian field and laboratory PFAS toxicology studies (Newsted et al. 2005; Custer et al. 2012, 2014, 2019; Tartu et al. 2014; Groffen et al. 2019; Dennis et al. 2020, 2021), which complicates interpretation of study results and introduces uncertainty among responses. A final factor to consider is that environmental PFAS mixture concentrations and ratios vary (Anderson et al. 2016; East et al. 2021), making them challenging to mimic in laboratory-based exposure studies. These

factors collectively complicate study design and data analysis and make reproduction of chronic PFAS mixture exposure results increasingly difficult to achieve (Goodrum et al. 2020).

Building on the current body of PFAS toxicology literature, the present study aimed to address these challenges and to provide chronic avian PFAS toxicology data for regulatory purposes and use in ERAs at PFAS-contaminated sites. Specifically, we sought to use hypothesis testing to determine whether chronic toxicity (based on reproduction, growth, or survival endpoints) occurred to a single bird species (representative of upland game birds) exposed to PFHxA or to PFOS mixed with PFHxA at environmentally relevant concentrations and mixture ratios.

From the PFAS exposures where toxicity was observed, we aimed to estimate a species-specific water concentration and average daily intake (ADI) no-observable–adverse effect level (NOAEL) and/or lowest-observable–adverse effect level (LOAEL) chronic toxicity value (CTV) threshold. Via dose–response modeling, we further aimed to provide 10 and 20% effect concentration values (EC10 and EC20, respectively) and corresponding ADIs for the variables producing monotonic dose responses. Finally, we sought to describe any interactions between the test chemicals when administered individually or as mixtures to compare to another oral avian PFAS exposure study (Dennis et al. 2020).

MATERIALS AND METHODS

Study design and equipment

Using methods and equipment described in a recent chronic avian oral PFAS toxicology study (Dennis et al. 2020), the present study used 26 breeding pairs (28 or 32 wk of age) of northern bobwhite quail (*Colinus virginianus*) commercially obtained from outdoor flight pens (Harper's Game Farm). Birds were randomly assigned to a prelabeled section within the batteries on arrival, acclimated, photostimulated, weighed, and chemically exposed as previously described (Dennis et al. 2020) with minor modification (i.e., third weight measurement for adult birds postphotostimulation to distinguish between known growth trends and probable treatment effects). Adult birds were euthanized at 90 d postexposure (dpe), and juveniles were euthanized at 21 d posthatch

(dph) via CO_2 asphyxiation followed by cervical dislocation and placed in storage (–25 °C) for future necropsy, tissue residue analyses, and cataloging purposes. All procedures were approved by the Institutional Animal Care and Use Committee of Texas Tech University (protocol 19103-12).

Quail husbandry

Similar to northern bobwhite quail husbandry methods previously reported (Dennis et al. 2020), adults housed in quail breeding batteries and chicks housed in commercial brooders were observed for general well-being twice daily. Laboratory conditions were recorded once daily to ensure a moderately consistent exposure environment. The temperature and relative humidity (mean ± standard error of the

mean) were monitored daily (68.6 ± 0.1 °C and 30.4 ± 1.1%). All treatment groups were represented in each brooder, and offspring were treated the same throughout the 21-dph observation period (Dennis et al. 2020).

Test chemicals, exposure concentrations, and ADI

The test chemicals (i.e., neat undecafluorohexanoic acid [PFHxA] liquid and heptadecafluorooctanesulfonic acid potassium salt [PFOS]) were purchased from Wellington Laboratories and Sigma-Aldrich at reported purities of ≥97 and 98% and tested analytical purities of 100 and 98%, respectively (Supplemental Data, S1). Following the methods of Dennis et al. (2020), the stock exposure solutions were made in Optima™ liquid chromatography-mass

spectrometry-grade water (Thermo Fisher Scientific), and dilutions were made from the stock solutions using distilled water to reach each nominal exposure concentration and/or mixture ratio (0.1, 1.0, and 5 ng/mL PFHxA and 0.1, 1.0, and 20 ng/mL 4:1 PFOS:PFHxA). The chemically characterized and verified exposure solution concentrations and mixture ratios (Supplemental Data, S1) were used to orally expose adult northern bobwhite quail via drinking water for a duration of 90 d.

The exposure concentrations and ratios included were specifically chosen to bracket PFAS concentrations and mixture ratios present in surface water samples (Filipovic et al. 2015; Anderson et al. 2016; East et al. 2021) for environmental and biological

relevance. As in Dennis et al. (2020), at the end of the study (90 dpe), the amount of water consumed per bird was estimated (i.e., total per pair divided in half), and the ADI of each chemical of interest at each exposure level was calculated in units of micrograms of toxicant per kilogram of body weight per day.

Egg collection, incubation, and hatching

In an effort to continue to reduce the traditional number of experimental animals used in avian reproductive toxicology studies and yet maintain sufficient statistical power (Organisation for Economic Co-operation and Development 1984; Office of Chemical Safety and Pollution Prevention 2012; Dennis et al. 2020), a total of 52 adult *C. virginianus* were used in the present study. Eggs were

collected, incubated, and hatched (Dennis et al. 2020), with an increased relative humidity range (65–75%) during hatching. All hatched offspring (limited thermoregulatory ability) remained in the hatcher utilizing absorbed nutrients for 24 ± 12 h posthatch and were then transferred to a brooder, where their beak was touched to the water source once for initial reference to enhance survival once outside the hatcher (Dennis et al. 2020).

Endpoints, hypothesis testing, and threshold derivation

The biological endpoints studied (reproduction, growth, and survival) and statistical methods employed to characterize the chronic toxicity of PFHxA and a binary PFOS and PFHxA mixture to northern bobwhite quail mirrored endpoints and

analytical methods described in detail elsewhere (Dennis et al. 2020), with the addition of logistical dose–response modeling. For hypothesis testing, each treatment group consisting of 3 replicate pairs from both exposures was compared to the same control group consisting of 6 replicate pairs. Specific response variables were similarly analyzed (with the addition of adult growth rate) using R statistical software (Ver 4.0.2; R Development Core Team 2020; packages plyr, phia, psych, nlme, car, FSA, multcompView, lsmeans, multcomp, ggplot2, rcompanion, dplyr, drc, lattice, Rmisc, ez, DescTools, mvtnorm, survival, TH.data). Parametric testing assumptions were met, and statistical analyses included Pearson's chi-squared tests, Fisher's exact tests, 2-sample *t* tests (paired and unpaired), linear

modeling coupled with an analysis of variance (ANOVA), and linear mixed-effects modeling for repeated measurements (i.e., mean weight at 3 time points) coupled with 2- or 3-factor ANOVAs (type 3, for unbalanced designs). Pearson's chi-squared tests and Fisher's exact tests that resulted in a treatment effect were followed by pairwise post hoc analysis without *p* value adjustment. Any ANOVAs that showed a main effect were subjected to multiple comparisons of means testing using Dunnett contrast post hoc analyses when mean response variables were unequal among treatments. Repeated measures ANOVAs showing a main effect were followed by a post hoc comparison of least square means using a Tukey *p* value adjustment. All 2- and 3-factor ANOVA results that produced an interaction (which often

obscure the main effect results) were further analyzed using the phia package in R.

From hypothesis testing we estimated species-specific NOAEL and/or LOAEL CTVs for each endpoint (reproductive, growth, and survival) from each exposure (PFHxA alone and a binary PFOS:PFHxA mixture) that produced a significant response compared to the control mean. When a significant adverse health effect was present compared to the control group at all 3 treatment levels of an exposure, whether or not the magnitude of response simultaneously increased with treatment level, we established an LOAEL CTV at the lowest treatment level but not an NOAEL CTV for that exposure. When a significant adverse health effect was present at only the highest treatment level of the

exposure relative to the controls, we established an LOAEL CTV at the highest treatment level and an NOAEL CTV at the middle treatment level for each exposure accordingly.

Dose–response analyses and ECx prediction

To provide data useful to risk assessors that also satisfy varying opinions regarding hypothesis testing and regression analysis (Allard et al. 2010; Green et al. 2013; McCarthy et al. 2017; Hill et al. 2018; Divine et al. 2019; Conder et al. 2020; Goodrum et al. 2020), we fitted all endpoints producing a statistical effect relative to the controls to a nonlinear dose–response model (Ritz et al. 2019; drc package; R Development Core Team 2020) and reported the predicted EC10

and EC20 values for each variable that was effectively modeled (see Thursby et al. 1997; Suter et al. 2000, 2005; Field et al. 2002). In the absence of monotonicity, it remains difficult to accurately model dose–response data and subsequently predict EC*x* estimates from the modeled exposure. However, from the EC*x* concentrations obtained, we further estimated a corresponding ADI using average bird weight and water consumed per bird per day from each exposure to support the estimated CTVs.

