



Perfluoroalkyl Contaminant Exposure and Effects in Tree Swallows Nesting at Clarks Marsh, Oscoda, Michigan, USA

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Abstract

A site in northeastern Michigan, Oscoda Township, has some of the highest recorded exposure in birds to perfluorinated substances (PFASs) in the United States. Some egg and plasma concentrations at that location exceeded the lowest reproductive effect threshold established for two avian laboratory species. The objectives of this study were to determine whether there were reproductive effects or physiological responses in a model bird species, the tree swallow (*Tachycineta bicolor*), associated with this extremely high exposure to PFASs. The lack of exposure above background to other contaminants at this site allowed for an assessment of PFAS effects without the complication that responses may be caused by other contaminants. A secondary objective was to determine the distribution of PFASs in multiple tissue types to better understand and interpret residues in different tissues. This can best be done at highly exposed locations where tissue concentrations would be expected to be above detectable levels if they are present in that tissue. There were no demonstrable effects of PFAS exposure on reproduction nor on most physiological responses.

Perfluoroalkyl substances (PFASs) are manmade fluorinated compounds that have high thermal, chemical, and biological inertness. They have been used in a wide variety of applications including stain-, oil-, and water-resistant coatings for fabric and papers, as well as, for many industrial applications including as fire-fighting foams (European Food Safety Authority 2008). Contamination by PFASs in the vicinity of fire suppression training facilities is now a well-known problem (Place and Field 2012), but effects on birds have not been studied. The contaminants of concern at fire suppression training facilities are the film-forming firefighting foams that often contain fluorinated surfactants. These

chemicals reduce surface-tension and offer superior capabilities compared with other formulations of fire extinguishing chemicals.

Clarks Marsh (44°26'39.14"N, 83°23'35.66"W), which is on the south side of the former Wurtsmith Air Force Base in Oscoda, Michigan (MI), is known for extensive PFAS contamination in ground and nearby surface waters (Bermejo et al. 1997; Moody et al. 2003). These PFASs originated from firefighting foams that were used during a 25-year period for training purposes. While the groundwater plume has been characterized, and a “do not eat” advisory for all fish caught in Clarks Marsh was issued in 2012, this is the first information published on exposure in birds from that area, as well as, the first characterization of PFASs in birds from firefighting foam sources. There have been publications on exposure and effects of PFASs at other types of point sources including manufacturing plants (Custer et al. 2013, 2014; Dauwe et al. 2007; Groffen et al. 2017, 2019; Lopez-Antia et al. 2017), as well as numerous publications on the worldwide distribution of PFASs in avian tissues (including Butt et al. 2010; Giesy and Kannan 2001).

The objectives of this study were to quantify exposure to, and possible effects of PFASs in tree swallows (*Tachycineta bicolor*) nesting at Clarks Marsh. Tree swallows are a model avian species, which have been used extensively

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in ecotoxicological studies across the United States and Canada (Custer 2011). Swallows feed on the aerial stage of benthic aquatic invertebrates, so concentrations in their tissues directly reflect contaminants in sediments that are bioavailable via the benthic invertebrate food web. They feed within ~0.5 km of their nest boxes (Stapleton and Robertson 2006) and therefore reflect local rather than regional contamination. Perfluorinated chemicals have been detected in swallow tissues across the Upper Midwest and Great Lakes regions (Custer et al. 2012, 2014, 2017a). Whereas eggs or plasma often is the tissue of choice, we also quantified concentrations in liver, brain, and diet to understand more fully the differences in accumulation amongst tissue types. Finally, we assessed whether there were reproductive effects or physiological responses associated with PFAS exposure.

Methods and Materials

Sample Collections Procedures

Tree swallow nest boxes ($n=22$) were placed in a 1 ha grassy field, 0.3 km above Clarks Marsh in the spring of 2014 and monitored for that and the following 3 years. Nest boxes were checked weekly starting in mid-May and continuing until late June, which is the time period when swallows are nesting in the area. The number of eggs laid in each nest, the number that hatched, and the number of nestlings that survived until 12 days of age were quantified. During early incubation, 2 eggs per clutch, hereafter called an egg sample, were collected for contaminant analysis, from which 10 randomly selected egg samples per year were analyzed for 13 PFAS in 2014, 2015, 2016, and 2017, including 9 perfluorocarboxylic acids (PFCAs; perfluorobutanoate [PFBA], CAS# 375-22-4; perfluoropentanoate [PFPeA], 2706-90-3; perfluorohexanoate [PFHxA], 307-24-4; perfluoroheptanoate [PFHpA], 375-85-9; perfluorooctanoate [PFOA], 335-67-1; perfluorononanoate [PFNA], 375-95-1; perfluorodecanoate [PFDA], 335-76-2; perfluoroundecanoate [PFUnA], 2058-94-8; and perfluorododecanoate [PFDoA], 307-55-1), and 4 perfluorosulfonates (PFSAs, perfluorobutane sulfonate [PFBS], 375-73-5; perfluorohexane sulfonate [PFHxS], 355-46-4; perfluorooctane sulfonate [PFOS], 1763-23-1; and perfluorooctane sulfonamide [PFOSA], 754-91-6). In 2014 and 2015, 12-day old nestlings were collected from the same nest boxes where eggs were collected, euthanized by decapitation (AVMA 2013), and plasma analyzed for the same suite of 13 PFASs. In 2015 only, liver and brain tissues were also analyzed from these same individuals to quantify the distribution of PFASs amongst different tissue types. In all 4 years, food from the stomach was removed, pooled across multiple nest boxes ($n=1$ or 2 pools per year depending on mass of food available), and analyzed for PFASs.

For the physiological responses (see further methods below), a subsample of the liver was excised from the nestlings collected for PFAS analyses and the liver tissue snap frozen in liquid nitrogen shortly after collection. The blood sample from those same nestlings was centrifuged to separate the plasma from the red blood cells (RBCs). The RBCs were fixed with Ham's F-10 freezing media and then snap frozen in a liquid nitrogen charged vapor shipper until analyzed. A subsample of the remaining plasma was snap frozen in liquid nitrogen and analyzed for two thyroid hormones (triiodothyronine [T3] and thyroxine [T4]). Both thyroid glands were excised from the nestling in all years, placed in a cryotube, and snap frozen in liquid nitrogen until analyzed for T3 and T4.

Chemical and Physiological Analyses

The PFASs were analyzed by SGS-AXYS, formerly AXYS Analytical Services, Sidney, British Columbia, Canada. Any use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the U.S. Government. See Custer et al. (2017a) for full details, but in brief, after thawing, samples were extracted by solid phase extraction using weak anion exchange cartridges. Eluates were spiked with recovery standards and analyzed by liquid chromatography–mass spectrometry (LC–MS/MS) and analyzed on a high-performance liquid chromatograph reversed phase C18 column coupled to a triple quadrupole LC–MS/MS. Lab blanks and spikes were analyzed at a rate of one per analytical batch (analytical run), ~6% of samples. Concentrations (ng/mL plasma and ng/g for all other tissue types, wet wt.) are expressed as geometric mean and 95% confidence interval (CIs) in tables and text. Total PFASs are the sum of all congeners that were detected. Percent recovery averaged 100.3%.