Regardless of the complexity involved in characterizing the chronic toxicity of PFAS, it remains imperative to estimate environmentally relevant toxicological reference values (TRVs) corresponding to observed toxicity in a model organism for

each chemical exposure studied (whether via hypothesis testing deriving a CTV representative of an NOAEL and/or an LOAEL threshold or via nonlinear regression modeling utilizing an EC x value) to inform chemical regulation and for comprehensive ERA of chemically contaminated areas (Green et al. 2013 ; Hill et al. 2018 ; Goodrum et al. 2020). Because chronic exposure data are limited for most PFAS, we aimed to provide the CTVs and/or EC x values necessary for TRV derivation and completion of comprehensive PFAS ERAs for the PFAS and PFAS mixtures presently studied.

RESULTS AND DISCUSSION

Study design and ADI

It is known that northern bobwhite quail populations in the wild typically only require a water source during drought, reproduction, and significant growth (Hiller et al. 2009; Johnsgard 2017; Texas Parks and Wildlife Department: Bobwhite Quail Management 2017). Thus, for the best possible environmental relevance, a key objective was to determine how exposure of adult northern bobwhite quail during a typical reproductive season (~90 d) via drinking water to these PFAS (i.e., at environmental concentrations and ratios) influenced adult and juvenile growth, egg laying and viability, and offspring survival. On completion of our study, all required quality controls were passed (Supplemental Data, S2) for a valid avian reproductive toxicology study (see Organisation for

Economic Co-operation and Development 1984; Office of Chemical Safety and Pollution Prevention 2012; Valverde-Garcia et al. 2018). Similar to a previous northern bobwhite quail toxicology study (Dennis et al. 2020), ongoing incidents of pecked metatarsals and stripped feathers between pairs in all pens occurred throughout the present study; however, most afflictions were not severe enough to require separation of pairs or application of antibiotic ointment. When separation was necessary (wound healing, up to 48 h), antibiotic ointment was applied once to wounds, and accommodations were made to track individual water consumption during separation.

Based on the verified exposure concentrations, the consumption of drinking water, and the average weight, an ADI was estimated for each bird, and an average was calculated for each treatment level (Table 1). The ADIs for the birds in the present study represent probable environmental exposure ranges currently reported (Flipovic et al. 2015; Anderson et al. 2016; East et al. 2021) and align with our previous ADI values (Dennis et al. 2020) for both the PFOS and the 1.2:1 PFOS:PFHxS mixture exposures over the course of the study.

Table 1. Verified exposure concentration and mixture ratio, body weight by sex, water consumption, and average daily intake for adult northern bobwhite quail (*Colinus virginianus*) from chronic (90-d) oral

perfluorohexanoic acid (PFHxA) or perfluorooctane sulfonic acid:PFHxA exposure via drinking water[a]

Exposure concentration[b]	Sex	Body weight (kg)	Water consumption (mL/bird/d)
Control	F	0.222 ± 0.003	33.9 ± 1.9
	M	0.212	

Exposure concentration[b]	Sex	Body weight (kg)	Water consumption (mL/bird/d)
	M	0.012 0.208 ± 0.016	

[a] Means ± standard error.

[b] Verified PFOS to PFHxA mixture ratios are in parentheses.

PFHxA = perfluorohexanoic acid; PFOS = perfluorooctane sulfonic acid; ADI = average daily intake; NA = not available.

Egg collection, incubation, and hatching

In the present study, egg laying began 2 d prior to PFAS exposure and between 12 and 26 d following photostimulation. In total, we collected 1710 eggs during the 90-d study. A total of 731 eggs were incubated (i.e., set), ranging from 26 to 34 eggs incubated/pair (Table 2). All incubated eggs were laid from 5 to 63 dpe. Of the total 571 chicks hatched, 478 were included in growth analyses (Table 3). Exclusions were due to either chick death prior to the end of the 21-d observation ($n = 19$) or loss of wing band during posthatch observation ($n = 74$). Of the total number of eggs collected for treatment analyses, 28 were too damaged to incubate. The majority of the damaged/cracked eggs

were laid either in the first week of egg production also coinciding with the first week of PFAS exposure (mostly by treatment hens) or beginning 56 d post–initial egg production to the end of the present study by one control pair. To further determine if the damaged treatment eggs were associated with increasing PFAS residues, future residue analysis is planned.

Table 2. Reproductive performance summary for northern bobwhite quail (*Colinus virginianus*) from chronic (90-d) oral perfluorohexanoic acid (PFHxA) and perfluorooctane sulfonic acid:PFHxA mixture toxicity studies

Reproductive parameter	Control (n = 6)	PFHxA low (n = 3)	PFHxA middle (n = 3)
Eggs laid	452	217	208
Eggs set	184	91	89
Viable embryos	183	89	88
Live 21-d embryos	167	79	83
Hatchlings	148	71	67
21-d-old survivors	145	70	65
21-d-old survivors/hen ± SE	24.2 ± 2.4	23.3 ± 0.7	21.7 ± 0.03
Eggs laid/hen ± SE	75 ± 3	72 ± 15	69 ± 1

PFHxA = perfluorohexanoic acid; PFOS = perfluorooctane sulfonic acid; SE = standard error.

Table 3. Northern bobwhite quail (*Colinus virginianus*) treatment means ± standard error from post hoc pairwise statistical comparisons among perfluorohexanoic acid (PFHxA) and perfluorooctane sulfonic acid:PFHxA mixture exposures relative to controls

Reproductive variable	Control (*n* = 6)	PFHxA low (*n* = 3)	PFHxA middle (*n* = 3)	PFI hig (*n* =

Reproductive variable	Control (n = 6)	PFHxA low (n = 3)	PFHxA middle (n = 3)	PFH hig (n =
Adult wt change (g)	6.8 ± 2.3	5.5 ± 2.9	13.4 ± 13.3	7.7 3.5
Adult growth rate (g/d)	0.075 ± 0.026	0.061 ± 0.062	0.149 ± 0.105	0.0 ± 0.0
Cracked eggs (%)	10.8 ± 9.5	0.0*	1.1 ± 1.1*	0.0
Fertility rate (%)	99.5 ± 0.5	98.0 ± 2.0	98.9 ± 1.1	10 0
Hatching success (%)	80.3 ± 6.2	79.0 ± 7.4	75.3 ± 1.0	81 2.9

* Significantly different from control mean.

PFHxA = perfluorohexanoic acid; PFOS = perfluorooctane sulfonic acid.

Endpoints, statistical analyses, thresholds, and dose responses

Adult weight change and growth rate

We examined adult northern bobwhite quail body weight and growth rate during reproduction using measurements over 3 time points (initial, postphotostimulation, and posteuthanization) to confirm the known weight and growth trends and to establish if chronic PFAS exposure affected these trends in reproducing northern bobwhite quail. This

measurement scheme was adopted because 1) sex affects northern bobwhite quail weight change during reproduction in laboratory testing regardless of treatment, 2) female northern bobwhite quail typically weigh more than male northern bobwhite quail during the reproductive season in wild populations, and 3) northern bobwhite quail growth is positively associated with weight gain (Guthery and Koerth 1992; US Environmental Protection Agency 1993; Hiller et al. 2009; Office of Chemical Safety and Pollution Prevention 2012; Johnsgard 2017; Dennis et al. 2020). Because northern bobwhite quail were obtained as young adults, they were likely still growing throughout the present study; thus, heavier postphotostimulation weights than initial weights were expected and confirmed ($t_{47} = -1.93$, $p = 0.030$),

irrespective of sex. Also, because of egg production in females and increased physical activity in males during reproduction, untreated females were expected and found to be heavier (~5 g) than untreated males postphotostimulation (prior to PFAS exposure). However, contrary to anticipated, females were not statistically heavier than males ($t_{46} = 0.848$, $p = 0.201$) immediately following photostimulation, and control females were not significantly heavier than control males ($t_{10} = 0.128$, $p = 0.450$) at the end of the study (Guthery and Koerth 1992; US Environmental Protection Agency 1993; Hiller et al. 2009; Office of Chemical Safety and Pollution Prevention 2012; Johnsgard 2017; Dennis et al. 2020). The absence of difference between the sexes was likely, in part, an artifact of the large variation

observed in individual postphotostimulation weights. This coupled with possible introduced stress from the additional handling to obtain postphotostimulation/preexposure weights could have influenced the results that contrasted with those of a similar northern bobwhite quail chronic oral PFAS study (Dennis et al. 2020).

To determine if there was a main effect of treatment on adult northern bobwhite quail mean weight among groups and to confirm the weight trends over time and between the sexes, we fit a linear mixed-effects model with a general correlation structure (accounting for the repeated measures design) to a 3-factor ANOVA and found a main effect of time on northern bobwhite

quail weight during reproduction ($F_{1,34}$ = 12.9, p = 0.001). This result was expected because weight is positively associated with time and adult quail were still growing throughout the present study period. There was no effect of sex ($F_{1,34}$ = 0.022, p = 0.882) or treatment (Table 3; $F_{6,34}$ = 0.596, p = 0.731) on adult northern bobwhite quail weight, and no interactions were found between time, sex, and/or treatment among the groups (both PFHxA and the PFOS:PFHxA mixture). The absence of effect between the sexes was again likely in part due to the large individual variation in mean weights among groups and the additional animal handling.