Reproductive success, quantified as percent hatching, was quantified as were three physiological responses: ethoxyresorufin-*O*-dealkylase (EROD) activity, genetic damage in red blood cells, measured as the amount of deoxyribonucleic acid (DNA) in each cell, and two thyroid hormone levels measured in both plasma and the gland itself. See Custer et al. (2017b) for detailed methodologies for EROD and DNA-CV (coefficient of variation); in brief, for EROD, liver tissue was thawed, homogenized, buffered, and then centrifuged. The resulting pellet was resuspended before analysis. Results are presented as pmol/product/min/mg microsomal protein. For nuclear DNA content, RBCs were thawed, added to a citrate buffer solution, and lysed. The nuclear DNA was stained with a propidium iodide solution and the DNA content of 10,000 cells quantified on a Becton–Dickinson flow cytometer. Each sample was run twice, and an average used in the analyses. Internal chicken standards were

run with each batch. Data are presented as the DNA-CV for 10,000 RBCs.

For plasma thyroid hormones, total T3 and T4 were analyzed using the AccuBind ELISA Total T3 and Neonatal-T4 AccuBind ELISA kits (Monobind Inc., Lake Forest, CA). The manufacturer's protocol was modified such that samples and standards were assayed in an identical plasma-serum matrix. For T3 analysis, plasma was diluted 1:2.4 in a solution containing 17% human hormone-free serum (Monobind) and 83% 4X stripped chicken plasma (SCP: BioreclamationIVT, Westbury, NY). Standards for T3 were diluted in the same solution. Plasma for T4 analysis was diluted 1:4 in 4X SCP. Dried standards included in the T4 kit were replaced with ones in serum (Monobind), which were further diluted in 4X SCP. The assays were then run following the remainder of the manufacturer's protocol. The respective sample matrices for the T3 and T4 assays were used to determine the limit of detection (LOD) for each year of the study (mean LOD = 0.07 ng/mL for T3 and 1.13 ng/mL for T4). The lowest calibration standard consistently exhibiting a CV < 15% was selected as the reporting limit (RL) for each year of the study (mean RL = 0.13 ng/mL for T3 and 1.50 ng/mL for T4). Concentrations were determined using a 4-parameter model. Each sample was assayed in duplicate and samples were reanalyzed if the CV was > 20%. Intra-assay CVs were $4.10 \pm 0.53\%$ for T3 and $4.20 \pm 0.38\%$ for T4. Inter-assay variation was 9.05% for T3 and 12.87% for T4, based on two reference samples.

Thyroid glands were digested with Pronase solution (Calbiochem, Millipore Sigma) and extracted with ethanol (McNabb et al. 2004). For T3 analysis, an aliquot of the ethanol extract was dried on a CentriVap (Labconco) and reconstituted in a solution of 4X SCP (83%) and human hormone-free serum (17%). For glandular T4 analysis, an aliquot of ethanol extract was diluted 1:10 to 1:80 using 4X SCP such that the final concentration of plasma was 92.6% and of ethanol was 7.4%. Standards for both T3 and T4 were diluted in the same matrices as the samples and assayed as described above. The mean LODs were 0.09 ng T3/mL and 1.30 ng T4/mL, and mean RLs were 0.16 ng T3/mL and 1.76 ng T4/mL. Intra-assay CVs averaged $2.93 \pm 0.32\%$ and $3.53 \pm 0.38\%$ for T3 and T4, and inter-assay variation was 7.14% and 7.82% for T3 and T4.

Statistical Analyses

Among year differences in tissue concentrations of total PFASs in eggs and plasma and among matrix differences in 2015 only (eggs, plasma, liver, and diet) were analyzed using analysis of variance (ANOVA) on log-transformed concentrations using R (R Core Team 2015, Ver 3.1.3). Half the detection limit was substituted for nondetectable values in statistical analyses and measures of central

tendency when < 100% but $\geq 50\%$ of samples had total PFASs detected. Means, when P values were < 0.05, were separated using Tukey's honestly significant differences. Correlation between arcsine-transformed percent hatching and natural log of total PFAS in eggs was analyzed using Excel (Microsoft).

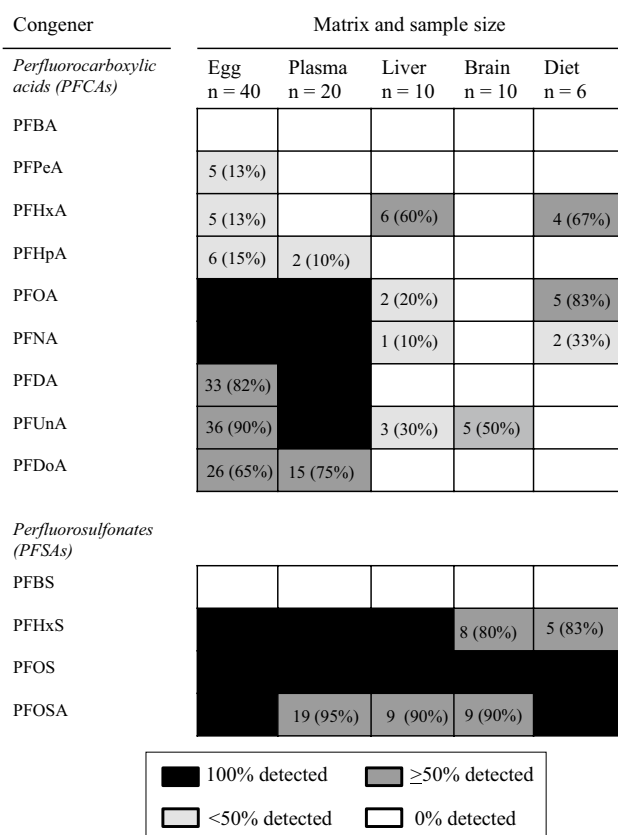
To compare PFAS patterns among groups (among years for eggs and plasma, and among tissues in 2015), multivariate analyses were conducted using Primer E (Ver. 6.1.6). This analysis included nonmetric multidimensional scaling (NMDS) plots for visualizations, analysis of similarity (ANOSIM) for differences among groups, and similarity percentages (SIMPER) to quantify which PFASs were important drivers when there were significant among group differences (Clarke and Warwick 2001). Analyses were done on log transformed concentration data using Bray Curtis distances. For the multivariate analyses, nondetected values were given a value of zero if there was at least one sample with detectable concentrations of total PFASs but < 50% of samples with detectable exposure, otherwise nondetects were given half the detection limit. If a congener was not detected in any sample, it was not included in the multivariate analyses. The size of R indicates the degree of difference in ANOSIM tests. Clear differences among groups are evident when R is ≥ 0.4 . There is some support for differences when R is between ≥ 0.3 and < 0.4, and groups barely differ when R is < 0.3 (adapted from Clarke and Warwick 2001). The P value indicates when groups differ significantly.