To further characterize the chronic effects of PFAS exposure on the growth of northern bobwhite quail during reproduction, we

calculated adult growth rates over the course of treatment (grams per day), ran a 2-factor ANOVA (type 3) on the calculated growth rates, and found that there was no main effect of treatment or sex (Table 3; $F_{6,34}$ = 0.870, p = 0.527; $F_{1,34}$ = 1.38, p = 0.248). There was in addition no confounding interaction found between treatment and sex ($F_{6,34}$ = 1.06, p = 0.405) on growth rates after chronic exposure to these PFAS treatments. Therefore, contrary to our previous study (Dennis et al. 2020), chronic oral avian exposure to the concentrations and compositions of the PFAS presently selected did not affect weight or growth during reproduction.

This was an interesting outcome because compared with the Dennis et al. (2020) study

showing toxicity due to exposure to a mixture of PFOS and PFHxS, the same result did not occur in females exposed to a mixture of PFOS and PFHxA or to females exposed solely to PFOS or PFHxA at similar concentrations. These collective study results demonstrate that chronic exposure to similar concentrations of single PFAS and even simple PFAS mixture solutions does not necessarily produce the same adverse health outcomes. These findings confound the identification of specific interactions occurring between the individual PFAS within the mixtures and continue to complicate the characterization of chronic oral PFAS toxicity to birds.

Egg production and fertility

Egg production among individual female northern bobwhite quail in the present study represented a range of 43 to 90 eggs laid/hen or 0.48 to 1 egg/hen/d, with 70.7 ± 1.4 eggs laid/hen or 0.79 ± 0.02 eggs laid/hen/d averaged over treatments (Table 2). The daily production range bracketed the range (0.59–0.69 egg/hen/d) found by Dennis et al. (2020). Both the average number of eggs laid/hen and eggs laid/hen/d (Table 2) were statistically similar among treatment groups ($F_{6,17} = 0.434$, $p = 0.846$; $F_{6,17} = 0.440$, $p = 0.842$), showing no treatment effect of chronic oral exposure to either PFHxA or a mixture of PFOS and PFHxA at these treatment levels on northern bobwhite quail egg production. These results were similar to previous chronic avian PFAS exposure study

results with PFOS and mixtures of PFOS and PFHxS (Dennis et al. 2020).

Using Fisher's exact test for independence with simulated *p* values (based on 2000 replicates), we found differences in both the percentage of cracked eggs laid and fertilized eggs relative to the total number incubated among treatments (Table 3; $p < 0.001$, $p < 0.001$). Pairwise post hoc analysis (without *p* value adjustment) showed that the control group laid more cracked eggs than the treatment groups except for the 0.58 ng/mL PFOS:PFHxA (2.1:1) mixture group. This suggests that exposure to the PFAS solutions studied had no adverse effect on the physical integrity of northern bobwhite quail eggs. Pairwise post hoc analysis of northern bobwhite quail egg fertility (i.e., viable

embryos/egg set) indicated that hens in the 26.1 ng/mL PFOS:PFHxA (2.8:1) mixture group had a lower fertility rate (Table 2) than other treatment groups, including the control group (Table 3; $p < 0.001$). From hypothesis testing, we found that exposure to PFHxA administered alone at the studied concentrations had no observable effect on northern bobwhite quail egg fertility. We determined that egg fertility had decreased by 20% relative to the controls as a result of chronic oral exposure to the PFOS:PFHxA mixture at an LOAEL exposure concentration CTV of 26.1 ng/mL PFOS:PFHxA (2.8:1), corresponding to an LOAEL ADI CTV of 4.455 $\mu g \times kg\ body\ weight^{-1} \times d^{-1}$. In addition, northern bobwhite quail egg fertility was relatively unaffected at an NOAEL exposure concentration CTV of 0.58 ng/mL PFOS:PFHxA

(2.1:1), corresponding to an NOAEL ADI CTV of 0.0800 μg × kg body weight^{-1} × d^{-1} (Table 4).

Table 4. Mean chronic toxicity value estimates ± standard error representative of an no-observable–adverse effect level and/or an lowest-observable–adverse effect level chronic toxicity threshold for northern bobwhite quail (*Colinus virginianus*) chronically orally exposed to perfluorohexane sulfonic acid (PFHxA) and a perfluorooctane sulfonic acid:PFHxA mixture

Exposure	Endpoint	Specific response	NO exp con CTV

PFHxA	Reproduction	Fertility	NA
		Hatching success	NA
	Survival	Juvenile survival 21 dph	NA
	Growth	Embryonic development	NA
		Juvenile wt 21 dph	NA
		Juvenile growth rate	NA

NOAEL = no-observable–adverse effect level; LOAEL = lowest-observable–adverse effect level; PFHxA = perfluorohexane sulfonic acid; PFOS = perfluorooctane sulfonic acid; CTV = chronic toxicity value; ADI = average daily intake; dph = days posthatch; NA = not available.

Dose–response modeling (Figure 1; log-normal, 2 parameters, drc package; R Development Core Team 2020) of the proportion of fertilized to incubated eggs relative to the control mean (0.995) predicted EC10 and EC20 values of 11.5 and 19.0 ng/mL, respectively, for northern bobwhite quail chronically exposed to the 2.1 to 2.8:1 PFOS:PFHxA mixture (Table 5). These predicted EC10 and EC20 values agree with the exposure concentrations (0.58 and 26.1 ng/mL) producing both the NOAEL and

LOAEL thresholds that represent the estimated CTVs for the PFOS:PFHxA mixture. Furthermore, the estimated corresponding ADIs (1.69 and 2.79 μg × kg body wt^{-1} × d^{-1}) from the predicted effective concentrations were similar to the estimated ADI CTVs derived from hypothesis testing. These results support both the ecological relevance of the hypothesis-testing results and the significance of the logistical regression modeling outcomes for chronic oral PFAS mixture exposure effects on northern bobwhite quail fertility.

Figure 1

Open in figure viewer | PowerPoint

Dose–response curve of the proportion of fertilized to incubated eggs from chronic oral exposure to 2.1 to 2.8:1 perfluorooctane sulfonic acid and to perfluorohexanoic acid mixture solutions. Gray shading represents the 95% confidence interval for the predicted model.

Table 5. Dose–response model predicted effective concentration estimates ± standard error representative of an *x*% effective concentration for northern bobwhite quail (*Colinus virginianus*) chronically orally exposed to perfluorohexane sulfonic acid (PFHxA) and a perfluorooctane sulfonic acid:PFHxA mixture

Exposure	Endpoint	Specific response	EC*x*
PFHxA	Reproduction	Fertility	10

Exposure	Endpoint	Specific response	ECx
		Hatching success	10
			20
	Survival	Juvenile survival 21 dph	10
			20
	Growth	Embryonic development	10
			20
PFOS:PFHxA	Reproduction	Fertility	10

Exposure	Endpoint	Specific	ECx

“Undefined” indicates a negative predicted lower limit. SE = standard error; PFHxA = perfluorohexane sulfonic acid; PFOS = perfluorooctane sulfonic acid; EC*x* = *x*% effective concentration; NA = not available; dph = days posthatch.

Interestingly, this result differs from a previous chronic PFAS exposure study outcome regarding this endpoint (Dennis et al. 2020), where no fertility effects were observed in northern bobwhite quail when exposed to PFOS in a mixture with PFHxS. In addition, no adverse fertility effects were observed that were due to PFOS or PFHxA single-chemical administration at similar exposure concentrations (Dennis et al. 2020;

present study), suggesting differences in the absorption and distribution of individual PFAS when coexposed. These results indicate that PFAS mixtures play an important role in chronic oral PFAS toxicity. This outcome also demonstrates that chronic environmental exposure to complex PFAS mixtures may not simply produce additive toxic responses.

Hatching success

Avian hatching success can vary widely among control birds (up to 45%) during reproductive studies (Organisation for Economic Co-operation and Development 1984; Newsted et al. 2005; Office of Chemical Safety and Pollution Prevention 2012; Valverde-Garcia et al. 2018; Dennis et al. 2020), possibly influencing results concerning this variable. Hatching

success of northern bobwhite quail also varies widely in the natural setting depending on maternal or paternal incubation, temperature, and precipitation (up to 40%; see Sandercock et al. 2008; Rolland et al. 2011). Because of exclusion of failed nests prior to calculation for consistency in reporting, hatching success among field studies may be overestimated and less varied once averaged. This method contrasts laboratory methods that include total eggs incubated (many without regard to internal cracking seen only via candling), possibly lowering hatching success and increasing variation relative to field studies (see Dabbert et al. 1997; Newsted et al. 2005; Dennis et al. 2020). However, untreated northern bobwhite quail in the present study had greater hatching success (i.e., hatchlings/eggs

set; Table 2) than that of controls in previous PFAS reproductive studies (Dabbert et al. 1997, 65%; Newsted et al. 2005, 59%; Dennis et al. 2020, 64%).

Contrary to previous laboratory studies (Newsted et al. 2005; Dennis et al. 2020) and similar to several environmental exposure studies (Custer et al. 2012, 2014; Tartu et al. 2014; Groffen et al. 2019), a negative association was found between treatment and hatching success (Table 3; χ^2 [6] = 34.7, p < 0.001). Post hoc pairwise analyses (without p value adjustment) showed that hatching success was lower in the 26.1 ng/mL PFOS:PFHxA (2.8:1) mixture treatment than in any other group, including controls (p < 0.001) but was not affected (p = 0.924) by even the highest single-chemical treatment

(6.10 ng PFHxA/mL) relative to the controls. From hypothesis testing we found that exposure to PFHxA administered alone at these concentrations had no observable effect on hatching success. We further determined that hatching success had decreased by 23% relative to the controls at an LOAEL exposure concentration CTV of 26.1 ng/mL PFOS:PFHxA (2.8:1), corresponding to an LOAEL ADI CTV of 4.455 $\mu g \times kg\ body\ weight^{-1} \times d^{-1}$. Moreover, we found that northern bobwhite quail hatching success was relatively unaffected at an NOAEL exposure concentration CTV of 0.58 ng/mL PFOS:PFHxA (2.1:1), corresponding to an NOAEL ADI CTV of 0.0800 $\mu g \times kg\ body\ weight^{-1} \times d^{-1}$ (Table 4).

Dose–response modeling (Figure 2; Brain-Cousens, 4 parameters, fixed upper limit at 0.80, drc package; R Development Core Team 2020) of the proportion of hatched to incubated eggs relative to the control mean (0.80) predicted EC10 and EC20 values of 5.56 and 10.7 ng/mL, respectively, for northern bobwhite quail hatching success when chronically exposed to the PFOS:PFHxA (2.1–2.8:1) mixture (Table 5). These predicted effective concentration values align with the PFAS mixture concentrations (0.58 and 26.1 ng/mL) producing the NOAEL and LOAEL chronic toxicity thresholds, respectively, for PFOS:PFHxA mixture exposure effects on northern bobwhite quail hatching success. The estimated corresponding ADIs (0.82 and 1.57 μg × kg body wt^{-1} × d^{-1}) from the predicted effective concentrations were,

again, similar to the estimated ADI CTVs derived from hypothesis testing. These results further support the precision of both CTV and EC*x* estimates for the PFAS mixture exposure.

Figure 2

Open in figure viewer | PowerPoint

Dose–response curve of the proportion of hatched eggs to eggs set from chronic oral exposure to 2.1 to 2.8:1 perfluorooctane sulfonic acid and to perfluorohexanoic acid mixture solutions. Gray shading represents the 95% confidence interval for the predicted model.

A comparison of this variable between similar northern bobwhite quail chronic oral exposure studies demonstrates that

coexposure of PFAS is an important factor influencing hatching success (Dennis et al. 2020; present study). For example, stimulatory hatching effects (i.e., possibly beneficial effects) were previously observed by Dennis et al. (2020) in northern bobwhite quail exposed to a PFOS:PFHxS (1.2–1.8:1) mixture (at 22.9 and 0.958 ng/mL ∑PFAS), but adverse effects on hatching were seen in northern bobwhite quail exposed to a PFOS:PFHxA (2.8:1) mixture (at 19.3 ng PFOS and 6.8 ng PFHxA/mL; i.e., 26.1 ng/mL ∑PFAS). Moreover, hatching success was not affected in northern bobwhite quail exposed to PFOS or PFHxA alone at similar exposure concentrations (Dennis et al. 2020; present study), again suggesting differences in the absorption and distribution of the individual PFAS when coexposed, indicating that PFAS

mixtures play an important role in chronic oral PFAS toxicity to avian receptors. This outcome also demonstrates that exposure to complex PFAS mixtures may not simply produce additive toxic responses.

The large natural variation in hatching success, 2 orders of magnitude difference between NOAEL and LOAEL values that are both within the realm of environmental possibility, and exposure to PFAS as mixtures in the environment could collectively explain the variation witnessed among avian field studies (Custer et al. 2012, 2014, 2019; Tartu et al. 2014; Groffen et al. 2019). These factors could also partially explain the discrepancy noted between field and laboratory studies (Custer 2021) with regard to the association observed between select PFAS

concentrations in eggs and avian hatching success. This variation among avian study outcomes on hatching success highlights the importance of clear reporting in the absence of consistent or updated avian PFAS toxicology guidelines (Organisation for Economic Co-operation and Development 1984; Office of Chemical Safety and Pollution Prevention 2012) and should encourage the examination of a broad range of reproductive health variables (i.e., not solely hatching success) when investigating chronic oral PFAS toxicity.

Arrested embryonic development

As a continuation of our efforts to investigate arrested embryonic development (Dennis et al. 2020), we examined the internal contents (Macklin et al. 2019) of each incubated egg

that did not hatch to determine whether arrested development occurred randomly during incubation or whether PFAS exposure during development affected this variable. Statistical investigation (one-factor ANOVA, type 3) revealed that average day of arrested embryonic development (i.e., arrested development day) was unequal among treatments (Table 3; $F_{6,137} = 3.13$, $p = 0.007$). Post hoc analyses using Dunnett contrasts with single-step p value adjustment showed that northern bobwhite quail embryos arrested development earlier in the 26.1 ng/mL PFOS:PFHxA (2.8:1) group than controls ($p = 0.012$). From hypothesis testing, we found that arrested development was relatively unaffected by in ovo exposure to only PFHxA at the present parental oral exposure concentrations. We further

determined that the stage at which northern bobwhite quail embryos ceased to develop was reduced by nearly 7 d in northern bobwhite quail embryos chronically exposed in ovo to a parental LOAEL exposure concentration CTV of 26.1 ng/mL PFOS:PFHxA (2.8:1), corresponding to an LOAEL ADI CTV of 4.455 μg × kg body weight^{-1} × d^{-1}. In addition, we found no observable difference in arrested development with respect to controls chronically exposed in ovo to a parental NOAEL concentration CTV of 0.58 ng/mL PFOS:PFHxA (2.1:1), corresponding to an NOAEL ADI CTV of 0.0800 μg × kg body weight^{-1} × d^{-1} (Table 4).

Dose–response modeling (Figure 3; log-logistic, 3 parameters, fixed upper limit at 18 d, drc package; R Development Core

Team 2020) of the average northern bobwhite quail arrested embryonic development day relative to controls (18 d) predicted EC10 and EC20 values of 0.697 and 2.31 ng/mL, respectively, when chronically exposed in ovo via parental oral exposure to the 2.1 to 2.8:1 PFOS:PFHxA mixture (Table 5). The undefined (i.e., negative) lower limit of the EC10 and EC20 estimates was likely reflective of the goodness of fit of the model. However, these estimates are within the chronic parental oral exposure concentration range (0.58–26.1 ng/mL) producing the PFOS:PFHxA mixture NOAEL and LOAEL chronic toxicity thresholds for average day of northern bobwhite quail arrested embryonic development. Furthermore, the estimated corresponding ADIs (0.10 and 0.34 μg × kg body wt^{-1} × d^{-1})

from the predicted effective concentrations were similar to the estimated ADI CTVs derived from hypothesis testing. The overlap in values supports the precision of both CTV and EC*x* estimates for the presently studied PFAS mixture.

Figure 3

Open in figure viewer | PowerPoint

Dose–response curve of the mean embryonic arrested development day from chronic oral exposure to 2.1 to 2.8:1 perfluorooctane sulfonic acid and to perfluorohexanoic acid mixture solutions. Gray shading represents the 95% confidence interval for the predicted model.

In a similar northern bobwhite quail exposure study with PFOS and a simple mixture of PFOS and PFHxS, Dennis et al. (2020) showed that PFOS may have affected arrested embryonic development to a greater degree (whether earlier or later arrest) when administered as a single compound versus when administered in a mixture with PFHxS. Conversely, the present study found that a PFOS and PFHxA mixture affected arrested embryonic development (earlier arrest) but that exposure to PFHxA alone did not. This is noteworthy because it appeared as though PFHxS antagonized the toxic effects of PFOS when mixed, whereas PFHxA mixed with PFOS presently did not (Calabrese 1990; Seed et al. 1995; Dennis et al. 2020). Lacking PFHxS exposure data, we cannot confirm the interaction between PFOS

and PFHxS; however, the similar functional groups likely competed for binding at targets (Calabrese 1990; Seed et al. 1995). These collective outcomes support the conclusions that northern bobwhite quail embryos are sensitive to in ovo PFOS exposure and that the severity and direction of the response vary depending on both exposure concentration and co-occurrence of other PFAS (Dennis et al. 2020). Furthermore, if PFHxA underwent similar absorption, distribution, metabolism, and excretion (ADME) processes regardless of coexposure with PFOS and if PFOS did not interact or compete with PFHxA during the ADME processes postexposure, then our results would indicate that multiple mechanisms of PFAS toxicity exist in northern bobwhite quail embryos as a result of in ovo exposure.

Furthermore, these outcomes demonstrate that chronic oral PFAS exposure may not simply induce an additive individual PFAS toxic response in birds with respect to complex mixtures.