For physiological response endpoints, responses were compared between Oscoda and reference locations (Root River [43°45'53.19", 91°21'56.45"] in 2015, and Star [46°01'28.73", 89°28'13.15"] and Plum Lakes [45°59'25.64, 89°33'36.00"] in 2016 and 2017). These reference sites were similar ecologically, and the same field and chemical methodologies were used as outlined above. Two-way ANOVAs (year and site) were run first and then, because there were no significant differences among the three reference sites for the physiological responses, two-way ANOVAs were then done comparing Oscoda to the reference sites combined. This provided a larger sample size for the reference locations.

Results

Exposure

Two PFASs, PFBA and PFBS, were not detected in any sample matrix (Fig. 1). Only PFOS was detected in all samples in all tissue matrices. The other two PFASs, PFHxS and PFOSA, were detected in > 80% of samples of all matrices. Brain tissue had the least number ($n=4$) of PFASs detected. In addition to the three PFASs detected in brain tissue, only one PFCA, PFUnA was detected, and that was in five of ten



Number in box is number with detectable concentrations of the number analyzed for matrices when <all or >none detected

Fig. 1 Frequency of detections for 13 perfluoroalkyl contaminants in tree swallow tissues at a site near Oscoda, Michigan, 2014–2017

samples (Fig. 1). Liver and diet samples tended to have <50% detection rates for each congener, except for PFHxA, which was detected in 60% of samples in both liver and diet, PFOA in 80% of the diet samples, and PFOS in 100% of both tissues. Detection limits (Table 1) were similar among years, but tissue types with smaller mass, such as liver, brain, and diet tended to have higher detection limits than eggs or plasma.

There were no significant differences ($P=0.083$) in concentrations of total PFAS in mean egg concentrations among years, which ranged between 554 and 954 ng/g (Table 2). In 2015, plasma total PFAS was ~50% lower than in 2014 ($P<0.001$). Concentrations of total PFASs were similar between eggs and plasma (Table 2) in 2015, with liver, brain, and diet concentrations significantly, and between 2.5 and 5 times lower, than in either egg or plasma ($P<0.001$).

On an individual congener basis, PFOS followed by PFHxS dominated the concentrations of congeners (Table 3). Concentrations of PFOS averaged 662 ng/g wet wt. in eggs. Six of 40 eggs exceeded the 1000 ng/g toxicity reference value threshold established for laboratory quails (*Colinus virginianus*) and mallards (*Anas platyrhynchos*, Newsted et al. 2005); two of those eggs samples were from 2014, and four were from 2016. All ten plasma samples from 2014, but only two of 10 in 2015 exceeded the plasma threshold (Newsted et al. 2005). Perfluorooctane sulfonate comprised 90.1% (eggs), 78.7% (plasma), 90.1% (liver), 95.2% (brain), and 90.0% (diet) of the total PFASs. In plasma, which was slightly less dominated by PFOS, concentrations averaged 856 ng/mL and exceeded other congeners, except of PFHxS, by a factor of > 65. Concentrations of PFHxS were of secondary importance in the other tissues as well.

While total PFASs in eggs did not differ among years, when the profile of individual congeners was analyzed in a

Table 1 Detection limits (ng/g wet wt. or ng/mL [plasma]) for tree swallow samples collected near Oscoda, MI 2014–2017

PFAS congener	Matrix and year											
	Eggs				Plasma		Liver	Brain	Diet			
	2014	2015	2016	2017	2014	2015	2015	2015	2014	2015	2016	2017
PFBA	2.04 ^a	1.69	0.58	0.53	0.50	0.64	1.19	1.09	2.69	1.21	0.54	0.52
PFPeA	0.60	0.60	0.58	0.52	0.50	0.64	1.19	1.09	3.10	1.60	0.50	1.09
PFHxA	0.52	0.60	0.58	0.52	0.50	0.64	1.19	1.09	3.10	2.36	0.63	1.04
PFHpA	0.52	0.60	0.58	0.52	0.50	0.64	1.19	1.09	3.10	1.59	0.79	0.52
PFOA	0.52	0.60	0.58	0.52	0.50	0.64	1.19	1.09	0.52	1.12	0.50	0.52
PFNA	0.52	0.60	0.58	0.52	0.50	0.64	1.19	1.09	0.52	1.12	0.50	0.52
PFDA	0.52	0.60	0.58	0.52	0.50	0.64	1.19	1.09	0.52	1.12	0.50	0.52
PFUnA	0.52	0.60	0.58	0.52	0.50	0.64	1.19	1.09	0.52	1.12	0.50	0.52
PFDoA	0.52	0.60	0.58	0.52	0.50	0.64	1.19	1.09	0.52	1.12	0.50	0.52
PFBS	1.05	1.20	1.16	1.05	1.04	1.28	2.37	2.18	2.54	2.24	1.00	1.04
PFHxS	1.42	1.20	2.41	1.05	2.28	1.28	2.37	2.18	10.7	2.24	1.00	1.04
PFOS	10.5	1.20	5.60	1.09	6.91	2.47	2.37	2.18	10.3	2.24	1.00	1.29
PFOSA	0.70	0.82	0.58	0.52	0.50	0.64	1.42	1.31	0.62	1.35	0.50	0.52

^aDL = 1.71 without 1 sample with a DL 5 times greater than rest

Table 2 Geometric mean and 95% confidence intervals (CI) for total perfluorinated chemicals in tree swallow tissues, Oscoda, Michigan

Matrix year	<i>n</i>	Total PFASs (ng/g wet wt.)	95% CI	Min	Max	
Eggs						
2014	10	908 a ^a		692–1192	483	1781
2015	10	623 a	A ^b	502–773	423	1012
2016	10	954 a		713–1277	443	1721
2017	10	554 a		415–739	270	1040
Plasma						
2014	10	1649 a ^a		1349–2017	1187	2530
2015	10	731 b	A	513–1042	387	1521
Liver						
2015	10	237	B	185–304	136	392
Brain						
2015	10	149	B	120–184	91	253
Diet						
2014	2	190 ^c		30.7–1183	165	220
2015	2	141	B	15.4–1286	118	167
2016	1	166		–	–	–
2017	1	143		–	–	–

^aMeans sharing same lower-case letter within matrix type are not significantly different among years, $P=0.083$ (egg) and $P<0.001$ (plasma)

^bMeans sharing same capital letter are not significantly different among tissue types (2015 only), $P<0.001$

^cDiet samples were pools from multiple nest boxes and were not tested for among year differences

multivariate context, there was separation among years, i.e., the congener profile shifted among years (global $R=0.472$) with 2015 differing the most from 2017, and 2014 and 2016 being intermediate (Fig. 2, upper). In plasma, both total PFAS concentrations and the congener profiles varied between 2014 and 2015 (global $R=0.436$, lower) the only 2 years with data for this matrix. While PFOS was the dominant congener from a concentration perspective, it was rarely the congener that was responsible for the difference among years (Table 4). In eggs, PFUnA and PFOSA were the

primary drivers for among year differences, with between 15.4 and 24.7% of the differences attributed to these two congeners. For plasma, PFOA (21.4%), followed by PFOSA (18.5%) were the two primary drivers.