Pipped-only proportion

Contrary to previous observations (Dennis et al. 2020), there was no effect due to chronic in ovo exposure at any treatment level, on either the proportion of embryos pipping among the unhatched (Table 3; Fisher's exact test for independence, $p = 0.066$) or the proportion of incomplete versus complete pipping among those pipped ($p = 0.679$). Therefore, unlike PFOS when administered alone and similar to the 1.2:1 PFOS:PFHxS mixture (Dennis et al. 2020), we found that in ovo exposure to PFHxA and a PFOS:PFHxA

mixture at the presently tested concentrations and ratios had no adverse effect on pipping under laboratory conditions. This outcome supports our previous conclusion that the chronic in ovo toxicity of PFOS (based on reduced pipping ability) is reduced when exposed in a PFAS mixture versus when exposed to PFOS alone at environmental concentrations (Dennis et al. 2020) and is therefore not necessarily additive in avian response with respect to PFAS co-exposure.

Juvenile survival rate

Offspring from all treatment groups were incubated, hatched, and housed together. The average juvenile stocking density (145 cm^2/bird) was within the stocking density range (129–258 cm^2/bird) of our previous

study (Dennis et al. 2020) in which juvenile survival was not affected among treatments (91–99%). However, in the present study, juvenile survival (i.e., 21-d survivors/hatchlings) was affected by treatment ($p = 0.004$). Pairwise post hoc analysis showed that mean 21-d juvenile survival (88%) was lower in offspring from the 26.1 ng/mL PFOS:PFHxA (2.8:1) parental exposure group than all other groups (Table 3; $p < 0.001$) but that 21-d juvenile survival was not affected by in ovo exposure to PFHxA. We also determined that 21-d juvenile survival was reduced on average by 9% compared to controls in northern bobwhite quail offspring chronically exposed in ovo to a parental oral LOAEL exposure concentration CTV of 26.1 ng/mL PFOS:PFHxA (2.8:1), corresponding to a parental LOAEL

ADI CTV of 4.455 $\mu g \times kg$ body weigh $t^{-1} \times d^{-1}$. Moreover, we found no di fference in 21-d juvenile survival with respect to controls chronically exposed in ovo to a parental NOAEL exposure concentration CTV of 0.58 ng/mL PFOS:PFHxA (2.1:1), corresponding to a parental NOAEL ADI CTV of 0.0800 $\mu g \times kg$ body weight $^{-1} \times d^{-1}$ (Table 4).

Dose-response modeling (Figure 4; log-logistic, 4 parameters, fixed lower and upper limits [0.50 and 0.97], drc package; R Development Core Team 2020) of the average survival probability of juvenile northern bobwhite quail 21-dph relative to controls (97%) predicted EC10 and EC20 values of 12.7 and 17.1 ng/mL, resp ectively, when chronically exposed in ovo via parental oral exposure to the 2.1 to 2.8:1 PFOS:PFHxA

mixture (Table 5). It is possible that the undefined lower limit of the EC10 and EC20 estimates for this variable is reflective of the large confidence interval describing the model (Figure 4 and Table 5). However, these effective concentrations align with the chronic parental oral exposure concentrations (0.58 and 26.1 ng/mL) producing the PFOS:PFHxA mixture NOAEL and LOAEL chronic toxicity thresholds for the average survival probability of juvenile northern bobwhite quail at 21 dph. The estimated corresponding ADIs (1.87 and 2.51 $\mu g \times kg\ body\ wt^{-1} \times d^{-1}$) from the predicted effective concentrations were similar to the estimated ADI CTVs derived from hypothesis testing. Agreement between these values supports both CTV and EC*x* estimates for the presently studied PFAS mixture.

Figure 4

Open in figure viewer | PowerPoint

Dose–response curve for proportion of juveniles surviving to 21 dph from chronic oral exposure to 2.1 to 2.8:1 perfluorooctane sulfonic acid and to perfluorohexanoic acid mixture solutions. Gray shading represents the 95% confidence interval for the predicted model.

This result was remarkable because juvenile survival was not affected in northern bobwhite quail offspring exposed in ovo to PFOS or PFHxA administered alone or to PFOS coadministered with PFHxS but was affected when exposed at similar exposure concentrations to PFOS coadministered with

PFHxA (Dennis et al. 2020; present study). These collective study outcomes suggest the possible presence of a possible synergistic reaction between PFOS and PFHxA in the mixture with respect to the northern bobwhite quail juvenile survival response resulting from chronic in ovo exposure via parental oral exposure to the PFOS:PFHxA mixture.

Offspring weights and growth rate

Generally, decreased juvenile weight as nestlings (8 dph) or at fledging (ready to fly) and slower juvenile growth rates are predictors of late fledging and reduced recruitment success and survival to adulthood in many avian species (Magrath 1991; Keller and Van Noordwijk 1994; Dawson et al. 2005;

Schwagmeyer and Mock 2007). Therefore, population-level adverse effects could occur as a result of decreased juvenile weight or growth rate. To investigate these variables, we accounted for time (i.e., 0, 7, and 21 dph) as it relates to growth using a repeated measures design (weights taken at 3 time points) by fitting a linear mixed-effects model with general structure correction to a 2-factor ANOVA (type 3).

Results initially showed no effect of treatment (χ^2 [6] = 1.37, $p = 0.968$), a main effect of time (χ^2 [2] = 8718, $p < 0.001$), and an interaction between treatment and time (χ^2 [12] = 92.4, $p < 0.001$) on offspring mean weight. The main effect of time was expected because of the known positive association between growth and days posthatch. Further,

post hoc analysis of the main effect of time on offspring weight (averaged over the levels of treatment with Tukey's *p* value adjustment) confirmed that offspring at 21 dph were heavier than those at both 7 and 0 dph ($p < 0.001$, $p < 0.001$), respectively. Interaction analyses (using phia package in R) indicated that though heavier over time, weight was similar among treatments at hatch and at 7 dph ($p = 0.968$, $p = 0.137$) but unequal among treatments at 21 dph ($p < 0.001$) relative to the controls. Based on the interaction between time and treatment, offspring weight was associated with treatment only at 21 dph.

Additional investigation confirmed (one-factor ANOVA, type 3) that offspring weight at 21 dph was unequal among treatments (*F*

$_{6,471}$ = 9.70, $p < 0.001$). Post hoc exploration (multiple comparisons of means, Dunnett contrasts with single-step p value adjustment) revealed that offspring exposed in ovo to all PFHxA treatment levels (0.10, 1.18, and 6.10 ng/mL) and all 2.1 to 2.8:1 PFOS:PFHxA mixtures (0.06, 0.58, and 26.1 ng/mL) had decreased weights at 21 dph relative to control offspring (Table 3; $p < 0.001$, $p < 0.001$, $p < 0.001$, $p = 0.005$, $p < 0.001$, $p = 0.007$, respectively). Through hypothesis testing we determined that offspring weight at 21 dph decreased by up to 5.6 g because of chronic in ovo exposure to either PFHxA or the PFOS:PFHxA mixture at a parental LOAEL exposure concentration CTV of 0.10 ng/mL PFHxA and 0.06 ng/mL PFOS:PFHxA (2.7:1), corresponding to LOAEL ADI CTVs of 0.0149 and 0.0082 μg × kg body weight^{-1} × d^{-1},

respectively. The magnitude of this effect was larger in northern bobwhite quail exposed to the single chemical versus the mixture at all exposure concentrations (Table 3). Because the effect was seen at the lowest treatment levels, an NOAEL CTV could not be established for offspring weight at 21 dph for either exposure.

To further examine possible treatment effects on growth posthatch (using a one-factor ANOVA, type 3), an average growth rate (grams per day) was calculated for each juvenile bird. Because offspring in the present study were growing and northern bobwhite quail growth is positively associated with time, northern bobwhite quail offspring should have exhibited similar increased growth rates over the 21-d

posthatch observation. Conversely, the average growth rate per day of juvenile northern bobwhite quail was unequal among treatments ($F_{6,471} = 9.70$, $p < 0.001$). Post hoc analysis using Dunnett contrasts for multiple comparisons (with single-step p value adjustment) revealed that offspring from all 3 single-chemical exposure levels (0.10, 1.18, and 6.10 ng/mL PFHxA) and from the middle mixture exposure level (0.58 ng/mL PFOS:PFHxA [2.1:1]) grew slower than controls (Table 3; $p < 0.001$, $p < 0.001$, $p < 0.001$, $p < 0.001$, respectively). From hypothesis testing for the single-chemical exposure, we found that juveniles grew up to 0.24 g/d slower than controls because of chronic in ovo exposure to PFHxA at a parental LOAEL exposure concentration CTV of 0.10 ng/mL PFHxA (corresponding to an

LOAEL ADI CTV of 0.0149 μg × kg body wt^{-1} × d^{-1} [Table 4]). Because the adverse health effect was seen at the lowest PFHxA exposure level tested, an NOAEL and corresponding CTVs for the single-chemical exposure could not be established for juvenile growth rate. Furthermore, because the adverse health effect was observed at only the middle exposure level for the PFAS mixture (Table 3), neither NOAEL and LOAEL thresholds nor corresponding CTVs could be established for the PFOS:PFHxA mixture for juvenile growth rate.

The dose responses could not be logistically modeled for either exposure using the drc package in R for the juvenile growth rate and offspring weight at 21-dph responses; therefore, we could not report EC*x* values for

these variables. Lack of a model fit was likely due to the decreased magnitude of effect at the highest treatment levels of both exposures relative to the lowest treatment levels, which confounded the causal relationship and the ability to predict the EC*x* estimates. This complication illustrates an important difference between hypothesis testing and dose–response modeling with regard to characterizing ecologically relevant, low-dose chemical toxicity involving nonmonotonic dose–response data. In the present study, hypothesis testing provided PFAS LOAEL CTVs for these endpoints useful for both TRV derivation and ERAs of PFAS-contaminated sites, whereas dose–response modeling failed to provide EC*x* estimates useful for the same purposes. Lack of a reliable logistical model fit did not, however,

negate the presence of an adverse health effect relative to the controls. Rather, we assume that this reflected cellular repair mechanisms as a biological reaction to the increasing exposure concentrations. To confirm the ecological relevance of these statistical outcomes, we calculated the magnitudes of the effects (3.6–12.1%) between treatment and control means and further suggest that even though the magnitude of the response did not increase with concentration and a logistical dose response could not be modeled for these 2 variables, the significant response observed at all 3 treatment levels reflected a biologically relevant treatment-related effect. Furthermore, if 10 to 20% is considered the ecological and/or biological relevance marker (Thursby et al. 1997; Suter et al. 2000, 2005;

Field et al. 2002), then these results indicate that possible adverse growth effects in northern bobwhite quail offspring could be present in wild northern bobwhite quail populations currently exposed to similar PFAS solutions.

These results collectively demonstrate that in ovo exposure to both individual PFAS and PFAS mixtures is an important factor influencing the processes involved in avian offspring growth and development. For example, at similar exposure concentrations, stimulatory effects were observed in northern bobwhite quail offspring exposed in ovo to 0.375 ng/mL PFOS:PFHxS (1.2:1) and to 0.216 and 0.596 ng/mL PFOS alone (see Dennis et al. 2020). Conversely, PFHxA alone and the PFOS:PFHxA mixture showed an

adverse effect on northern bobwhite quail offspring weight and growth rate at concentrations at low as 0.10 ng/mL PFHxA and 0.06 ng/mL PFOS:PFHxA (2.7:1). This again indicates that PFAS mixture exposure in ovo plays an important role in characterizing the chronic oral PFAS toxicity to avian ecological receptors. These outcomes combined are remarkable in that they illustrate that PFAS mixtures may potentially alter the mechanisms involved in the absorption and distribution of individual PFAS when co-exposed and consequently their toxic potential, demonstrating that chronic oral avian toxicity may not necessarily be simply additive with respect to PFAS mixtures.

CONCLUSIONS

We provided these chronic oral PFAS toxicology data to inform both future PFAS regulation and ecotoxicological risk to terrestrial receptors at PFAS-contaminated areas. To our knowledge, this collection of work was the first to chronically expose avian receptors orally to binary PFAS mixtures via drinking water and to characterize the threshold of chronic oral toxicity for an avian species to ecologically relevant water exposure concentrations of PFOS, PFHxA, 1.2:1 PFOS:PFHxS, and 2.1 to 2.8:1 PFOS:PFHxA. The estimated water exposure concentration CTV representative of an LOAEL threshold corresponding to chronic oral PFAS toxicity in northern bobwhite quail exposed to PFHxA and a PFOS:PFHxA mixture was 0.10 ng/mL for PFHxA and 0.06 ng/mL for the 2.7:1 PFOS:PFHxA mixture, corresponding

to an adult LOAEL ADI CTV of 0.0149 and 0.0082 μg × kg body weight^{-1} × d^{-1}, respectively. The adverse health effects observed that could occur in wild populations at these water exposure and ADI levels include reduced offspring body weight at 21 dph and/or slower growth rates from hatch to 21 dph, both of which could manifest as relevant population-level effects. The predicted EC10 and EC20 values for reproductive and survival endpoints ranged from 0.70 to 19.0 ng/mL, corresponding to an ADI of 0.10 to 2.79 μg × kg body weight^{-1} × d^{-1}. The theoretical EC10 and EC20 values and corresponding ADIs are similar to the NOAEL and LOAEL exposure concentration and ADI CTVs, supporting both the ecological relevance of the CTV estimates and the

significance of the EC*x* estimates presently provided.

These collective study outcomes reinforce our previous conclusions that multiple mechanisms of chronic toxicity may exist in birds chronically exposed to PFAS depending on the dose and co-occurrence with other PFAS and that an interacting or competing effect may be present in PFAS mixtures that potentially alters the ADME of individual PFAS when coexposed. Furthermore, our results demonstrate that interactions occur among the individual PFAS present in a PFAS mixture to alter the potential toxicity of the individual PFAS present in the mixture to exposed bird populations. Therefore, chronic oral PFAS toxicity to avian receptors, represented as simply the sum of the individual toxicities of

the measured PFAS, may not be a suitable method for assessing chronic PFAS mixture exposure risk to avian receptors at PFAS-contaminated sites. These novel chronic PFAS toxicology study outcomes for terrestrial vertebrates suggest that even the most current publications that derived PFAS TRVs for use in ERAs at PFAS-contaminated sites may have underestimated the chronic oral toxicity of select PFAS and their mixtures to terrestrial ecological receptors (e.g., upland game birds) and could possibly benefit from reassessment.

Finally, the class of adverse health effects and the nonmonotonic relationships produced in northern bobwhite quail chronic oral exposure reproductive toxicology studies support the notion that PFAS are endocrine-

disrupting compounds. As such, hypothesis testing may provide information for risk assessors that logistical modeling cannot. Therefore, best practice when analyzing chronic exposure results should include hypothesis testing followed by logistical dose–response modeling for a broader picture of chronic oral PFAS exposure toxicity risk to ecological receptors. Future study should include additional exposure concentrations in areas of multiple curve inflections to fit a more robust model and more readily estimate EC*x* values to support CTV estimates from hypothesis testing.

Supplemental Data

The Supplemental Data are available on the Wiley Online Library at https://doi.org/10.1002/etc.5135.

Acknowledgment

We appreciate the practical support we received from B. Dabbert at Quail Tech Alliance (without which we would have had many fewer chicks to analyze because of the lack or loss of a wing band). This project was funded by the Strategic Environmental Research and Development Program (ER-2627). This project was approved by the Texas Tech University Institutional Animal Care and Use Committee (protocol 19103-12). The authors are unaware of any existing conflicts of interest.

Author Contributions Statement

N.M. Dennis assisted in study design; led the study; collected, processed, and analyzed the

data according to approved and modified study methods; and wrote the full manuscript; F. Hossain and A. Karnjanapiboonwong assisted with bird husbandry; S. Subbiah assisted with bird husbandry and provided PFAS analytical support; M.L. Dennis assisted with bird husbandry and commented on the draft manuscript; C. McCarthy offered working advice on ERA and provided comment on the draft manuscript. W.A. Jackson and J.P. Crago commented on the draft manuscript; C.G. Heron provided PFAS analysis of exposure water; J.A. Field supervised PFAS analytical testing; C.J. Salice secured project funding, assisted in study design, provided expertise in the area of ecotoxicology, and commented on the draft manuscript; T.A. Anderson secured project funding, assisted in study

design, provided guidance in the area of environmental analytical toxicology for data analyses, and commented on the draft manuscript.

Open Research

Data Availability Statement

Data, associated metadata, and calculation tools are available from the corresponding author (n.dennis@ttu.edu).

This article has earned an Open Data badge for making publicly available the digitally shareable data necessary to reproduce the

reported results. The data are available at https://doi.org/10.6084/m9.figshare.14256446 . Learn more about the Open Practices badges from the Center for Open Science: https://osf.io/tvyxz/wiki .

Supporting Information

This article includes online-only Supplemental Data.

Filename	Description

Filename	Description
etc5135-sup-0001-PFAS_chronic_tox_SI_revised.docx 27 KB	Supporting information.

Please note: The publisher is not responsible for the content or functionality of any supporting information supplied by the authors. Any queries (other than missing content) should be directed to the corresponding author for the article.