There were differences in how the PFAS congeners partitioned amongst tissue types (Fig. 3). There was separation among tissues in congener composition (Fig. 3), with egg and plasma completely separating in multivariate space, but being more similar to each other than to either the liver, brain, or diet. For differences in congeners among tissues,

Table 3 Geometric mean and 95% confidence intervals for PFASs (ng/g wet wt. or ng/mL [plasma]) detected in at least 50% of tree swallow tissue samples, Oscoda, Michigan 2014–2017

	Egg	Plasma	Liver	Brain	Diet
Sample size	40	20	10	10	6
<i>Perfluorocarboxylic acids</i>					
PFHxA	35ND ^a –0.92	20ND	1.29 (0.72–2.30)	10ND	2.87 (1.10–7.42)
PFOA	2.29 (1.85–2.86)	13.1 (9.27–18.4)	8ND–1.52	10ND	1.10 (0.73–1.65)
PFNA	2.25 (1.89–2.68)	4.34 (3.68–5.12)	9ND–0.80	10ND	4ND–0.94
PFDA	1.14 (0.86–1.52)	1.72 (1.49–1.99)	10ND	10ND	6ND
PFUnA	2.03 (1.42–2.91)	2.96 (2.38–3.69)	7ND–1.40	0.80 (0.60–1.06)	6ND
PFDoA	0.74 (0.56–0.96)	0.67 (0.51–0.89)	10ND	10ND	6ND
<i>Perfluorosulfonates</i>					
PFHxS	55.1 (46.3–65.6)	206 (153–278)	17.8 (10.8–29.3)	4.46 (2.49–8.00)	8.13 (3.77–17.6)
PFOS	662 (577–760)	856 (655–1120)	209 (166–265)	142 (115–174)	138 (105–183)
PFOSA	7.38 (5.93–9.20)	3.16 (2.07–4.83)	5.21 (2.73–9.95)	1.96 (1.31–2.92)	5.82 (2.71–12.6)

^aNumber preceding ND is number not detected followed by maximum concentration found in the other samples

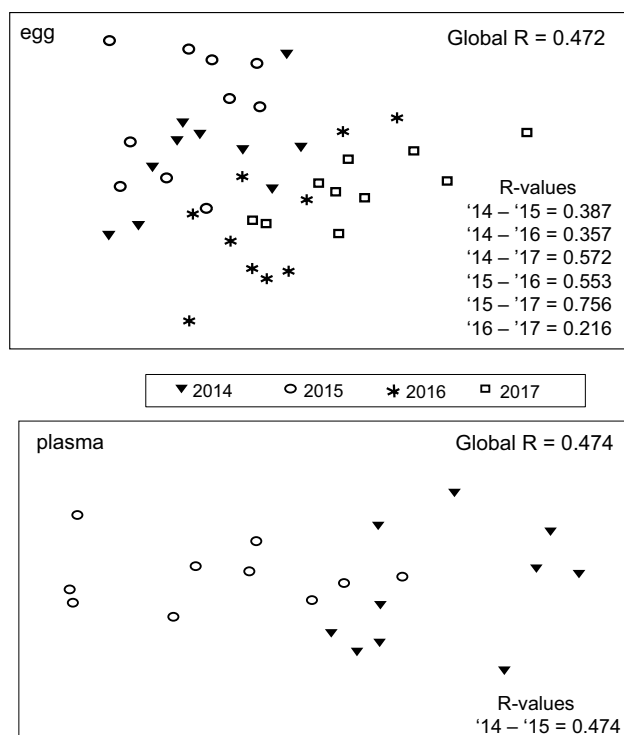


Fig. 2 Nonmetric multidimensional scaling plots of perfluoroalkyl contaminants among years in eggs and plasma of tree swallows nesting near Oscoda, Michigan, 2014–2017. R indicates the degree of pattern difference in ANOSIM tests. Clear differences are evident when $R \geq 0.4$. There is some support for differences when R is ≥ 0.3 to < 0.4 , and groups barely differ when R is < 0.3

PFHxS was the primary driver in seven of the ten comparisons (18.4–29.3% of the differences), PFUnA in two comparisons (19.3–19.5%), and PFOSA for one comparison (liver vs. diet, 21.8%; Table 4). Perfluorooctane sulfonate often was not even among the top 4 congeners driving the differences among tissue types except in 4 of 12 comparisons, despite it always being the most dominant in concentration. Instead PFHxS, PFHxA, PFOA, and sometimes PFOSA (Table 4) were the dominant drivers.

Effects

Reproduction was followed closely between 2014 and 2016. Egg laying began in 2014, the first year that the nest boxes were in place, on May 14. Egg laying began a few days earlier, on May 11 and May 12, in 2015 and 2016 (Suppl. Fig. 1). In most years there were two peaks in nest initiation: the first around May 15 and 16, and a second on May 21. During 2014, 24% of the female birds were first-breeding-season females whereas the percentage of first-year females dropped to 10% and 7% in the following 2 years. Clutch size was similar among years with the modal clutch size being 5 in 2014 and 2016 and 6 in 2015 (Suppl. Fig. 2). There were

no 7-egg clutches in 2014, but there were 13 in 2015 and 5 in 2016. The percent of eggs laid that hatched, not including eggs that were collected for chemical analyses, was 92, 84, and 83% in 2014, 2015, and 2016 at Oscoda and 67, 71, and 80% at the reference locations, respectively (Table 5). The percentage of successful nests, those that hatched at least 1 egg, was 95, 88, and 95% for the 3 years at Oscoda and 67, 73, and 87% at the reference locations, respectively. There was no correlation ($r = 0.037$) between transformed total PFAS exposure and percent hatching. There was also no correlation ($r = -0.009$) if untransformed data for total PFAS and percent hatching were used.

For the physiological response variables, there was a significant year effect for both EROD and DNA-CV, but no difference between Oscoda and the reference sites, nor was there a significant interaction (EROD $P = 0.002$ [year], $P = 0.741$ [site], $P = 0.740$ [interaction]; DNA-CV $P < 0.001$ [year], $P = 0.826$ [site], $P = 0.826$ [interaction]). Ethoxyresorufin-*O*-dealkylase was higher in 2017 compared to the other 2 years at both Oscoda and the reference sites, while DNA-CV was lower in 2017 compared with the other 2 years (Table 6). There were no significant differences in either T3 or T4 concentration in thyroid glands (T3, $P = 0.821$ [year], $P = 0.127$ [site], $P = 0.127$ [interaction]; T4, $P = 0.817$ [year], $P = 0.288$ [site], $P = 0.287$ [interaction], nor in plasma T4 ($P = 0.867$ [year], $P = 0.385$ [site], $P = 0.385$ [interaction]). For plasma hormones for T3, however, site and the interaction term were significant ($P = 0.114$ [year], $P = 0.048$ [site], $P = 0.048$ [interaction] Table 6). In 2015, plasma T3 was significantly higher at Oscoda than at the reference location.

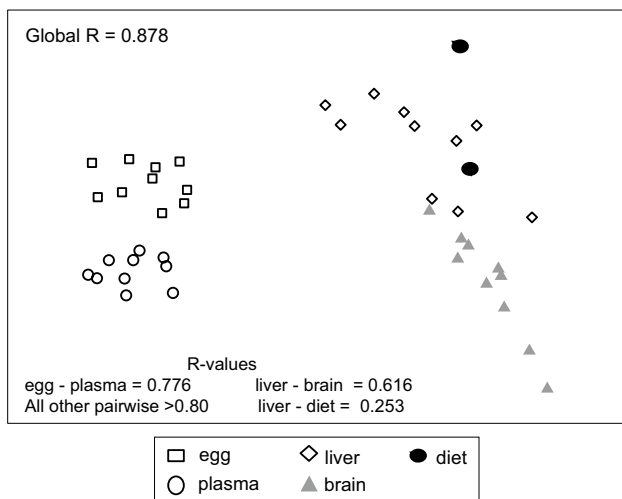
Discussion

Exposure

Exposure to PFASs in eggs at Oscoda, MI was extremely high, but not as high as reported in great tits (*Parus major*) nesting near a perfluorochemical manufacturing plant in Antwerp, Belgium. Median egg concentrations of PFOS there were 10,380 ng/g wet wt. (Groffen et al. 2017) compared with 727 ng/g at Oscoda. The maximum value in eggs in Belgium was 69,218 ng/g compared with a maximum at Oscoda of 1781 ng/g in eggs. The extreme exposure in Belgium was quite localized, however, and was > 80 times less in as little as 1 km from the manufacturing plant site (max. concentration = 782 ng/g wet wt. at Vlietbos). Tree swallow eggs and plasma collected at other locations in Minnesota (MN) and Wisconsin (WI) were 2 to 6 times lower than levels of total PFASs at Oscoda. Mean total PFAS concentrations in tree swallow eggs and plasma at Pigs Eye Lake, MN, located near a dump

Table 4 Principal PFASs drivers (> 10% of dissimilarity or top 4 drivers) in multivariate separations among years for tree swallow eggs and plasma, and among matrices (2015 only), Oscoda, Michigan

Tissue and comparison	<i>R</i> value ^a	Percent of dissimilarity					
		PFUnA	PFOSA	PFOA	PFDA	PFHxS	Other PFASs
Eggs							
2014 versus 2015	0.387	17.3	10.8	10.1			
2014 versus 2016	0.357	12.5	15.4	11.9	11.9	11.0	
2015 versus 2016	0.553	24.7	13.6			11.4	PFDoA- 11.2
Plasma							
2014 versus 2015	0.474		18.5	21.4		15.2	PFOS = 17.5
2015							
Egg versus plasma	0.78	12.6	17.0	14.7		19.6	PFDoA- 10.2
Egg versus liver	0.98	19.5		11.0	11.4		PFNA = 11.0
Egg versus brain	1.00	14.5		11.3		19.6	PFOS = 12.0
Egg versus diet	0.11	19.3				12.0	PFOS = 13.1 PFHxA = 11.1
Plasma versus liver	1.00	11.6		17.4		19.0	PFNA = 13.0
Plasma vs brain	1.00			18.9		29.7	PFNA = 13.5 PFOS = 12.1
Plasma versus diet	1.00	12.4		12.3		20.2	PFOS = 12.4 PFNA = 12.2
Liver versus brain	0.62	11.9	21.4			29.3	PFHxA = 21.2
Liver versus diet	0.25		21.8			17.3	PFHxA = 22.4 PFOS = 15.8
Brain versus diet	0.81	13.5	15.6	12.9		18.4	PFHxA = 32.0

^aBetween year pairs**Fig. 3** Nonmetric multidimensional scaling plot of perfluoroalkyl contaminants among tissue types of tree swallows nesting near Oscoda, Michigan, 2015. *R* indicates the degree of pattern difference in ANOSIM tests. Clear differences are evident when $R \geq 0.4$. There is some support for differences when R is ≥ 0.3 to < 0.4 , and groups barely differ when R is < 0.3

site used, in part, by a nearby PFAS manufacturing plant, were 227–418 ng/g (eggs) and 352–437 ng/mL (plasma, Custer et al. 2014) compared with 954 and 1649 ng/mL at Oscoda in the present study. At 69 locations across the

Great Lakes, excluding Oscoda, mean total PFASs in nestling plasma were highly variable and ranged between a geometric mean of 15.8 up to 581.9 ng/mL (Custer et al. 2017a). The highest concentrations in the Great Lakes region were observed at a site (581.9 ng/mL at Wild Rice Lake, MN) located down gradient from an airport where firefighting training exercises may have been conducted (Custer et al. 2017a).

Mean background exposures for total PFAS in WI and MN are between 6.35 and 20.6 ng/g in eggs and between 13.7 and 76.5 ng/mL in plasma (Custer et al. 2014). At Great Lakes locations not associated with Areas of Concern, i.e., background for the Great Lakes region, total PFASs in nestling plasma were somewhat higher, between 48.4 and 223.1 ng/mL (Custer et al. 2017a). A number of these non-AOC “reference” sites were still within highly industrialized and urbanized landscapes, which are common in the vicinity of the Great Lakes and which may account for this slight elevation. In addition to PFAS point sources, such as airports with fire suppression activities (Oscoda and Wild Rice Lake) or manufacturing plants (sites along the upper Mississippi River near the Twin Cities), other significant but uncharacterized sources appear to exist in some of the highly industrialized and urbanized watersheds of the Great Lakes (Custer et al. 2017a).