REFERENCES

Agency for Toxic Substances and Disease Registry. 2018. Toxicological profile for

perfluoroalkyls. Draft for public comment, *Atlanta*, GA, USA.
Google Scholar

Allard P, Fairbrother A, Hope BK, Hull RN, Johnson MS, Kapustka L, Mann G, McDonald B, Sample BE. 2010. Recommendations for the development and application of wildlife toxicity reference values. *Integr Environ Assess Manag* **6**: 28– 37.
Wiley Online Library | CAS | PubMed | Google Scholar

Anderson RH, Long GC, Porter RC, Anderson JK. 2016. Occurrence of select perfluoroalkyl substances at US Air Force aqueous film–forming foam release sites other than fire-training areas: Field-validation of critical fate and transport properties. *Chemosphere* **150**: 678– 685.
Crossref | CAS | PubMed | Web of Science® | Google Scholar

Buck RC, Franklin J, Berger U, Conder JM, Cousins IT, De Voogt P, Jensen AA, Kannan K, Mabury SA, van Leeuwen SP. 2011. Perfluoroalkyl and polyfluoroalkyl substances in the environment: Terminology, classification, and origins. *Integr Environ Assess Manag* **7**: 513– 541. Wiley Online Library | CAS | PubMed | Google Scholar

Calabrese EJ. 1990. *Multiple Chemical Interactions*. CRC, Boca Raton, FL, USA. Google Scholar

Calabrese EJ. 2009. Getting the dose–response wrong: Why hormesis became marginalized and the threshold model accepted. *Arch Toxicol* **83**: 227– 247. Crossref | CAS | PubMed | Web of Science® | Google Scholar

Conder J, Arblaster J, Larson E, Brown J, Higgins C. 2020. Guidance for assessing the ecological risks of PFASs to threatened and endangered species at aqueous film forming foam-impacted sites. Department of Defense, Strategic Environmental Research and Development Program Project ER18-1614. Alexandria, VA, USA.
Google Scholar

Custer CM. 2021. Linking field and laboratory studies: Reproductive effects of perfluorinated substances on avian populations. *Integr Environ Assess Manag*, in press. https://doi.org/10.1002/ieam.4394
Wiley Online Library | PubMed | Web of Science® | Google Scholar

Custer CM, Custer TW, Delaney R, Dummer PM, Schultz S, Karouna-Renier N. 2019. Perfluoroalkyl contaminant exposure and effects in tree swallows nesting at Clarks

Marsh, Oscoda, Michigan, USA. *Arch Environ Contam Toxicol* **77**: 1– 13.
Crossref | CAS | PubMed | Web of Science® | Google Scholar

Custer CM, Custer TW, Dummer PM, Etterson MA, Thogmartin WE, Wu Q, Kannan K, Trowbridge A, McKann PC. 2014. Exposure and effects of perfluoroalkyl substances in tree swallows nesting in Minnesota and Wisconsin, USA. *Arch Environ Contam Toxicol* **66**: 120– 138.
Crossref | CAS | PubMed | Web of Science® | Google Scholar

Custer CM, Custer TW, Schoenfuss HL, Poganski BH, Solem L. 2012. Exposure and effects of perfluoroalkyl compounds on tree swallows nesting at Lake Johanna in east central Minnesota, USA. *Reprod Toxicol* **33**: 556– 562.
Crossref | CAS | PubMed | Web of Science® | Google Scholar

Dabbert CB, Mitchell RB, Oberheu DT. 1997. Northern bobwhite egg hatchability and chick immunocompetence following a field application of clopyralid. *Bull Environ Contam Toxicol* **58**: 801– 806.
Crossref | CAS | PubMed | Web of Science® | Google Scholar

Dawson RD, Lawrie CC, O'Brien EL. 2005. The importance of microclimate variation in determining size, growth and survival of avian offspring: Experimental evidence from a cavity nesting passerine. *Oecologia* **144**: 499– 507.
Crossref | PubMed | Web of Science® | Google Scholar

Dennis NM, Karnjanapiboonwong A, Subbiah S, Rewerts JN, Field JA, McCarthy C, Salice CJ, Anderson TA. 2020. Chronic reproductive toxicity of perfluorooctane sulfonic acid and a simple mixture of

perfluorooctane sulfonic acid and perfluorohexane sulfonic acid to northern bobwhite quail (*Colinus virginianus*). *Environ Toxicol Chem* **39**: 1101– 1111.
Wiley Online Library | CAS | PubMed | Web of Science® | Google Scholar

Dennis NM, Subbiah S, Karnjanapiboonwong A, Dennis ML, McCarthy C, Salice CJ, Anderson TA. 2021. Species- and tissue-specific avian chronic toxicity values (CTVs) for perfluorooctane sulfonate (PFOS) and a binary mixture of PFOS and perfluorohexane sulfonate. *Environ Toxicol Chem* **40**: 899– 909.
Wiley Online Library | CAS | PubMed | Web of Science® | Google Scholar

Divine C, Zodrow JM, Frenchmeyer M, Dally K, Osborn E, Anderson P. 2019. Approach for assessing PFAS risk to threatened and endangered species. Strategic Environmental Research and Development

Program Project ER18-1653. Alexandria, VA, USA.
Google Scholar

East A, Anderson RH, Salice CJ. 2021. Per- and polyfluoroalkyl substances (PFAS) in surface water near US Air Force bases: Prioritizing individual chemicals and mixtures for toxicity testing and risk assessment. *Environ Toxicol Chem* **40**: 859–870.
Wiley Online Library | CAS | PubMed | Web of Science® | Google Scholar

Field LJ, MacDonald DD, Norton SB, Ingersoll CG, Severn CG, Smorong D, Lindskoog R. 2002. Predicting amphipod toxicity from sediment chemistry using logistic regression models. *Environ Toxicol Chem* **21**: 1993–2005.
Wiley Online Library | CAS | PubMed | Web of Science® | Google Scholar

Filipovic M , Woldegiorgis A , Norström K , Bibi M, Lindberg M , Österås AH . 2015 . Historical usage of aqueous film forming foam: A case study of the widespread distribution of per fluoroalkyl acids from a military airport to groundwater, lakes, soils and fish . Chemosphere 129 : 39 – 45 .
Crossref | CAS | PubMed | Web of Science® | Google Scholar

Flynn RW , Chislock MF , Gannon ME , Bauer SJ, Tornabene BJ , Hoverman JT , Sepúlveda MS . 2019 . Acute and chronic e ffects of per fluoroalkyl substance mixtures on larval American bullfrogs (Rana catesbeiana). Chemosphere 236 : 43 – 50 .
Crossref | Web of Science® | Google Scholar

Flynn RW , Hoskins TD , Iacchetta M , de Perre C, Lee LS , Hoverman JT , Sepulveda MS . 2020 . Dietary exposure and accumulation of per- and poly fluoroalkyl substances alters

growth and reduces body condition of post-metamorphic salamanders. *Sci Total Environ* **765**:142730.
Crossref | PubMed | Web of Science® | Google Scholar

Flynn RW, Iacchetta M, de Perre C, Lee L, Sepúlveda MS, Hoverman JT. 2021. Chronic per-/polyfluoroalkyl substance exposure under environmentally relevant conditions delays development in northern leopard frog (*Rana pipiens*) larvae. *Environ Toxicol Chem* **40**: 711– 716.
Wiley Online Library | CAS | PubMed | Web of Science® | Google Scholar

Giesy JP, Kannan K. 2001. Global distribution of perfluorooctane sulfonate in wildlife. *Environ Sci Technol* **35**: 1339– 1342.
Crossref | CAS | PubMed | Web of Science® | Google Scholar

Goodrum PE , Anderson JK , Luz AL , Ansell GK . 2020 . Application of a framework for grouping and mixtures toxicity assessment of PFAS: A closer examination of dose additivity approaches . Toxicol Sci 179 : 262 – 278 .
Crossref | Web of Science® | Google Scholar

Greaves AK , Letcher RJ , Sonne C , Dietz R , Born EW . 2012 . Tissue-speci fic concentrations and patterns of per fluoroalkyl carboxylates and sulfonates in East Greenland polar bears . Environ Sci Technol 46 : 11575 – 11583 .
Crossref | CAS | PubMed | Web of Science® | Google Scholar

Green JW , Springer TA , Staveley JP . 2013 . The drive to ban the NOEC/LOEC in favor of ECx is misguided and misinformed . Integr Environ Assess Manag 9 : 12 – 16 .

Wiley Online Library | CAS | PubMed | Web of Science® | Google Scholar

Gro ffen T , Lasters R , Lopez-Antia A , Prinsen E, Bervoets L , Eens M . 2019 . Limited reproductive impairment in a passerine bird species exposed along a per fluoroalkyl acid (PFAA) pollution gradient . Sci Total Environ 652 : 718 – 728 .
Crossref | PubMed | Web of Science® | Google Scholar

Guthery FS , Koerth NE . 1992 . Substandard water intake and inhibition of bobwhite reproduction during drought . J Wildl Manage 56 : 760 – 768 .
Crossref | Web of Science® | Google Scholar

Haukås M , Berger U , Hop H , Gulliksen B , Gabrielsen GW . 2007 . Bioaccumulation of per- and poly fluorinated alkyl substances

(PFAS) in selected species from the Barents Sea food web . Environ Pollut 148 : 360 – 371 .
Crossref | CAS | PubMed | Web of Science® | Google Scholar

Hill CE , Myers JP , Vandenberg LN . 2018 . Nonmonotonic dose–response curves occur in dose ranges that are relevant to regulatory decision-making . Dose Response 16 :1559325818798282.
Crossref | Web of Science® | Google Scholar

Hiller TL , Felix AB , Guthery FS . 2009 . Association of northern bobwhites with surface water in the semi-arid Texas panhandle . Wilson J Ornithol 121 : 135 – 140 .
Crossref | Web of Science® | Google Scholar