Table 5 Reproductive success for tree swallows nesting above Clarks Marsh, Oscoda Michigan and reference locations between 2014 and 2017

Reproductive elements	Oscoda			Reference		
	2014	2015	2016	2015	2016	2017
Number of clutches	19	24	20	3	11	23
Ave. clutch size	5.4	5.7	5.3	4.33	5.8	5.7
Number of successful nests ^a	18 (95%)	21 (88%)	19 (95%)	2 (67%)	8 (73%)	20 (87%)
Percent hatching ^b	91.7	83.8	82.8	66.7	70.5	79.5
Fate of unhatched eggs						
Infertile		2	2		1	
Missing	1	3	7		4	2
Dead embryo						
¼ development			2			
½ development			3			7
¾ development	1	2	1			2
Full term			1			
Fate of clutches						
Abandoned	1 clutch of 4 eggs	1 clutch of 5 eggs			1 clutch of 3 eggs	1 clutch of 3 eggs
Depredated		1 (bear)		1 (wren)	1 (unk. ^c)	1 (unk.)
Percent with dead embryo	5%	0%	10%	0%	0%	13%

^aSuccessful nest is one that hatched at least 1 egg; percentages in parentheses

^bPercent of eggs laid that hatched. Does not include eggs collected for chemical analysis

^cUnk = unknown predator

Table 6 Means and 95% CIs (in parentheses) for ethoxyresorufin-*O*-dealkylase (EROD, pmol/product/min/mg), chromosomal damage (DNA-CV), and thyroid hormone levels (T3 and T4, ng/mL) in tree swallows nesting above Clarks Marsh, Oscoda Michigan in 2015, 2016, and 2017

Physiological response and year	Oscoda (<i>n</i> = 10, 6, 4) ^a	Reference (<i>n</i> = 3, 8, 16) ^a
EROD		
2015	22.53 b ^b (10.45–34.61)	19.44 b ^b (2.35–36.52)
2016	34.06 ab (21.33–46.78)	11.59 b (7.98–15.19)
2017	53.68 a (37.23–70.12)	35.57 a (23.38–45.76)
DNA-CV		
2015	2.72 a ^c (2.00–3.44)	2.52 a ^c
2016	2.31 a (2.09–2.52)	2.31 a (2.11–2.51)
2017	1.56 b (1.22–1.90)	1.60 b (1.54–1.66)
Thyroid hormones		
Plasma T3 (all years combined)		
2015	1.10 A ^d (0.69–1.51)	0.18 B (0.64–1.10)
2016	0.86 (0.67–1.04)	0.84 (0.64–1.04)
2017	0.84 (0.74–0.94)	0.91 (0.80–1.02)
Plasma T4		
	10.1 (8.74–11.44)	12.73 (11.67–13.79)
Glandular T3		
	1.71 (1.20–2.22)	2.76 (2.41–3.10)
Glandular T4		
	104.0 (80.41–127.5)	382.6 (329–436.2)

^aSample size per year, except *n* = 1 for DNA-CV in 2015 and *n* = 14 for DNA-CV in 2017

^bMeans sharing same lower case letter are not significantly different within location (*P* = 0.002)

^cMeans sharing same lower case letter are not significantly different within location (*P* < 0.001)

^dMeans sharing same upper case letter are not significantly between Oscoda and Ref (*P* = 0.048)

There was high among-year variability in exposure to PFASs at Oscoda with mean values ranging between 554 ng/g (2017) and 954 ng/g (2016) in eggs and between 731 and 1649 ng/mL in plasma. The diet samples varied

in a pattern similar to the egg samples, providing corroboration that these annual differences were correct and that avian tissue concentrations are a result of exposure via ingestion of food. This wide annual variation is not

unusual at highly contaminated sites. For example, on the Housatonic River, MA total polychlorinated biphenyl (PCBs) means in eggs ranged between 41.7 µg/g wet wt. (1998) and 100.9 µg/g (1999), whereas 2000 was intermediate (67.5 µg/g, Custer et al. 2003). Possible reasons for these differences included: variation between arrival time at a site and commencement of egg laying and/or differential exposure due to varying prey items emerging from the contaminated sediments. On the Housatonic River; for example, there was a more than tenfold difference in concentrations of PCBs in prey (2.79 µg/g and 31.90 µg/g at Holmes Rd site, C. Custer, unpublished data) collected at two different times in 1999. Although we do not know the invertebrate species that comprised those two samples, it is plausible that the prey species differed over that timeframe or they emerged from different sediment deposits that may have been differentially contaminated. At two other sites on the Housatonic River, there was a sevenfold difference in PCB concentrations in the diet within the same year, which may have reflected the heterogeneous distribution of contaminants in the sediment or the redistribution of contaminated sediments after high-water events.

Congener Distribution

The types and proportions of PFASs found in great tit eggs at a plant in Belgium (Groffen et al. 2017), a point source location, were generally similar to those found in tree swallow eggs at Oscoda. Total PFASs in eggs were dominated by PFOS (> 95% of the total PFASs) at both locations. One difference between these two locations was that PFNA was detected in only 42% of samples in Belgium but 100% of samples in Oscoda. This difference may have resulted from the relatively high detection limit (1.8 ng/g) in Belgium for PFNA compared with the present study (0.52–0.60 ng/g), hence some samples fell below the detection limit in Belgium. At a third known, point-source location, along the Upper Mississippi River near the Twin Cities, MN and WI, PFOS also dominated tree swallow eggs although to a slightly lesser extent (90%, Custer et al. 2014). Similar to observations in the present study, PFHxS also ranked second in concentration in eggs in Belgium, yet only ranked 7th out of 8 congeners along the Upper Mississippi River (Custer et al. 2014).

Differences in dominant congeners between study locations were also found in plasma, although the proportions were slightly different. While PFOS still dominated in the plasma, representing 75–80% of total PFASs at both Oscoda and the Upper Mississippi River (Custer et al. 2014), the second ranked congener at Oscoda was PFHsX (19%), which was ranked only fifth on the Upper Mississippi River (Custer et al. 2014); PFOA was the second ranking total PFAS on

the Upper Mississippi River (20% of total PFAS) compared to only 1% at Oscoda. These variations in proportions may have resulted from different products being manufactured or used at the point sources.

At locations without identifiable point sources, total PFASs were lower (Custer et al. 2014), but PFOS was still the dominant congener in tree swallow plasma. The level of PFOS dominance, however, seemed to be related to whether the site was located on a river with wastewater effluent sources. In isolated lakes in MN and WI, where atmospheric deposition was putatively the primary source of PFASs, PFOS accounted for only 30–40% of total PFASs in plasma (Custer et al. 2014, 2017a), whereas in rivers where there were wastewater treatment plants, the proportion of PFOS was larger (67–87%). In other bird species exposed to mainly non-point sources, such as in the Norwegian arctic, PFOS still dominated total plasma PFASs but to a lesser extent (57%, glaucous gulls [*Larus hyperboreus*], Verreault et al. 2005); this was similar to tree swallows at non-point-source locations. In herring gulls (*Larus argentatus*) nesting in Saginaw Bay, MI (Gebbinck and Letcher 2012), PFOS comprised 70% of total PFASs perhaps indicating some wastewater treatment or other point source influences there. Perfluorohexane sulfonate was the second most abundant plasma congener in the herring gulls, while in glaucous gull plasma PFUnA was the second most prevalent congener followed by perfluorotridecanoic acid (not measured in the current study nor in herring gulls). More work might be useful to determine whether the congener profile could help to determine sources of PFASs (Custer et al. 2014; Rüdell et al. 2011).

Whereas PFOS was always the dominant congener, > 90% for all tissue types except for plasma in the current study (78.7%), it was rarely the primary driver in the multivariate analyses of differences among years or tissue types. This was true even when multivariate analysis focused on percent composition rather than concentration (data not shown). As a result, differences between tissues were driven by other, less dominant congeners, which varied more among tissue types or years.

Tissue Distribution

There was little consistency amongst studies in PFASs distribution within different bird species. For example, tree swallows had three times more PFOS in plasma compared to liver tissue, glaucous gulls had similar concentrations in those two tissues, and herring gulls had three times less PFOS in plasma compared with liver tissue (Gebbinck and Letcher 2012; Verreault et al. 2005). Exposure of the nestling tree swallows to very high levels of PFOS in the present study (> 150 ng/g in diet) occurred over a 12-day period, which would have been sufficient time for elevation of

plasma concentrations, but not in liver. In herring gulls, the plasma and liver tissues were from adult birds, so plasma levels would be indicative of current exposures while the liver concentrations may have reflected a cumulative and longer-term measure. Because herring gulls are long lived and spend most of the year in and around the Great Lakes, they may have had time to build up concentrations in their liver tissues even though their daily intake may have been much lower. As has been observed in glaucous gulls (eggs and adult plasma, Verreault et al. 2005), nestling tree swallows in the present study had ~ equal concentrations of PFOS in eggs and plasma. In contrast, herring gulls had 2.6 times higher concentrations of PFOS in eggs compared to adult plasma (Gebbinck and Letcher 2012). It is uncertain why herring gulls differed from either tree swallows or glaucous gulls, but perhaps they were excreting accumulated PFAS into the eggs. Whereas swallows are income breeders (Ward and Bryant 2006), herring gulls may be more capital-breeders, i.e., using stored resources to create eggs rather than current dietary resources.

Total PFASs often are below the detection limit in individual tissues. For example, levels in brain, muscle, and adipose tissue in herring gulls were mostly below the detection limit in part because of relatively low overall levels of exposure to total PFASs (~ 100 ng/g in eggs and < 40 ng/g in blood; Gebbinck and Letcher 2012). A similar lack of detectable PFAS congeners were found in muscle (63% of PFAS congeners not detected in at least 50% of samples), kidney (43%), and liver tissues (47%) in common guillemot (*Uria aalge*) in the Baltic Sea even though exposure was considerably higher (Holmström and Berger 2008). Despite very high exposure in the current study, most PFCAs, such as PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, and PFDoA were not detected in brain tissue, and were either not detected or detected in < 50% of liver samples. It may be safe to say, therefore, that despite high total PFAS exposure, little of the PFCAs accumulated in liver, brain, muscle, or adipose tissue. The PFASs, however, except for PFBS, were detected in nearly all the tree swallow tissues analyzed; PFHxS and PFOSA were detected in 80–90% of tree swallow brain tissue and 90–100% of liver tissue in Oscoda, most likely because of the overall high exposure at that location. These sulfonates were not detected, however, in muscle or liver tissues in common guillemot (Holmström and Berger 2008), even with quite high PFAS exposures. Further distribution studies should be done at highly contaminated sites to understand the tissue dynamics in various bird species.

From an analytical perspective, data from the current study suggest the use of whole egg or plasma as the best sampling matrix to determine exposure because of the wide number of PFAS congeners that can be detected in these tissues even when exposure is more modest.

Concentrations in both tissues were high compared with other tissues and allowed for nearly all congeners to be in the detectable range (this study, Custer et al. 2013; Gebbinck and Letcher 2012; Holmström and Berger 2008; Verreault et al. 2005). Concentrations in liver, brain, and diet were low or not detected in swallows at Oscoda compared with eggs or nestling plasma. Effects of detection limits relative to the overall exposure level should be carefully considered when selecting a sampling matrix and interpreting data.

A water treatment plant was built in 2016 to intercept the PFAS-contaminated groundwater plumes leaving the air force base, but no immediate effect of this was seen in the tree swallow tissues. The reason for this lack of effect could be that because contaminants from the sediments are transferred into the benthic aquatic insects and then into the birds (Custer et al. 2017a), there is little short-term involvement of water in this process. For vertebrate species, the main route of exposure is via the food consumed rather than water that is drunk or which passes over the gills (for mercury, see Boening 2000).

Trophic Level Considerations

The PFASs are proteinophilic and are known to biomagnify in higher trophic level animals of the food web (Kelly et al. 2009). Perfluorooctane sulfonate and PFHxS were 2.2 and 1.5 times higher in liver tissue than in diet of tree swallows. In contrast, magnification factors between food (cod) and liver concentrations of PFOS for black guillemots (*Cepphus grille*) and glaucous gulls from the Barents Sea were 5.2 and 17.9 (Haukås et al. 2007). Guillemots and glaucous gulls represent the fourth and fifth trophic levels, whereas swallows are a second trophic level species, which likely accounts, at least in part, for the difference in magnification. We observed higher magnification factors between diet and plasma than for diet and liver for PFOS and PFHxS both at Oscoda (factor of 5–7 times) and in a previous study along the Mississippi River and nearby lakes (6–7 times higher, Custer et al. 2014). These results agree with observations in mammals demonstrating the high affinity of PFOS and other congeners for plasma proteins (Han et al. 2003; Beesoon and Martin 2015). One of the congeners that had a different pattern was PFHxA which was detected more frequently in diet (67% of samples), than in eggs (13%) or plasma (0%) and therefore diet may be a better matrix for monitoring environmental PFHxA levels than avian tissues. This pattern difference may also be true for other PFAS congeners with fewer than six (sulfonates) or eight carbons (acids).