Jensen AA , Le ffers H . 2008 . Emerging endocrine disrupters: Per fluoroalkylated

substances . Int J Androl 31: 161 – 169.
Wiley Online Library | CAS | PubMed | Web of Science® | Google Scholar

Johnsgard PA. 2017. The North American Quails, Partridges, and Pheasants. Zea E-Books, Lincoln, NE, USA.
Crossref | Google Scholar

Keller LF, Van, Noordwijk AJ. 1994. Effects of local environmental conditions on nestling growth in the great tit Parus major. Ardea 82: 349 – 362.
Web of Science® | Google Scholar

Leeson A, Anderson RH, Thompson T. 2021. Managing aqueous film–forming foam (AFFF)–derived PFAS at Department of Defense sites. Environ Toxicol Chem 40: 23.
Wiley Online Library | CAS | PubMed | Web of Science® | Google Scholar

Macklin KS , Blake JP , Hess JB , Berry WD , Voitle RA , Hess TA. 2019 . Bobwhite quail embryo development. ANR-1410. Alabama Cooperative Extension System, Auburn University, Auburn, AL, USA.
Google Scholar

Magrath RD . 1991 . Nestling weight and juvenile survival in the blackbird, Turdus merula . J Anim Ecol 60: 335 – 351 .
Crossref | Web of Science® | Google Scholar

McCarthy C , Kappleman W , DiGuiseppi W . 2017 . Ecological considerations of per- and poly fluoroalkyl substances (PFAS) . Curr Pollut Rep 3: 289 – 301 .
Crossref | CAS | Web of Science® | Google Scholar

McCarthy CM , Roark SA , Middleton ET . 2021a . Considerations for toxicity

experiments and risk assessments with PFAS mixtures . Integr Environ Assess Manag , in press. https://doi.org/10.1002/ieam.4415
Wiley Online Library | PubMed | Web of Science® | Google Scholar

McCarthy CM , Roark SA , Wright D , O'Neal K , Salice CJ , Muckey B , Stanaway M , Rewerts JN, Field JA, Anderson TA . 2021b . Toxicological response of Chironomus dilutus in single chemical and binary mixture exposure experiments with six per fluoralkyl substances . Environ Toxicol Chem , in press. https://doi.org/10.1002/etc.5066
Wiley Online Library | Web of Science® | Google Scholar

Moody CA , Hebert GN , Strauss SH , Field JA . 2003 . Occurrence and persistence of per fluorooctanesulfonate and other per fluorinated surfactants in groundwater at a fire-training area at Wurtsmith Air Force

Base, Michigan, USA . J Environ Monit 5: 341 – 345 .
Crossref | CAS | PubMed | Web of Science® | Google Scholar

Newsted JL , Jones PD , Coady K , Giesy JP . 2005 . Avian toxicity reference values for per fluorooctane sulfonate . Environ Sci Technol 39: 9357 – 9362 .
Crossref | CAS | PubMed | Web of Science® | Google Scholar

Office of Chemical Safety and Pollution Preventio n. 2012 . Ecological e ffects test guidelines. EPA 712-C-023. Washington, DC.
Google Scholar

Organisation for Economic Co-operation and Development . 1984 . Test No. 206: Avian reproduction test. OECD Guidelines for the Testing of Chemicals . Paris, France.
Google Scholar

Organisation for Economic Co-operation and Development . 2006 . Current approaches in the statistical analysis of ecotoxicology data: A guidance to application . Series on Testing and Assessment . Paris, France.
Google Scholar

R Development Core Team . 2020 . R: A Language and Environment for Statistical Computing, Ver 4.0.2. R Foundation for Statistical Computing, Vienna, Austria.
Google Scholar

Rewerts JN , Christie EC , Robel AE , Anderson TA, McCarthy C , Salice CJ , Field JA . 2021 . Key considerations for accurate exposures in toxicological assessments of per fluorinated carboxylates and sulfonates . Environ Toxicol Chem 40: 677 – 688 .
Wiley Online Library | CAS | PubMed | Web of Science® | Google Scholar

Ritz C , Jensen SM , Gerhard D , Streibig JC . 2019 . Dose-Response Analysis Using R . CRC, Boca Raton, FL, USA.
Google Scholar

Rolland V , Hostetler JA , Hines TC , Percival HF , Oli MK . 2011 . Factors in fluencing reproductive performance of northern bobwhite in south Florida . Eur J Wildl Res 57 : 717 – 727 .
Crossref | Web of Science® | Google Scholar

Salice CJ , Anderson TA , Anderson RH , Olson AD . 2018 . Ecological risk assessment of per fluooroctane sulfonate to aquatic fauna from a bayou adjacent to former fire training areas at a US Air Force installation . Environ Toxicol Chem 37 : 2198 – 2209 .
Wiley Online Library | CAS | PubMed | Web of Science® | Google Scholar

Sandercock BK , Jensen WE , Williams CK , Applegate RD . 2008 . Demographic sensitivity of population change in northern bobwhite . J Wildl Manage 72 : 970 – 982 .
Wiley Online Library | Web of Science® | Google Scholar

Schwagmeyer PL , Mock DW . 2008 . Parental provisioning and o ffspring fitness: Size matters . Anim Behav 75 :291–298.
Crossref | Web of Science® | Google Scholar

Seed J , Brown RP , Olin SS , Foran JA . 1995 . Chemical mixtures: Current risk assessment methodologies and future directions . Regul Toxicol Pharmacol 22 : 76 – 94 .
Crossref | CAS | PubMed | Web of Science® | Google Scholar

Strategic Environmental Research and Development Program . 2016 . Ecotoxicity of

per fluorinated compounds: FY 2016 statement of need. ERSON-16-02. Alexandria, VA, USA.
Google Scholar

Suski JG , Salice CJ , Chanov MK , Ayers J , Rewarts J , Field J . 2021 . Sensitivity and accumulation of per fluorooctanesulfonate (PFOS) and per fluorohexanesulfonic acid (PFHxS) in fathead minnows (Pimephales promelas) exposed over critical life stages of reproduction and development . Environ Toxicol Chem 40 : 811 – 819 .
Wiley Online Library | CAS | PubMed | Web of Science® | Google Scholar

Suter II GW , Efroymson RA , Sample BE , Jones DS . 2000 . Ecological Risk Assessment for Contaminated Sites . CRC, Boca Raton, FL, USA.
Google Scholar

Suter II GW , Norton SB , Fairbrother A . 2005 . Individuals versus organisms versus populations in the de finition of ecological assessment endpoints . Integr Environ Assess Manag 1:397–400.
Google Scholar

Tartu S , Gabrielsen GW , Blévin P , Ellis H , Bustnes JO , Herzke D , Chastel O . 2014 . Endocrine and fitness correlates of long-chain per fluorinated carboxylates exposure in Arctic breeding black-legged kittiwakes . Environ Sci Technol 48: 13504 – 13510 .
Crossref | CAS | PubMed | Web of Science® | Google Scholar

Temkin AM , Hocevar BA , Andrews DQ , Naidenko OV , Kamendulis LM . 2020 . Application of the key characteristics of carcinogens to per and poly fluoroalkyl substances . Int J Environ Res Public Health 17: 1668 .

Crossref | CAS | Web of Science® | Google Scholar

Texas Parks and Wildlife Department . 2017 . Bobwhite Quail Management. Austin, TX. [cited 2021 July 1]. Available from https://tpwd.texas.gov/landwater/land/habitats/high_plains/upland_game/bobwhite.phtml
Google Scholar

Thursby GB , Heltshe J , Scott KJ . 1997 . Revised approach to toxicity test acceptability criteria using a statistical performance assessment . Environ Toxicol Chem 16:1322–1329.
Google Scholar

Tornabene BJ , Chislock MF , Gannon ME , Sepúlveda MS , Hoverman JT. 2021 . Relative acute toxicity of three per-and poly fluoroalkyl substances on nine species

of larval amphibians. Integr Environ Assess Manag , in press. https://doi.org/10.1002/ieam.4391 .
Google Scholar

US Environmental Protection Agency . 2019 . EPA's per- and poly fluoroalkyl substances (PFAS) action plan , EPA 823-R-18-004 , Washington, DC .
Google Scholar

Valverde-Garcia P , Springer T , Kramer V , Foudoulakis M , Wheeler JR . 2018 . An avian reproduction study historical control database: A tool for data interpretation . Regul Toxicol Pharmacol 92 : 295 – 302 .
Crossref | PubMed | Web of Science® | Google Scholar

Van de Vijver KI , Ho ff P , Das K , Brasseur S , Van Dongen W , Esmans E , Reijnders P , Blust R, De Coen W . 2005 . Tissue distribution of

per fluorinated chemicals in harbor seals (Phoca vitulina) from the Dutch Wadden Sea . Environ Sci Technol 39: 6978 – 6984 .
Crossref | CAS | PubMed | Web of Science® | Google Scholar

Weir SM , Salice CJ . 2021 . Investigating potential toxic e ffects of pollutants on population growth rates and probability of extinction for a representative squamate . Ecotoxicology 30: 175 – 186 .
Crossref | CAS | PubMed | Web of Science® | Google Scholar