Reproduction

Oscoda presented as an ideal location for assessing the effects of PFAS on reproduction due to high exposure to PFASs but little exposure to any other chemical contaminant (Custer et al. 2017a). Reproductive success between 2014 and 2016 was good at Oscoda with between 83% and 92% of the eggs hatching and qualitatively better than reproduction at the reference locations. The daily probability of egg failure in 2014 was 0.006, which was less than the failure rate at > 85% of the other 69 monitored sites across the Great Lakes (Custer et al. 2018), indicating a lack of a reproductive effects associated with PFAS exposure as well. This lack of a reproductive effect is consistent with the toxicity reference values established by Newsted et al. (2005) for quails and mallards in a laboratory study where the predicted no-effect threshold concentrations of PFOS in plasma and eggs are ~ 1000 ng/g. The mean concentrations in plasma and eggs in the current study were below this threshold value, at 856 ng/mL and 662 ng/g. This contrasts with the reported impaired reproduction at some PFAS-contaminated sites along the upper Mississippi River and nearby locations where hatching success was between only 68% and 71%, and where hatching appeared to drop off beginning at ~ 150 ng/g wet wt. of total PFASs in eggs (Custer et al. 2012, 2014). The reason for this discrepancy might be (1) either the additive effects of other contaminants with PFASs at the upper Mississippi River sites, and/or (2) exposure to unmeasured contaminants at those sites. Polycyclic aromatic hydrocarbons have recently been found to be associated with reduced hatching success (Custer et al. 2018) and that class of contaminant was not measured in the upper Mississippi River studies. The results from the current studies are also consistent with great tit (*Parus major*) data from a PFAS-contaminated site in Belgium (Groffen et al. 2019). There was no difference in percent hatching or percent of successful nests (hatching at least 1 egg) amongst five sites along a gradient of PFAS contamination. The Belgium study did find a negative association, using Principal Component Analysis and a range of reproductive endpoints, including both hatching and nest success. This type of analysis was not performed in the current study.

Typically, the date of first egg is later the first year of nest box placement than in subsequent years. That delay was observed in this study and ranged between 2 and 4 days. A longer time spent in a contaminated environment before egg laying provides more time for the female to accumulate a contaminant before a portion of it is deposited into the egg. This seemed to be the case in the current study because PFAS concentrations in eggs and plasma were qualitatively higher in 2014 (initial year) than in 2015. A similar effect was observed in lower Green Bay, WI where later nesting pairs had higher exposure than earlier nesting pairs (Custer et al. 1998).

Physiological Responses

Corroborating this lack of reproductive effects of PFASs at Oscoda was the lack of any physiological responses in tree swallow nestlings that were associated with PFASs, both in the current and an earlier (Custer et al. 2017b) study. The physiological responses measured in the current study are commonly used and indicate whether there is sufficient exposure to xenobiotic substances to elicit a physiological response in the organism. Ethoxyresorufin-*O*-dealkylase is part of the cytochrome P450 detoxification pathway and is induced by a variety of hydrocarbon toxins, such as PCBs and polycyclic aromatic hydrocarbons, but also by some PFASs (Liu et al. 2013). The measurement of DNA content can indicate problems in the division of genetic material during mitosis. There has been little study, however, of whether there may be DNA damage associated with PFAS exposure. Finally, perfluorinated substances are known competitive binders of the thyroid hormone transport protein transthyretin (Weiss et al. 2009), and as a result can affect thyroid hormone levels in exposed organisms and affect many thyroid-dependent physiological responses including body temperature, heart rate, and metabolism. We observed no differences in EROD, DNA-CV, plasma T4, and glandular T3 or T4 between Oscoda and the reference locations. These results are consistent with a previous study at Oscoda, where neither EROD, six measures of oxidative stress, nor DNA-CV were associated with PFAS exposure when assessed in a multivariate context that also included other organic contaminants, such as PCBs, polybrominated diphenyl ethers, and other legacy contaminants (Custer et al. 2017b). Thyroid hormone concentrations were not measured in that earlier study. Liu et al. (2013) found that general toxicity of PFASs and specifically, effects on genotoxicity and EROD biomarkers, depends on fluorinated chain length and/or functional group of the congeners. The responses of these endpoints to mixtures are more complex, but our data suggest that the PFAS profiles at Oscoda are not dominated by inducers of genotoxicity, thyroid effects, nor EROD activity.

There were annual differences in EROD activity; 2017 was higher both at Oscoda and the reference locations than in other years. The annual differences in the current study are unlikely due to changes in contaminant exposure, because EROD activity differences in 2017 were similar at both Oscoda and the reference sites. Seasonal and annual variations in EROD activity have been reported previously for multiple organisms, including tree swallows (Lohnes 2006; Kammann et al. 2005). These variations have been found to reflect environmental temperature, sex-based differences in study animals, developmental stage, and other intrinsic and extrinsic factors (Sleiderink et al. 1995; Sanchez et al. 2008; Rainio et al. 2012). However,

the specific causes of the variation in the present study are unknown.

The difference in DNA-CV between 2017 and the previous 2 years resulted from a change in analytical instrumentation in 2017. Although the data between years were significantly correlated ($P < 0.001$), the instrument used in 2017 resulted in 0.73-fold less DNA-CV compared to the previously-used instrument (Chi-yen Tseng, person. commun. Baylor Univ.).

For the thyroid hormones both in plasma or glands, the only significant difference between locations or years was lower T3 at the reference location in 2015 compared to Oscoda in 2015. Plasma T3 seemed abnormally low at the reference site in 2015 (0.18 ng/mL). This data point should be used with caution, however, because the sample size was low ($n = 3$) in that year. The concentration of T3 at the reference location in 2015 was qualitatively lower than at the reference locations in 2016 or 2017 (0.84 and 0.91 ng/mL) as well, which may indicate that the 2015 data point should be used with caution. While the mean glandular T4 (382.6 ng/mL) at the reference location was 3.6 times higher than at Oscoda, the variation was quite large which accounted for the non-significant difference there. Additional work with thyroid hormones and their association with PFASs seems warranted.

Conclusions

While the exposure of tree swallows nesting at Oscoda, MI to PFASs was extraordinary, there were no demonstrable effects on reproduction nor most physiological responses. This lack of reproductive effect is consistent with the toxicity reference values established in the laboratory for quails and mallards. In contrast to PFASs found near research and manufacturing plants, where adverse reproductive effects have been correlated with elevated PFAS exposure, the PFASs used at air force bases are highly refined, i.e., meet rigid standards for purity and PFAS congener content, and at those types of locations effects were not found. This difference in composition may explain the variations in toxicity observed between these two types of point sources. Consistent with other avian studies, PFOS was always the dominant PFAS in the present study. Eggs and plasma were found to accumulate higher concentrations of PFASs than other tissues such as liver and brain, and consequently more congeners were above the detection limit. Total PFASs were 5–7 times more concentrated in plasma compared to diet samples indicating the bioaccumulation potential of PFASs. There was some annual variation in exposure, but this variation is similar to the variation found in other highly contaminated environments.

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